

HANDBOOK OF Processed Meats and Poultry Analysis

Edited by
LEO M.L. NOLLET
FIDEL TOLDRA



H A N D B O O K O F

**Processed Meats
and Poultry Analysis**

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Preface

Muscle foods include a vast number of foods including meat, poultry, and seafood. There is a wide range of processed meats and poultry. These products represent an important percentage of the total food consumption worldwide.

Handbook of Processed Meats and Poultry Analysis is the second handbook of a series of four books related to analysis techniques and methodologies for animal products. This book is a reference volume for the analysis of meat and poultry products. Owing to the variety of products and type of processes and treatments (curing, dry-curing, fermentation, cooking, smoking, etc.), these products need particular analytical methodologies, which are described in this book. These analyses are focused on technological, nutritional, and sensory quality, as well as the safety aspects related to processing.

This book contains 34 chapters.

Chapter 1 introduces readers to the topic of the book and the importance of analysis in meat and poultry products.

Chapters 2 through 10 (Part I) describe the analysis of technological quality including the use of noninvasive techniques, such as physical sensors, and techniques to follow up the process, the analysis of moisture and water activity, the analysis of main ingredients (Chapter 4), additives used for these types of products, and the progress of specific biochemical reactions of great importance for final quality (Chapters 5 through 10).

In Chapters 5 through 7, additives, preservatives, smoke flavorings, and colorants are fully detailed. Determination methods of biochemical reactions such as oxidation, proteolysis, and lipolysis are discussed in Chapters 8 through 10.

For information and detection methods regarding glycolysis, the reader is directed to the relevant chapter of the first handbook (chapter on glycolysis in the *Handbook of Muscle Foods*, First Edition).

Chapters 11 through 16 (Part II) deal with the analysis of nutrients in various products mentioned as affected by processing.

Chapter 11 discusses the composition and calories of processed meats and poultry. This chapter is followed by chapters on analysis methods for essential amino acids, omega-3 and trans fatty acids, minerals and trace elements, and vitamins. Finally, in Chapter 14 the reader finds information on methods to measure the antioxidant capacity of meat and meat products.

Chapters 17 through 21 (Part III) are related to the sensory quality of meat and poultry products and the description of the major analytical tools and most adequate methodologies to determine color, as affected by curing and heating; texture, as affected by proteases, drying, and heating; and flavor, as affected by enzymatic reactions, microbial fermentation, and heating.

Finally, Chapters 22 through 34 (Part IV) are devoted to safety, especially to analytical tools for the detection of pathogens, toxins, adulterants (typically in minced meat products like sausages), materials in contact with foods (in packaged products), and chemical toxic compounds (polychlorinated biphenyls [PCBs], amines, and nitrosamines) that can be added or generated during processing. Other chapters deal with mycotoxins, genetically modified organisms, and irradiated ingredients.

The reader will also find information in Chapter 30 on veterinary drug residues.

This handbook provides readers with a full overview of the analytical tools available for the analysis of meat and poultry products. It describes the role of these techniques and methodologies for processing control and evaluation of final nutritional and sensory qualities. This book also describes analytical methodologies to ensure the control of different safety concerns related to processing. In summary, readers will find the main available analytical techniques and methodologies for the analysis of meat and poultry products, its compounds, and its major characteristics.

The editors of this handbook would like to thank very cordially all the authors. This book is the result of their enthusiastic cooperation and help. They are appreciated for their scientific and in-depth knowledge of the different and diverse topics.

Fidel Toldrá and Leo M.L. Nollet

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Fidel Toldrá, PhD, is a research professor at the Department of Food Science, Agrochemistry and Food Technology Institute (CSIC), Valencia, Spain, and serves as European editor of *Trends in Food Science and Technology*, editor-in-chief of *Current Nutrition & Food Science*, and as a member of the Food Additives Panel of the European Food Safety Authority (EFSA). Dr. Toldrá has served as author, editor, or associate editor of 14 books on food chemistry and biochemistry and meat processing. Some recent titles are *Advanced Technologies for Meat Processing* (CRC Press, 2006); *Handbook of Food Science, Technology, and Engineering* (CRC Press, 2006); *Advances in Food Diagnostics* (Blackwell, 2007); *Handbook of Food Product Manufacturing* (Wiley, 2007); and *Handbook of Fermented Meat and Poultry* (Blackwell Publishing, 2007). He was awarded the 2002 International Prize for Meat Science and Technology, given by the International Meat Secretariat, during the 14th World Meat Congress held in Berlin. In 2008 he was elected Fellow of the International Academy of Food Science and Technology. He is also a member of the Executive Committee of the European Federation of Food Science and Technology (EFFoST). His research is focused on the (bio)chemistry and analysis of foods of animal origin.

Leo M.L. Nollet received his MS (1973) and PhD (1978) in biology from the Katolieke Universiteit Leuven, Belgium. Dr. Nollet is the editor and associate editor of numerous books. He edited for Marcel Dekker, New York—now CRC Press of Taylor & Francis Group—the first and second editions of *Food Analysis by HPLC* and *Handbook of Food Analysis*. The last edition is a three-volume book. He has also edited *Handbook of Water Analysis* (first and second editions) and *Chromatographic Analysis of the Environment* (third edition, CRC Press). With Dr. Toldrá he coedited two books published in 2006 and 2007: *Advanced Technologies for Meat Processing* (CRC Press) and *Advances in Food Diagnostics* (Blackwell Publishing). With M. Poschl he coedited *Radionuclide Concentrations in Foods and the Environment*, also published in 2006 (CRC Press).

Dr. Nollet has been coeditor of several books with Y.H. Hui and other colleagues, including *Handbook of Food Product Manufacturing* (Wiley, 2007); *Handbook of Food Science, Technology, and Engineering* (CRC Press, 2005); and *Food Biochemistry and Food Processing* (Blackwell Publishing, 2006). Finally, he edited *Handbook of Meat, Poultry, and Seafood Quality* (Blackwell Publishing, 2007). He is a member of the editorial advisory board of the Food Science & Technology series, CRC Press (Taylor & Francis, Boca Raton, Florida). He is head of the task group for affiliated publications of The European Federation of Food Science and Technology.

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PROCESSING CONTROL

I

Chapter 1

Introduction: Importance of Analysis in Meat Products

Hans Steinhart

Analysis of food serves mainly to define its quality. One distinguishes among process, nutritional, and sensory quality. According to DIN ISO 9000, the term quality is defined as the totality of features concerning the ability of a product to fulfill its requirements (International Institute for Standardization, 2005). The concept of food quality has, however, to be considered on a much broader basis, as the different demands of the manufacturer, the consumer, the surveillance agency, and the legislature must be taken into account in order to obtain healthy and safe products without neglecting the concepts of economy and ecology. Therefore, food must not be evaluated just for its safety and its nutritional value but also for its sensorial, technological, and even ideological (e.g., food of biodynamic origin) or religious value (e.g., kosher food). The consumer wants “healthy” products with high nutritional value in regard to macronutrients such as proteins, carbohydrates, fats, and fiber as well as such minor nutrients as vitamins and trace elements. Products should also be superior in taste, flavor, and texture. As consumer behavior, like society in general, is changing constantly, people tend to favor prepackaged food for convenient preparation and food with a long shelf life. Allergens, contaminants and residues, trans-fatty acids (TFA), genetically modified organisms (GMO), and prions are catchwords for things that are generally regarded as undesired components in food, especially in meat and meat products, by consumers; unfortunately, they cannot be totally avoided by the manufacturers. Although these undesired components constitute an underlying risk, avoiding microbial contamination is a far more important topic in ensuring food safety and food quality. All these factors add up to an ideal concept of food quality; at a minimum, effort has to be made to ensure maximum nutritional value and food safety, but the economic success of a product will surely also be affected by other factors, including (irrational) consumer expectations. Additionally, as nutritional research is gaining in importance, new analytical methods permit the analysis of biochemical pathways of minor food ingredients which can be considered to have positive effects for humans (e.g., ω -3 fatty acids); such

methods can also allow for detection of trace amounts of potentially hazardous components such as acrylamide, furan, or TFA. This means that the term food quality cannot be considered static; manufacturers and legislators have to consider the latest research results based on the progress in analytical methods. Analysis of meat and meat products is a complex process because it involves many totally different aspects of the problem. The most important decision to be made before one starts any analytical work is the question of what answer should be given through the analytical procedure. The statement of the problem defines the analytical method that can be used to get the best answer. Regarding meat, the most important questions in connection with analytical procedures are: processing control, composition of meat and meat products (nutritional value), sensory quality, and safety aspects.

Analytical properties are important tools to describe the quality of food. They can be classified into four groups:

1. Accuracy and representativeness of the analytical methods.
2. Precision, robustness, sensitivity, and selectivity of the methods. These attributes are closely related to a proper sampling of the material that is to be analyzed.
3. Expediency, cost-effectiveness, and personnel-related factors have a high practical relevance.
4. Interpretation of the qualitative and quantitative results. This is a major task in modern analysis of food. It is not sufficient to determine only qualitative and/or quantitative results; these results have to be put in the context of the quality of the whole food. If scientists do not properly interpret the results of their work, people or media who are not concerned with analysis may interpret these results from an emotional background. This can frequently lead to misinterpretation of the quality of the investigated food.

The accuracy of an analytical procedure relies strongly on the precision, robustness, sensitivity, and selectivity of the method chosen. It can be defined as the consistency of results of a procedure, or the mean of n results with a defined method and the value that is held true. The accuracy of a method is determined through its systematic aberrations. This means that accuracy is higher as errors in the analytical procedures are reduced. Representativeness depends on a couple of factors, the most important being the sampling of the food, the selected analytical procedure, and the client's information needs. It is important to state that an accurate result gained with an unrepresentative analytical method will produce a poor quality result.

The precision of a method is determined through repeated measurements using independent samples and calculation of the standard deviations of the mean value. The precision of a method is described by hazard mistakes.

The robustness of a method is its resistance to changes in results when applied to individual aliquots of the same sample to test the same analyte under slightly different experimental conditions. A method is more robust, for example, when it is possible to use a broader range of pH values as compared to another method which is only applicable by defining a narrow pH range.

The sensitivity of a method includes two different attributes. A method is considered sensitive if it is able to detect and to determine small amounts of an analyte in the food. This definition describes the two aspects, qualitative and quantitative analysis. Another aspect of sensitivity describes the ability of a method to discriminate between similar amounts of analytes in a food sample.

The selectivity of a method is defined as its ability to produce results which are strictly related to a certain analyte in a food sample. The signal determined is therefore exclusively determined by the properties of the analyte. Selectivity is high if there is no interference with other analytes

in the sample. Selectivity can be improved if the knowledge of the ingredients of a food is high, and if the matrix components which can interfere with the analyte are limited. The selectivity of a method can also be improved if only a few analytes need to be determined.

To ensure food safety and consumer expectations, precautions must be taken in terms of a total quality management system. Quality control must work on different levels and, therefore, different analytical procedures must be established for different steps of production. One must distinguish between fast process analyses with simple (inexpensive) methods, surveillance methods using elaborate analysis methods for qualification and quantification of residues and contaminants, and highly sophisticated methods for research purposes. Nevertheless, all analysis methods suit their purpose to enhance food quality, and effort is made to simplify research methods to be applicable in the production process as fast methods or even online methods.

One of the most important objectives in obtaining high-quality food is controlling for contaminants and residues. These compounds can either enter the food during production (e.g., hormones, pharmaceuticals, TFA, and monomers from coatings) or they can be formed during storage or processing of food (e.g., mycotoxins, botulinum toxins, acrylamide, and furan). These compounds have toxic effects in cell culture experiments, in animals, and in humans. Maximum limits have therefore been set by the legislative action.

Analysis methods must be sensitive enough to detect those contaminants in trace amounts; on the other hand, they must be selective enough to clearly identify these compounds free of matrix interference. Although GC-FID and HPLC-UVD/FLD methods are very sensitive, they are often lacking in selectivity. Selective sample preparation by liquid-liquid extraction or solid-phase extraction is advantageous but also tedious and expensive. That is why most common analysis methods for residues/contaminants tend to utilize HPLC-mass spectrometry (MS) methods with fast sample preparation techniques (mostly automated solid-phase extraction) based on single-quadrupol (or, for better selectivity and sensitivity, triple-quadrupol) devices. The selectivity of the triple-quadrupol devices allows compounds to be analyzed for their specific mass fragments at different polarities (positive/negative) even if co-eluting compounds are present. In multiple reaction monitoring (MRM) mode, the mass-spectrometer scans specifically for one daughter ion resulting from fragmentation of one parent ion. Simultaneous determination of most relevant compounds can be easily performed by HPLC-MS/MS (or, alternatively, GC-MS/MS) methods, though the latter are limited to volatile compounds. As quality control is also an important issue in surveillance laboratories, mass-spectrometric methods offer a higher reliability, especially when it comes to legal issues. Contaminated food is not allowed to be marketed and has to be destroyed, resulting in sometimes huge economic losses for the producer. Therefore, contaminated products are often mixed with other products to reach maximum allowable levels, and surveillance has to cope with such criminal activity by auditing the production facilities and using the most sensitive analysis methods for their work.

Labeling of ingredients is of special importance for consumer protection. European legislation demands the correct labeling of ingredients, possible allergens, and nutritional facts. For the consumer, labeling is very important in terms of the identity of food for two reasons. First, if products from different animal or plant sources were used for production (e.g., poultry and pig meat), the ingredients must be qualitatively and quantitatively analyzable in food. Second, consumers are very concerned about GMO in food. Distinguishing between different animals and plants can be done by electrophoresis of protein, but this has been shown to be insufficiently sensitive and selective. Immunological methods, such as the determination of different milk types by the method of Ouchterlony, are applicable for a specific purpose but are time consuming and lack sensitivity. Current methods are based on the polymerase chain reaction (PCR) method, which is constantly

improved to identify the smallest amount of different meat species or GMO ingredients in food products. Three different PCR techniques are currently utilized for species identification. Normal PCR requires specific primers for deoxyribonucleic acid (DNA) to be amplified, such as primers for the pig or cattle growth hormone sequence. As this single PCR may lack selectivity due to cross-reactivity, alternative methods, such as restriction fragment length polymorphism PCR (RFLP-PCR) or the terminal restriction fragment length polymorphism PCR (t-RFLP-PCR), have been developed. These methods amplify mitochondrial cytochrome b gene or other suitable genes, but unless separating these amplification products by electrophoresis as in normal PCR, they are cut by specific restriction enzymes (e.g., HAE III endonuclease) inside the amplification product (RFLP-PCR) or from the terminal end (t-RFLP-PCR). The obtained fragment pattern is characteristic for different species as these genes differ slightly (polymorphism). The t-RFLP-PCR, with its fluorescence-labeled amplification products, is an especially promising technique capable of distinguishing among 40 different species in low amounts (detection limit, 1–5%).

The need for manufacturers to efficiently control the production processes encourages the use of online and at-line methods for quality control. Product quality cannot be sufficiently controlled by recipes because it is not possible to check every single unit of a charge. Electronic sensors for moisture, pH value, and sugar content can now be easily installed on the basis of conductivity, refractometric, or polarometric measurements in homogenous samples. The use of electronic noses is convenient and capable of measuring changes in air composition to enable detection of mold-smitten stocks. These electronic sensors consist of metal oxide semiconductors, conducting polymer sensors, or quartz crystal microbalance sensors. These sensors can be easily contaminated by adsorbed molecules, to which the metal oxide sensors are especially sensitive. Nevertheless, these sensors have to be calibrated for specific targets and lack robustness; current applications are limited to detection of volatile compounds (e.g., flavor analysis).

Other fast methods have been developed for at-line analysis/production control, including NIR spectroscopy. Although these methods must be extensively calibrated, they are suitable for simple matrices to determine the content of macronutrient (proteins, fat, carbohydrates). It is possible to control for specific production standards, so tedious reference methods need only be used if deviations from quality standards occur during production. Laboratories are always in need of developing faster methods in order to save time and expense, and not only for pesticide or residue determination. Fatty acid determination for compliance with new labeling demands can be easily performed within 30 min by the Caviezel method. Fats are saponified, and instead of analyzing the fatty acid methyl esters, free fatty acids are analyzed using a packed column with automated quantification of results. This method is suitable for determination of butyric acid and total fat content but lacks in resolution for TFA. Trace elements can be determined by continuum sources for atom absorption spectroscopy with no need to change the lamp. Alternatively, element determination based on inductively coupled plasma-mass spectrometry will become more common as nutritional research results will show the necessity to control for composition and amount of trace elements.

This book gives within its 34 chapters a complete overview of all analytical aspects concerning meat and poultry analysis. The authors of the chapters are excellent specialists in meat and meat product analysis.

Chapter 2

Physical Sensors and Techniques

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2.1 Introduction

Currently, the most important objective for the industry is to develop and control the processes to produce food products with specific properties of quality, safety, and nutrition as demanded

by consumers. In this context, modern physical sensors play an important role for the industry and constitute an advance in controlling food properties. These sensors are mainly based on electromagnetic radiation, ultrasound, and resonance techniques. Some of the industry applications of these techniques are still being developed and a lot of effort is employed in obtaining in-line nondestructive and even noncontacting sensors for food quality measurements. The integration of these physical measurements with a deep study of the food or the process can produce useful information for food quality assessment. In this analysis it is important to consider the food as a complex system in order to study the microstructure changes, the interactions among food components, macromolecular changes, and also physicochemical changes, and to integrate all this knowledge in food quality improvement.

Dielectric spectroscopy occupies an important place among the modern measurement methods employed for chemical and physical analysis of materials in different research fields, including medicine, food technology, and material science. This technique allows the investigation of the relaxation process in a wide range of characteristic times (10^4 – 10^{-12} s). Although this method does not possess the selectivity of nuclear magnetic resonance (NMR) spectroscopy, it provides, in some cases, unique information about the food properties. Dielectric spectroscopy is sensitive to interfacial polarization and intermolecular polarization (dipole–dipole), and allows the acquisition of valuable information about the process and the food components, and also can be monitored.

Utilization of this technique, based on microwave (MW) radiation, for obtaining electromagnetic spectra in a wide range of frequencies requires an important equipment investment. Owing to this fact, and in view of industry application, it is necessary to undertake a preliminary study to delimit the range of frequencies and to diminish sensor costs. Moreover, this technique is difficult to apply in foods because of the high heterogeneity of samples and the changes suffered by the biological systems over time. Because of this, more research in this field is necessary.

The aim of this chapter is to present an overview of the physical sensors and techniques employed in food quality control, paying special attention to the electromagnetic radiation sensors as a promising method for online food control, and also to offer a detailed exposition of the current applications of these techniques in the food industry.

2.2 Nondestructive Online Food Analysis

2.2.1 Introduction

Food quality has to be defined with regard to changes in consumer expectations, legislative needs, and new developments in instrumental analysis [1]. The term “quality” is defined according to DIN ISO 9000 as the totality of features relevant to the ability of a product to fulfill its requirements [2].

To ensure food safety and to suit consumer expectations, caution has to be taken in terms of a quality management system. Quality control must act at different levels and, therefore, different control methods have to be established for different steps of production. A lot of effort is spent on simplifying quality control methods to be applicable in the production process as online methods. Online sensors operate directly in the process and obtain a real-time signal which can be related to quality parameters of the food.

A group of alternative technologies based on the application of electromagnetic energy has attracted special attention among academic and industrial communities. These technologies can be developed on a wide range of frequencies and have some advantages in comparison to traditional control methods. The main advantages are the fast acquisition and processing of data, and the fact that they are nondestructive and, in some cases some, even noncontact methods.

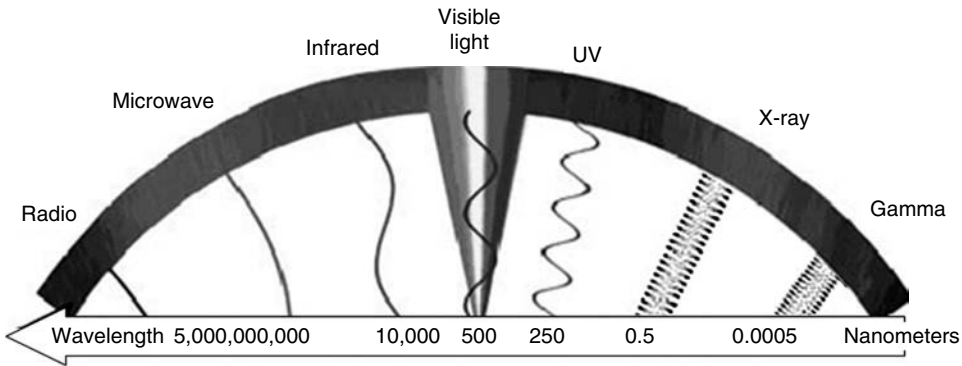


Figure 2.1 The electromagnetic spectrum.

Figure 2.1 shows the electromagnetic spectrum, which is characterized by the different kinds of radiation as a function of its wavelength and frequency. There are different kinds of sensors based on the interaction of the material with the electromagnetic waves: sensors using visible, ultraviolet, and infrared (NIR, NIT, FTIR, and thermography) waves, MWs, radio waves, X-rays, and high-frequency waves (NMR and electronic magnetic resonance).

Some applications have been in the market for many years, in particular for laboratory purposes. There are also numerous online examples: X-rays for foreign body detection, visible light sensors for color sensing or machine vision inspections, near-infrared (NI) sensors for quality inspection and temperature measurement, and MW sensors for measurement of water content [3].

2.2.2 Basic Principles

Dielectric spectroscopy determines the dielectric properties of a medium as a function of frequency. Complex permittivity (ϵ_r) is the dielectric property that describes the behavior of a material under the influence of an electromagnetic field [4–5]; it is defined by the following equation:

$$\epsilon_r = \epsilon' - j \cdot \epsilon'' \quad (2.1)$$

In this equation, $j = \sqrt{-1}$. The real part of complex permittivity is called the dielectric constant (ϵ'), and the imaginary part is called loss factor (ϵ''). The dielectric constant is related to the capacitance of the material and its ability to store energy; the dielectric loss factor is related to the absorption and dissipation of the electromagnetic energy. The subscript “r” indicates that the values are relative to air and, for this reason, the variable is dimensionless (Equation 2.2):

$$\epsilon_r = \epsilon^*/\epsilon_0 \quad (2.2)$$

in which the air permittivity is $\epsilon_0 = 88,542 \times 10^{-12}$ F/m.

There are different mechanisms affecting dielectric behavior. These mechanisms are divided into two classes—resonance and relaxation processes. The first happens when the applied electric field has a frequency that matches the natural oscillation frequency of the material and happens at high frequencies (infrared region). It includes electronic polarization and atomic polarization. Electronic polarization results from the displacement of electrons around the nuclei. Atomic polarization is due to relative displacement of atomic nuclei because of the unequal distribution of charge in molecule formation.

Relaxation phenomena occur at MW and radio frequencies and characterize, with the conductivity, the dielectric behavior of practically all tissues at these frequencies.

Dielectric properties of biological tissues result from the interaction among electromagnetic radiation and tissue constituents at the cellular level and at the molecular level.

Biological tissues are complex systems with a high number of dispersions. These systems cannot be reduced to a single dispersion caused by free water molecules because of the presence of macromolecules whose behaviors have to be taken into account. Dielectric permittivity takes very high values at low frequencies, decreasing in different steps (relaxations) when frequency increases. Its frequency dependence facilitates the search of a number of phenomena and, for this reason, the study of dielectric properties in biological tissues has recently received much attention in food technology [6]. Many authors have studied these dispersions in biological systems [7–12].

In biological systems, there are four main relaxation regions: α , β , δ , and γ (Figure 2.2). In their simplest form, each of these relaxation regions can be characterized by Debye equations (see Section 2.2.2.1). In these equations, the constants (ϵ_s and ϵ_∞) determine the beginning and the end of the dispersion changes. However, biological systems are characterized by their complexity, and it is very difficult to simplify relaxation phenomena using Debye equations.

The γ -dispersion, also called orientation polarization, is located near 20 GHz; it is due to the polarization of free water molecules. The β -dispersion, or interfacial polarization, is mainly due to the Maxwell–Wagner effect. This effect is produced due to interfacial phenomena on heterogeneous

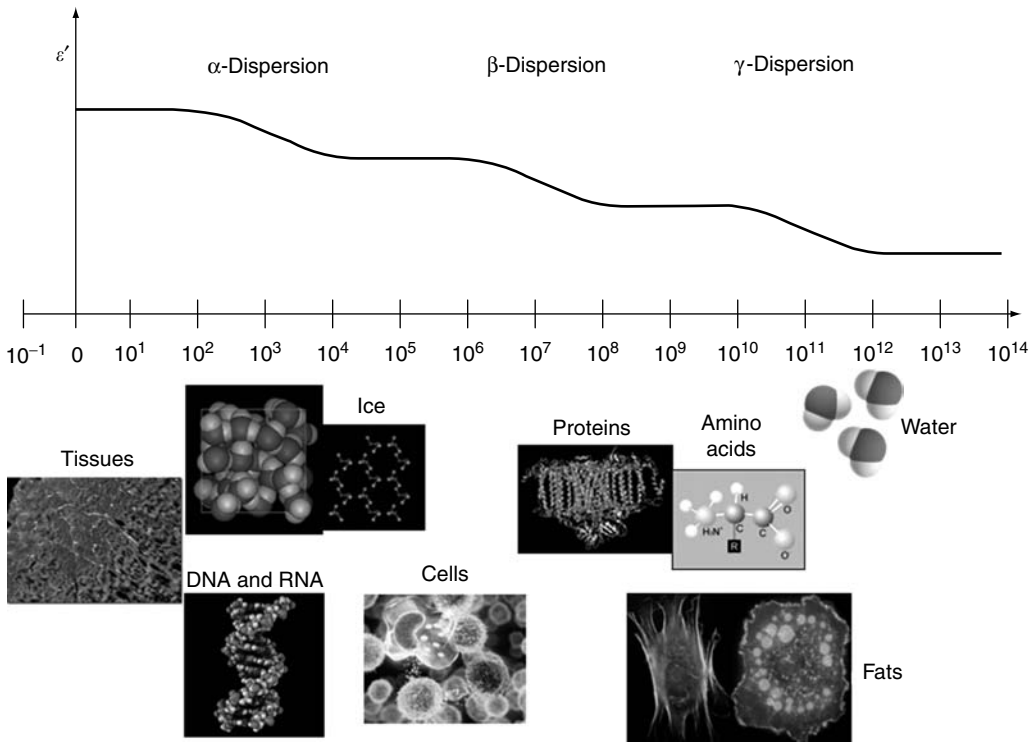


Figure 2.2 Schematic representation over the electromagnetic spectrum (in logarithm scale) of the different causes that contribute to dielectric constant in biological systems. The four relaxation regions (α , β , δ , and γ) that can be presented in these systems are also represented.

materials. This mechanism is due to the polarization of the cellular membranes, which act as a barrier to the ion fluxes between the intra- and extracellular liquids [6]. Moreover, other dispersions can be produced by proteins or other macromolecules at frequencies between the β and γ dispersions, depending on the size and charge of the molecules. The β -dispersion caused by proteins is smaller than the one caused by cellular membranes and appears as a small tail at the end of the β -dispersion caused by the membranes. Other dispersions are caused by small subcellular structures, such as mitochondria, cell nuclei, and other subcellular organelles. Since these structures are smaller in size than the surrounding cell, their relaxation frequency is higher but their total dielectric increment is smaller, contributing also to the small tail at the end of the large β -dispersion.

An additional relaxation (δ) is located between the β and γ dispersions. This relaxation is also caused in part by the rotation of amino acids, rotation of charged side groups of proteins, and relaxation of protein-bound water, which occurs somewhere between 300 and 2000 MHz [10]. It is important to highlight that the dielectric response of water molecules depends on the environment of the dipolar molecule. Bound water molecules have a different relaxation frequency from free water due to the fact that their movement (polarization) is limited by the union of these dipolar molecules to the substrate [13].

At lower frequencies, α -dispersion is produced, but its causes are still not clear. Some hypotheses have been advanced, such as relaxation of intracellular structures, relaxation of counterions, and relaxational behavior of membranes, but more research is necessary to clarify the causes of this relaxation phenomenon [10]. Conductivity of tissue increases similarly in several major steps symmetrical to the changes of the dielectric constant. Conductivity increases from a few millimhos per centimeter to nearly a thousand [10].

Only a perfect dielectric can store and release electromagnetic energy without absorbing it. The ϵ'' parameter is related to the absorption and dissipation of electromagnetic energy in other kinds of energy, including thermal [14]. These energy absorptions are caused by different factors, which depend on the structure, composition, and frequency.

In most dielectric measurement techniques it is impossible to separate the losses due to conduction from the ones due to polarization. Thus, the loss factor, ϵ'' , is expressed by

$$\epsilon'' = \epsilon_d'' + \epsilon_{\text{MW}}'' + \epsilon_c'' + \epsilon_a'' + \frac{\sigma}{\epsilon_0 \omega} \quad (2.3)$$

where

- ϵ_d'' = loss factor caused by the dipolar orientation or dipolar relaxation
 - ϵ_{MW}'' = loss factor due to the Maxwell–Wagner effect
 - ϵ_c'' = loss factor relative to electronic polarization
 - ϵ_a'' = loss factor caused by atomic polarization
 - $\sigma/\epsilon_0 \omega$ = loss factor due to effect of ionic conductivity
- σ , ϵ_0 , and ω = conductivity of the material, the dielectric constant in vacuum, and the angular frequency, respectively

The different mechanisms that contribute to the effective loss factor are shown in Figure 2.3.

- *Ionic conductivity.* Charged atoms and molecules (ions) are affected by the field at radio frequencies and the lowest MW frequencies. Such ions move based on the changes in the electric field. If ions do not find any impediment (e.g., aqueous solutions, conducting materials), ionic conductivity gives rise to an increment in effective losses. At these frequencies, the ionic losses are the main contributors to the loss factor (assuming ions to be present in the material) [15].

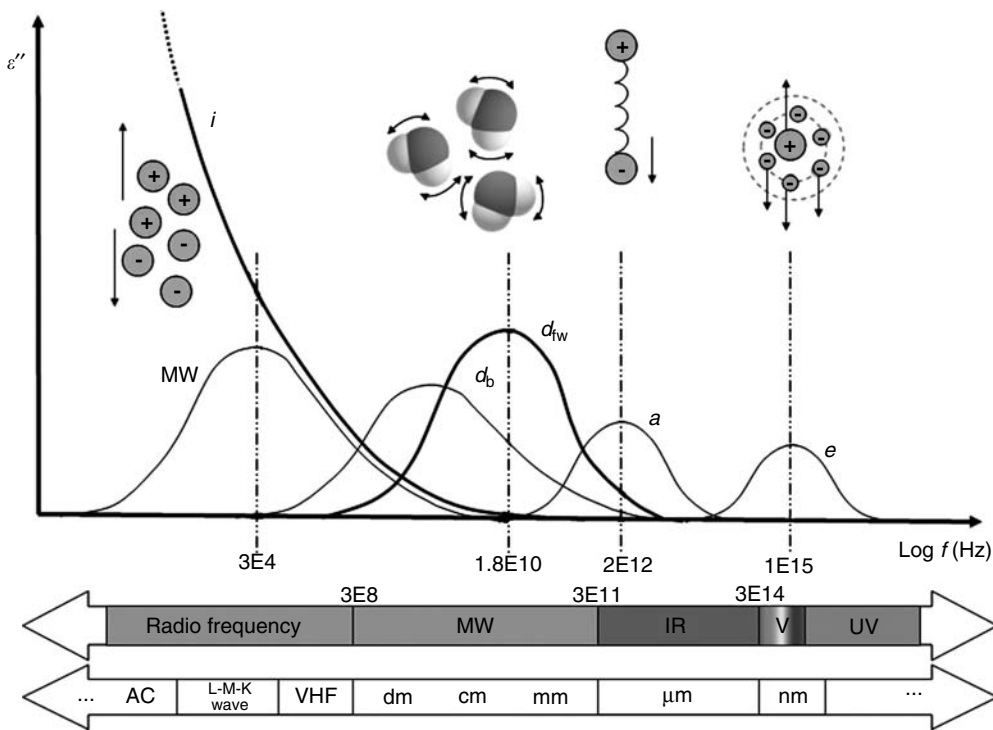


Figure 2.3 Schematic representation over the electromagnetic spectrum (in logarithmic scale) of the different effects that contribute to the effective loss factor. Where: *i* represents the ionic losses; MW means Maxwell–Wagner effect; d_{fw} is related to the dipolar losses of free water; d_b is related to the dipolar losses of bound water; *a* is related to the atomic losses; *e* is related to the electronic losses. (From De los Reyes, R., Castro-Giráldez, M., Fito, P., and De los Reyes, E., *Advances in Food Diagnostics*, Blackwell Publishing, Iowa, 2007.)

- **Maxwell–Wagner effect.** Foods are complex systems and usually present conducting regions surrounded by nonconducting regions; for example, foods with a cellular structure have cytoplasm (conducting region) surrounded by the membrane (nonconducting region). In these cases, ions are trapped by the interfaces (nonconducting regions) and, as the ion movement is limited, the charges are accumulated, increasing the overall capacitance of the food and the dielectric constant (Maxwell–Wagner polarization) [16]. This phenomenon is produced at low frequencies, at which the charges have enough time to accumulate at the borders of the conducting regions.

The Maxwell–Wagner loss curve versus frequency has the same shape as the dipolar loss curve. At higher frequencies, the charges do not have enough time to accumulate and the polarization of the conducting region does not occur. At frequencies above the Maxwell–Wagner relaxation frequency, both ionic losses and the Maxwell–Wagner effect are difficult to distinguish due to the fact that both effects exhibit the same slope.

- **Dipolar orientation.** Under a MW field, molecules with an asymmetric charge distribution (permanent dipoles) rotate to align with the electric field storing energy (dipolar polarization, orientation polarization, or γ -dispersion). The dipolar contribution to total losses

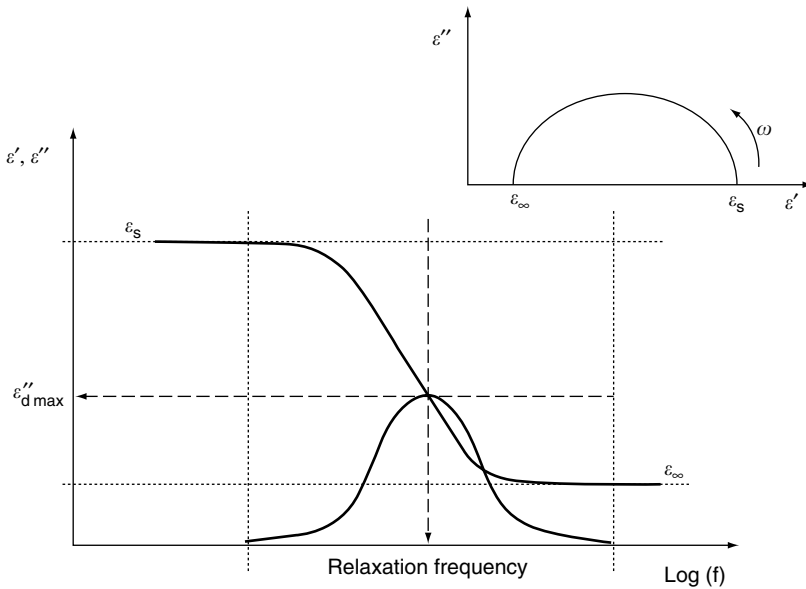


Figure 2.4 Representation of dipolar losses and dielectric constant versus logarithm of frequency following the ideal Debye model. Cole and Cole diagram is represented at the right top of the figure. (Adapted from Debye, P., *Polar Molecules*, The Chemical Catalogue Co., New York, 1929.)

occurs when a phase lag between the dipole alignment and MW field appears. When this phenomenon occurs, the material starts to lose stored energy and the dielectric constant decreases. When the frequency of the field is too high for dipole rotation, the dipolar losses end and the dielectric constant reaches a constant value [15].

The highest value of dipolar losses ($\epsilon''_{d \max}$) is produced at the relaxation frequency. The dipolar contribution to total losses is one of the most important at MW frequencies due to the fact that water is an abundant and common component of foods. Therefore, the dipolar orientation mechanism has been widely studied [17,18].

2.2.2.1 Debye Model as an Example of Relaxation Phenomena

The Debye model (1929) [17] can be used to describe permanent dipolar behavior in liquids and in polar molecular solutions in nonpolar solvents (Figure 2.4). The equation of this model is as follows:

$$\epsilon_r = \epsilon' - j\epsilon'' = \epsilon_\infty + \frac{\epsilon_s - \epsilon_\infty}{1 + j\omega\tau} \quad (2.4)$$

in which

ϵ_∞ = relative permittivity at very high frequencies

ϵ_s = static dielectric constant

τ = relaxation time in seconds

Based on Equation 2.4, it is possible to identify the dielectric constant and the loss factor:

$$\epsilon' = \epsilon_{\infty} + \frac{\epsilon_s - \epsilon_{\infty}}{1 + \omega^2\tau^2} \tag{2.5}$$

$$\epsilon'' = \frac{(\epsilon_s - \epsilon_{\infty})\omega\tau}{1 + \omega^2\tau^2} \tag{2.6}$$

The loss factor reaches its maximum value at relaxation frequency (f_c). Relaxation frequency can be related to relaxation time (τ) through the equation $f_c = 1/2\pi\tau$. In general, larger molecules have less mobility and higher relaxation times than smaller ones. Therefore, relaxation frequency diminishes when molecular weight increases [19].

2.2.2.2 Examples of Pork Meat Spectra

Some spectra of pork meat samples are shown in Figures 2.5 through 2.7. Figure 2.5 shows the spectra of pork meat samples (*longissimus dorsi*) at 24 h postmortem measured by an impedance analyzer, Agilent® 4294A (Agilent Technologies Company, United States), connected to a parallel plate fixture, Agilent® 16451B (from 40 Hz to 2 MHz), and a Vector Network Analyzer (VNA), Agilent® E8362B connected to a coaxial probe, Agilent® 85070E (from 500 MHz to 20 GHz). The measurements were made at 4°C following the direction of the meat fibers. Figure 2.6 shows the dielectric spectrum from 500 MHz to 20 GHz of the same samples in detail.

Some spectra (from 500 MHz to 20 GHz) of salted and raw pork meat samples are shown in Figure 2.7. It can be appreciated that the extremely high loss factor at low frequencies in salted samples is due to the relevant contribution of ionic losses to the total losses of the samples.

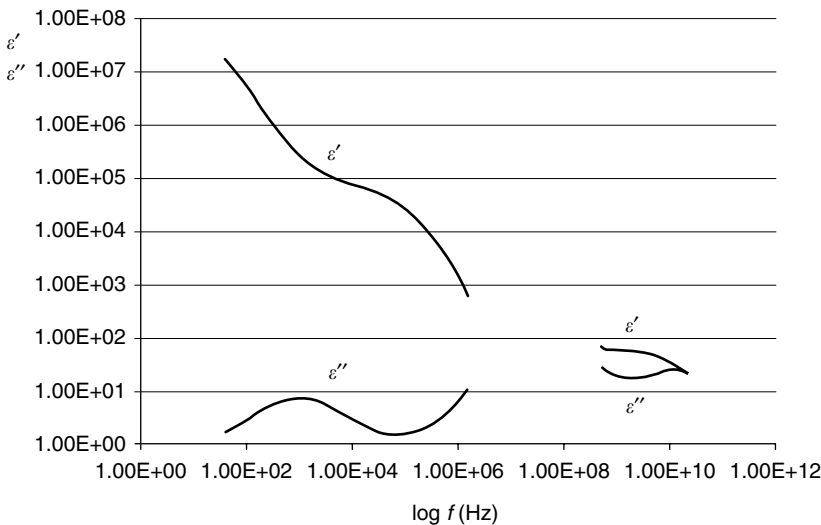


Figure 2.5 Dielectric spectra (40 Hz–2 MHz) from pork meat samples (*longissimus dorsi*) at 24 h postmortem measured at 4°C following the direction of the meat fibers. Frequency axis is in logarithmic scale.

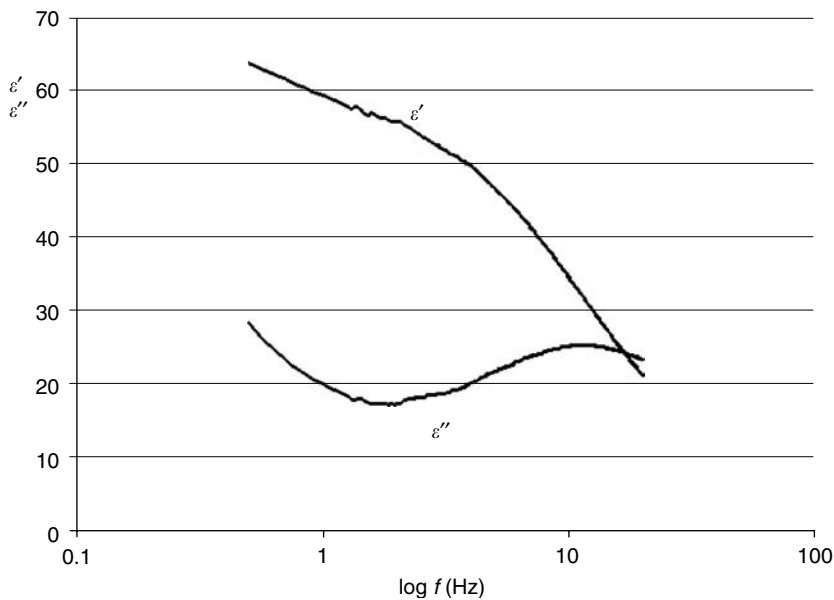


Figure 2.6 Dielectric spectra (500 MHz–20 GHz) from pork meat samples (*longissimus dorsi*) at 24 h postmortem measured at 4°C following the direction of the meat fibers. Frequency axis is in logarithmic scale.

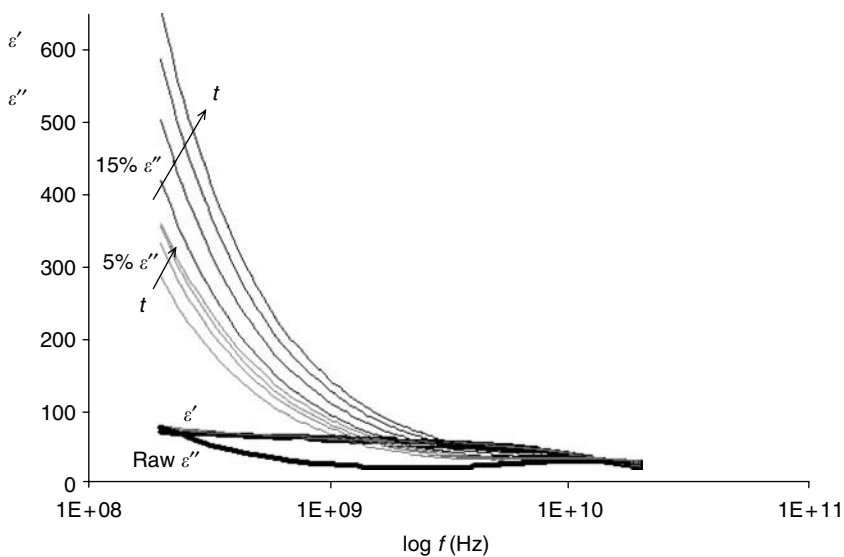


Figure 2.7 Dielectric spectra (500 MHz–20 GHz) from raw pork meat (black bold line) and pork meat samples at salting times (t) yielding from 2 to 8 h of immersing time into 5% (gray lines) and 15% (black lines) brine. The arrows beside t indicate the growth of the salting time. Frequency axis is in logarithmic scale.

2.2.3 Microwave Dielectric Spectroscopy

2.2.3.1 Overview of Microwave Measurement Techniques

The dielectric properties of foods can be determined by several techniques using different MW measuring sensors depending on the frequency range of interest, the type of target food, and the degree of accuracy required. VNA is very useful and versatile for detailed studies. At MW frequencies, generally about 1 GHz and higher, transmission line, resonant cavity, and free-space techniques have been very useful [20].

MW dielectric property measurement methods can be classified as reflection or transmission measurements, using resonant or nonresonant systems, with open or closed structures for sensing the properties of material samples [21].

Transmission measurements are made by at least two sensors: Emitter sensors send the signal through the material and receiver sensors capture the resulting signal. Reflected measurements are made when the same sensor emits and receives the signal after it is reflected by the material. The different measuring techniques are explained in the next section.

2.2.3.1.1 Parallel Plate Technique

The parallel plate method requires a thin sheet of material placed between two electrodes to form a capacitor (Figure 2.8). This method is also called the capacitance technique; it uses an LCR meter or impedance analyzer to measure capacitance and dissipation. This method is typically used at low frequencies (<100 MHz).

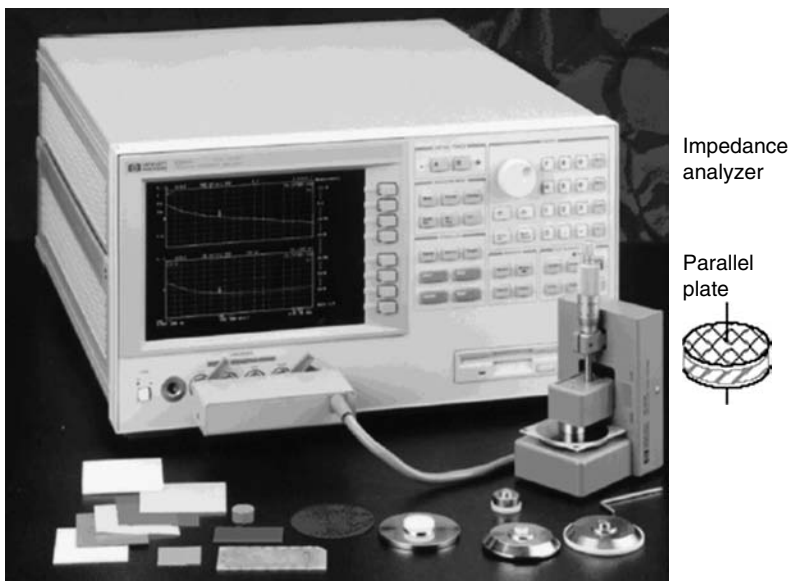


Figure 2.8 Impedance analyzer and parallel plate. (From Agilent Technologies Company, Agilent solutions for measuring permittivity and permeability with LCR Meters and Impedance Analyzers, Application Note 1369-1, United States, 2006.)

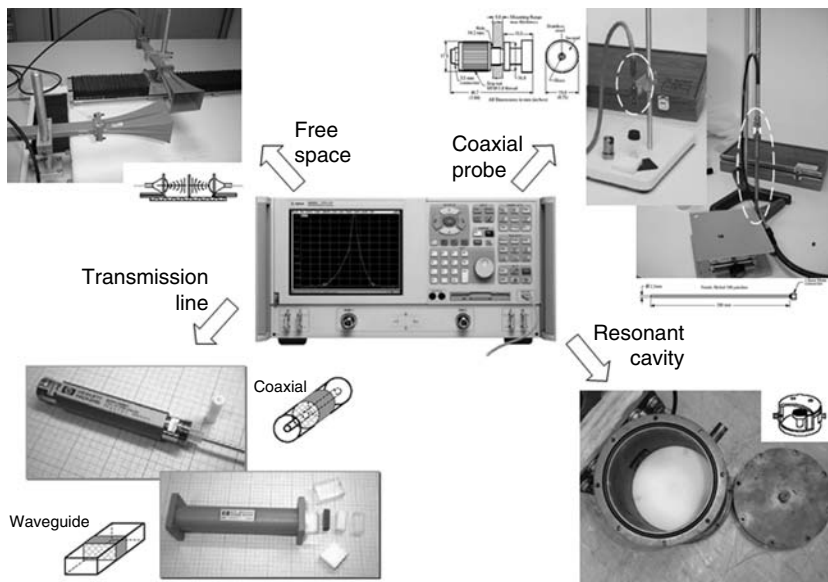


Figure 2.9 Overview of the measurement techniques, which can be used with a VNA. (From Agilent Technologies Company, United States and De los Reyes, R., Castro-Giráldez, M., Fito, P., and De los Reyes, E., *Advances in Food Diagnostics*, Blackwell Publishing, Iowa, 2007.)

2.2.3.1.2 Open-Ended Probe Technique

A typical coaxial probe system consists of a VNA, a coaxial probe (Figure 2.9), and an external computer and software to calculate permittivity from calibrated S-parameter measurements. This method is widely used for liquids or semisolids, although solids with a flat surface can also be measured, which makes it ideal for many foodstuffs. The material is measured by immersing the probe into the liquid or semisolid samples, or by touching the flat surface of the solid. This method is nondestructive, easy to use, and does not need sample preparation. It uses a frequency range from 200 MHz to 20 GHz and requires a sample thickness >1 cm [22]. The sample has to be thick enough to represent that it is endless. Air gaps or bubbles between a solid and the probe must be avoided since they produce errors. This is a reflection measurement method, in which the same coaxial probe emits and receives the signal.

2.2.3.1.3 Resonant Cavity Technique

Cavities are characterized by the central frequency (f_c) and quality factor (Q). Permittivity is calculated from the changes in these properties due to the presence of the sample. The sample is placed in the center of a waveguide that has been made into a cavity. The sample volume must be precisely known. The technique has good resolution for low loss materials and small samples. This technique uses a single frequency and is commonly used for measuring the dielectric properties of homogeneous food materials, since it is simple, accurate, and capable of operating at high temperatures [4,23–25].

2.2.3.1.4 Transmission Line

This method uses a waveguide or coaxial transmission line with a VNA. Free space is sometimes considered a transmission line technique. In this case at least two antennas are needed.

In waveguide and coaxial transmission line techniques, the most important factor is sample preparation. Rectangular samples and annular samples have to be prepared for waveguide and coaxial lines, respectively. ϵ' and ϵ'' can be determined by measuring the phase and amplitude of MW signals reflected from or transmitted through the target material. This method is useful for hard, machineable solids and requires a precise sample shape; it is therefore a destructive method.

2.2.3.1.5 Free-Space Technique

The free-space technique requires a large flat, thin, parallel-faced sample and special calibration considerations [22]. It does not need special sample preparation and presents certain advantages due to the fact that it is a nondestructive and noncontact measuring method, and can be implemented in industrial applications for online process control. This technique can also be used at high temperatures. The sample is placed in front of one or between two or more antennas. In the first case, the same antenna transmits and receives the signal. In the second case, there are two antennas, a transmitter and a receiver. The attenuation and phase shift of the signal are measured and the data are processed in a computer to obtain the dielectric properties. The usual assumption made for this technique is that a uniform plane wave is normally incident on the flat surface of a homogeneous material, and the sample size must be sufficiently large to neglect the diffraction effects caused by the edges of the sample [26]. This technique is useful for a broad frequency range, from the low MW region to mm-wave.

Tomography images can be made by using an antenna system and a reconstruction algorithm. Pixel definition is a function of the wavelength inside the food. Other techniques have been developed for medical applications, such as confocal microwave imaging (CMI), which focuses back-scattered signals to create images that indicate regions of significant scattering [27].

2.2.3.1.6 Time Domain Reflectometry Technique

Time domain reflectometry (also known as spectroscopy) methods were developed in the 1980s. This technique measures the complex permittivity of dielectric materials over a wide frequency range, from 10 MHz to 10 GHz. It is a rapid, accurate, and nondestructive method. It utilizes the reflection characteristic of the sample to obtain the dielectric properties. It must be emphasized that the sample size must be small and the material must be homogeneous.

2.2.3.1.7 Microstrip Transmission Line Technique

The effective permittivity, represented by a combination of the substrate permittivity and the permittivity of the material above the line, of a microstrip transmission line is strongly dependent on the permittivity of the region above the line. This effect has been utilized in implementing MW circuits and to a lesser extent on the investigation of dielectric permittivity (Figure 2.10).

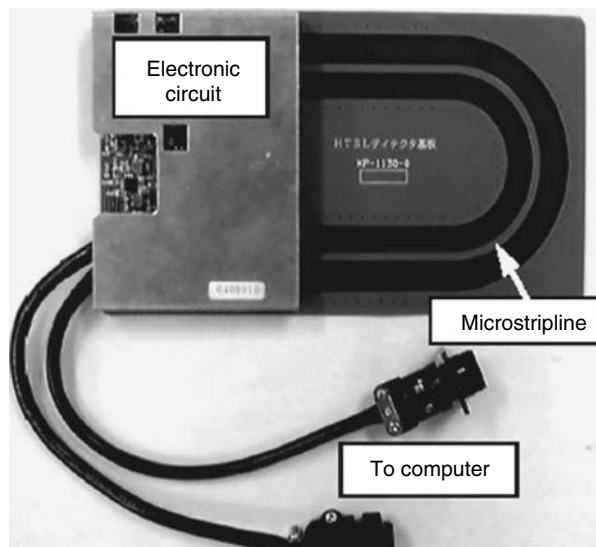


Figure 2.10 Microstrip transmission line.

2.2.3.2 Quality Control in Meat Products by Microwave Dielectric Spectroscopy

The food industry needs online methods to control the products and optimize processing; electromagnetic sensors, particularly MW sensors, offer a noninvasive solution. However, some foods are complex matrices with heterogeneous composition, and the determination of quality parameters using MW sensors is still a complex topic.

The permittivity and conductivity parameters are the properties which determine the propagation of an electromagnetic wave in biological tissue. Gabriel et al. [7–9] described these parameters in detail. It is important to point out that the limitation of most dielectric probes is the volume of the sample that interacts with the field. The volume has to be representative of the whole piece of meat, due to the fact that the electromagnetic parameters in this kind of tissue vary in a heterogeneous way. The dielectric properties of structured tissues such as meat products have been studied by many authors and the most important applications are presented in this section.

An important application of MWs in foods is the analysis of fish and meat freshness. After death, muscle is unable to obtain energy by the respiratory system; glycolysis contributes to energy generation by means of glycogen conversion to lactate. Glycolysis lowers the pH, bringing it closer to the isoelectric point of proteins and thus contributes to a decrease in water-holding capacity [28]. The level of glycogen stored in the animal at the time of slaughter affects the texture of the future marketed meat. For all these reasons, the dielectric properties are expected to change during *rigor mortis* [19].

A MW sensor for food structure evaluation based on a polarimetric MW method is being developed at Clermont-Ferrand INRA Centre (French National Institute for Agricultural Research). Their objective is to discriminate between fresh and frozen/thawed fish fillets and to monitor meat aging. Both applications are based on the reduction of muscle anisotropy of the tissues during processing; this reduction produces a change in the dielectric properties [29].



Figure 2.11 Distell meat fat meter. (From Distell Company, West Lothian, Scotland, <http://www.distell.com>, 2008.)

Another important application is the detection of added water, a classic fraud in the food industry. This application has been widely studied in fish, fish products, and meat using MW dielectric spectra [30–32].

It has been reported that it is possible to predict the fat composition in fish or minced meat using electromagnetic measurements [33–35]. The fat content in these foods is clearly related to the water content of the product, so that if one is known the other can be determined. A MW instrument that mainly consists of a microstripline is currently being marketed (Distell Company®, West Lothian, Scotland). This compact and nondestructive meter can measure fat fish [33], meat [34], and fish freshness [35] (Figure 2.11).

A number of recent studies have tried to relate changes in the dielectric properties (i.e., ϵ' and ϵ'') of meat to the denaturation status of its constituent structural proteins. These properties are composition dependent [36–37] and are influenced by water (free versus bound) and ionic (free versus bound) content of the food, among other factors [38]. The work of Tornberg et al. [39] and Hills et al. [40] has led to a better understanding of water binding in meat, and it is now usually sufficient to consider three states of water, namely “structural and bound water” (i.e., water hydrogen bonded inside the grooves and cavities of globular proteins), surface water (i.e., hydration water of the macromolecule which extends only one or two molecular layers from the surface of the biopolymer), and bulk water (i.e., the rest of the water). Li and Barringer [41] monitored changes in the ϵ'' of high-salt ham samples at MW frequencies and concluded that changes in ϵ'' corresponded to the denaturation temperature of actomyosin. In addition, Bircan and Barringer [42] monitored ϵ' and ϵ'' (at MW frequencies) in meat, fish, and poultry samples within the temperature range of 20–120°C and found that both ϵ' and ϵ'' increased at a temperature which appeared to match the differential scanning calorimetry (DSC) denaturation temperature of collagen in these foodstuffs.

Zhang et al. [43] recently published a study about the dielectric properties of two sample meat batters in the temperature range of 5–85°C at both radio and MW frequencies. However, measurements were only taken at intervals of 20°C and a number of nonmeat ingredients were present in the meat product. Thus, the effect of protein denaturation on dielectric properties may

Table 2.1 Composition and Dielectric Properties (27.12 MHz) of Some Kinds of Meats

<i>Species (Anatomical Location)</i>	<i>Type</i>	<i>Moisture (%)</i>	<i>Protein (%)</i>	<i>Fat (%)</i>	<i>Ash (%)</i>	<i>Salt (%)</i>	ϵ'	ϵ''
Beef (forequarter trimmings)	Lean	71.5	21.3	6.1	0.83	0.11	70.5	418.7
Lamb (leg)	Lean	73.0	21.9	3.6	1.48	0.14	77.9	387.2
Chicken (breast)	Lean	73.6	24.3	1.2	0.86	0.13	75.0	480.8
Turkey (breast)	Lean	74.5	24.1	0.4	0.98	0.08	73.5	458.4
Pork (back)	Fat	19.0	3.9	76.1	0.20	0.07	12.5	13.1

Source: Adapted from Lyng, J.G., Zhang, L., and Bruton, N.P., *Meat Sci.*, 69, 589, 2005.

be masked by nonmeat ingredients and missed because of the large temperature measurement interval. Therefore, Brunton et al. [44] monitored the changes in the dielectric properties of whole meat across a temperature range of 5–85°C, but at 1°C intervals, to determine if changes in the dielectric properties when measured at a finer temperature resolution could be related to changes in the denaturation state of the constituent structural proteins as monitored by DSC. In addition, changes in the rheological properties and juice loss of the whole beef muscle were measured as a function of temperature in an attempt to correlate changes in these properties with protein denaturation. A complete study of the dielectric properties of meats and ingredients used in meat products at MW and radio frequencies was also recently reported [45]. Some of the results given in this work are shown in Table 2.1.

Studies by Miura et al. [46] concluded that spectra analysis is a very useful tool for quality control of foodstuffs. Specifically, the authors studied the differences between raw, frozen, and boiled chicken at 25°C. They also studied the dielectric spectra of fish, vegetables, eggs, dairy products, and beverages.

The dielectric properties of turkey meat were measured at 915 and 2450 MHz [47]. The authors developed a number of equations to correlate the real and imaginary part of permittivity with temperature, moisture, water activity (a_w), and ash. Other equations were developed to model the dielectric properties of ham as a function of temperature and composition [48].

Clerjon et al. [49] tried to assess the feasibility of an online MW sensor for a_w measurement. Dielectric spectroscopy gives information on a molecule's chemical relations with its surroundings, whereas a_w is the thermodynamic measure linked with water bonding to the food matrix. The authors analyzed 45 model samples of animal gelatin gel of various water and NaCl contents (a_w from 0.91 to 1); the samples were characterized in terms of a_w and dielectric properties. This investigation provided correlations between a_w and some dielectric properties, such as relaxation frequency.

A Guided Microwave Spectrometer® (Thermo Electron Corporation, United States) has been developed for online measurement of multiphase products (Figure 2.12). This guide is used to measure moisture in raw materials such as corn, rice, and soybeans and in processed materials such as tomato paste and ground meat. It can also measure Brix, pH, and viscosity; acid in orange juice, soft drinks, mayonnaise, and tomato products; fat in ground meats, peanut butter, milk, and other dairy products; salt in mashed potatoes and most vegetable products; and, last, alcohol in beverages.

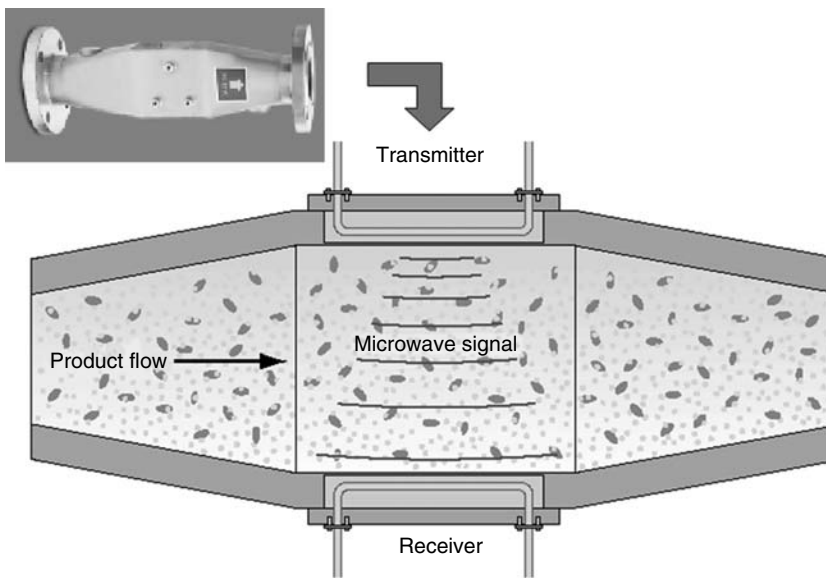


Figure 2.12 Guided microwave spectrometer and its operation scheme. (From Thermo Electron Corporation, United States, http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF_4001.pdf, 2008.)

2.2.3.3 Quality Control in Meat Products by Electrical Impedance Spectroscopy

In biological tissues such as meat, the impedance depends mainly on its structure and on ionic conductivity. These two variables change with meat aging. The high complexity of meat structure produces a strong electrical anisotropy [50]. This anisotropy was explained by the presence of muscle fibers filled with an electrolyte solution and surrounded by a selective membrane. Damez et al. [51] have studied the electrical anisotropy behavior of beef meat during maturation for the purpose of early assessment of meat aging.

Muscle is composed by muscle fibers surrounded by connective tissues. These various muscle components have different electrical and dielectric properties: The permittivity of connective tissue is very close to that measured in tendon [52]. Electrical properties depend on the physical and chemical parameters that determine the concentration and mobility of ions within the metabolic fluids. Electrically, meat can be simply represented by an array of highly elongated conducting cells isolated from each other by membranes [51]. Extracellular fluids and intracellular fluids can be described as electrolyte solutions. In muscles, Na^+ and Cl^- ions largely predominate in extracellular fluid, whereas in intracellular fluid, K^+ is the major cation, and phosphate and proteins are the major intracellular anions [53].

Both ionic force and osmotic pressure increase between death and *rigor mortis*. It is estimated that between 60 and 80% of the increase in osmotic pressure is driven by metabolites, and the rest by free inorganic ions not present in the cytoplasm before *rigor mortis* [54–56]. These ions, which are concentrated in organelles such as the sarcoplasmic reticulum and mitochondria, are released during membrane depolarization after the death of the animal [57]. Feidt and Brun-Bellut [58] showed that the release of Na^+ , K^+ , and Cl^- ions over time was not only pH-dependent but was

also directly affected by cell death, in particular the rupture of membranes. In addition, Mg^{2+} and Ca^{2+} are linked to proteins: Even when released from the sarcoplasmic reticulum after exhaustion of the adenosine triphosphate (ATP) and inactivation of membrane pumps, these two ions can still bind to proteins with which they have a strong affinity. The final quantities of free Mg^{2+} and Ca^{2+} thus appear to be mainly conditioned by pH. When the pH approaches the isoelectric point (pI) of myofibrillar proteins (i.e., pH 5.4), the protein's charges tend to be canceled and their capacity to adsorb cations decreases. A lower pH leads to further release of the two ions.

The use of electrical measurement in meat goes back to the 1930s with some pioneering works [50,59] that were the first to describe the basic electrical properties of meat. In the decade 1960–1970, much work was carried out in the medical field on the electric properties of biological tissues (skeletal muscles, cardiac muscle, skin, bone, etc.) [60–62]. These studies were designed to evaluate the structural and physiological integrity of these tissues.

2.2.3.3.1 Evaluation of pH

After the first works of Callow [59], most of the work on electrical impedance of meat published since the 1970s concerns the use of this variable to monitor the fall in pH or to evaluate ultimate pH, mainly in pork [63] but also in beef [64].

One of the most important quality problems in the pork industry is the production of pale, soft, and exudative (PSE) meats, which are characterized by pale color, soft texture, and a low water-holding capacity. This meat is related to fast changes in the pH, cellular breakdown, and an increment of the extracellular liquid; therefore, the behavior of PSE meat when subjected to an electric field will be different from that of the normal meat, at least at the beginning of the postmortem process.

In beef and pork, one quality problem is dark, firm, dry (DFD) meats with high pH and high susceptibility to microbiological problems. DFD meat is associated with membrane modifications and changes in the extracellular medium. Therefore, it will also affect the meat's electrical properties. Recent results show that electrical measurements do not permit early detection of DFD [65–68]. The difficulty of detecting PSE meats during the development of *rigor mortis* arises because during this time parameters such as pH and temperature are rapidly evolving and metabolic modifications sequentially affect structure and therefore electrical properties [69]. Impedance (conductivity) shows better ability to detect PSE meats once the final pH has been reached [67]. Some studies have been done by various authors at low frequencies using a conductimeter. PSE meat presented higher conductivity values at 45 and 60 min postmortem than normal meat, but due to the high values of standard deviation, it was not possible to reach any conclusions [70,71]. On the other hand, a relationship was found between impedance and water-holding capacity [72].

2.2.3.3.2 Evaluation of Fat Content

Since the 1980s, many studies have been made on the use of electrical properties to estimate fat content in animal carcasses or muscles. Fat is a good electrical insulator and plays an important role in meat tissues impedance. Some studies of body composition have been made on pork [73] and on beef and pork [74]. Slinger and Marchello [75] measured the electrical conductivity of bovine carcasses immediately after slaughter. The authors obtained a fat content estimation with an excellent accuracy ($R^2 = 0.95$). This was possible because just after slaughter there is no modification of membranes or extracellular compartments, and measurements were made at a stable temperature.

Intramuscular fat (IMF) plays an important role in the sensory quality of meat. Some authors [76,77] consider an IMF content of 2–3% in the *musculus longissimus dorsi* as optimal for the taste of pork. The established methods for postmortem ascertainment of IMF are extraction methods or near-infrared spectroscopy in a meat sample. Chemical fat extraction, however, is time consuming, and the sampling of meat compromises the trading value of the carcass. Methods enabling fast detection of IMF without an impairment of the carcass are necessary. The use of ultrasound imaging techniques seems promising. Prediction of muscle composition is also possible using electrical properties. Madsen et al. [78] described a specially adapted impedance measurement system (6 electrodes, 10 frequencies between 50 Hz and 50 kHz) for the prediction of IMF in beef. This patented portable apparatus (U.S. Patent 6265882) uses electrodes inserted in the muscle to estimate IMF in carcasses with measurements at several frequencies.

Altmann and Pliquet [79] presented a device to measure the impedance repeatedly during the passage of the probe through the muscle. The authors examined different times postmortem and various directions of probe insertion. A standardized method based on their results was used to predict IMF in pork and beef.

2.2.3.3.3 Evaluation of Tenderness

Meat-tenderizing biochemical and physicochemical processes occur during aging. These processes include the action of endogenous proteases on the structure of muscle fibers, a progressive increase of membrane water permeability, and the weakening of connective tissues.

Faure et al. [80] set out to evaluate the state of maturation by quantifying these effects. They proposed an approach based on the ratio of impedance at low frequency to that at high frequency. This impedance ratio decreases during refrigerated storage, but Lepetit et al. [81] showed that variation in its absolute value from one animal to another could be explained by variations in ion or fat content. Also, this impedance ratio cannot reliably indicate the state of maturation or destructuring of meat. Similar work reports on the ratio of capacity (the dielectric parameter reflecting the insulating state of the membranes) to electrical resistance [82]. In this case, the measured parameters are also affected by the adiposity of tissues.

Muscle is electrically anisotropic, meaning that muscle and thus meat exhibit changes in electrical properties according to the direction of the electrical fields in the sample. After *rigor mortis*, the electrical impedance of meat decreases linearly with the mechanical resistance of muscle fibers, and electrical anisotropy is a better predictor of muscle fiber strength than impedance alone [81]. A complementary approach has been presented by Byrne et al. [64], relating the electrical properties of muscle after cooking to the tenderness assessed by Warner–Bratzler shear force (WBSF). Their results showed there was no direct relationship between meat tenderness and simple electrical measurements.

The rate of aging in beef varies tremendously from one animal to another. The strength of muscle fibers can reach its minimum value within a few days, whereas it can take more than 2 weeks for the same muscle in another animal. It has been shown [83] that it is possible to select meats that age rapidly if the state of aging is known at 48 h postmortem. This will avoid long storage periods for already aged meats. The expected benefits in storage costs are about 50%. In this study the state of aging was measured with a destructive mechanical method, but this information could be obtained from a nondestructive sensor. A sensor using electrical impedance anisotropy was devised by Damez et al. [84] and has been patented [85].

2.2.4 Quality Control in Meat Products by Near-Infrared Spectroscopy

Near-infrared radiation (NIR) is a part of the electromagnetic spectrum radiation over the wavelength of visible light and below the MWs between 780 and 2500 nm [86]. Rapid screening techniques to determine quality characteristics of meat are of great interest to the industry [87]. In this respect, NIR is considered as one of the most important techniques in fast and nondestructive methods for online control of meat quality and safety [1]. NIR technology has been used to analyze and control some chemical, physical, and other properties of pork [65,87,88–90], beef [91–94], lamb [94], oxen [95,96], poultry [97,98], turkey [99], kangaroo [100], and treated meat [97,101].

When electromagnetic energy interacts with a sample at NI frequencies, some of this radiation is absorbed by the covalent bonds, and this absorption produces mechanical vibration of the molecules. The water molecule is one of the best-known examples of NIR absorbed by a vibrating molecule. The frequency of oscillation of any mode is dependent on the atomic masses and bond strengths of the –OH group. Mechanical vibration promotes molecular movement and produces friction and collision dissipation [102]. The types of absorption that dominate the NIR are hydrogenic absorptions such as –OH, –NH, C=O, and –CH vibrations [103]. Therefore, NIR techniques are useful to identify molecules by analysis of their constituent bonds and to determine some physical properties by the structural conformation of these molecules.

The NIR spectrum has coupled the absorption effect from the different molecular groups; therefore, multivariate data analysis is indispensable for the analysis of NI spectra. The strong overlap among the absorption waves, caused by overtones and combination bands of stretching and bending vibrations, hinders the interpretation of NIR spectra. Figure 2.13 shows an example of an NIR spectrum. It can be clearly observed as a result of overtones and combination bands that a major overlap exists among the peaks of the separate components. Multivariate analysis techniques are needed to filter the required information out from the spectrum.

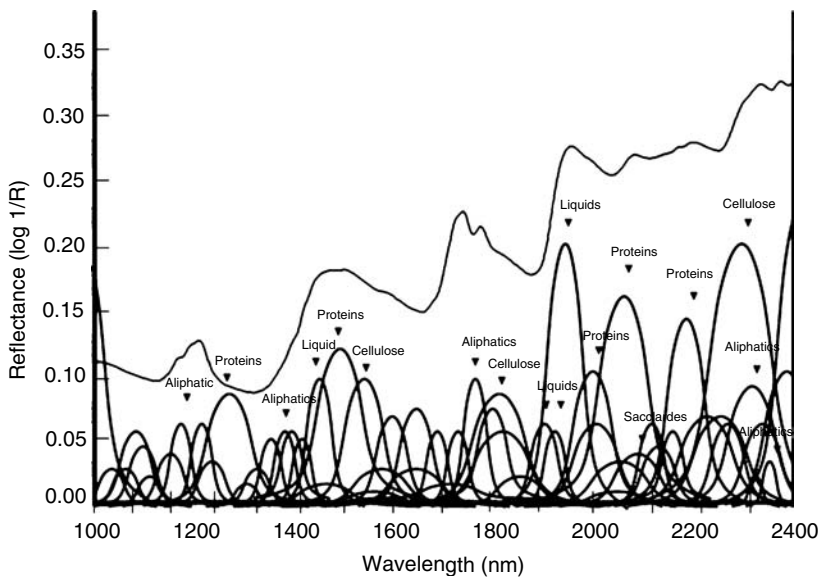


Figure 2.13 An example of NIR spectrum.

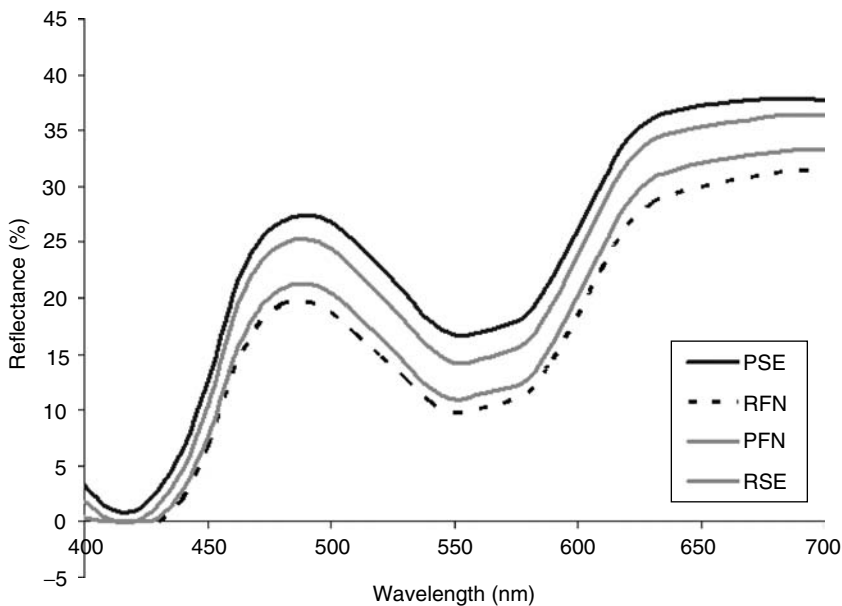


Figure 2.14 Visible spectrum of different samples of pork meat.

In meat analysis, NIR technology has been used to determine quality traits such as fat, water, protein, collagen content, and other characteristics [104–106]. In fat prediction, the content of oleic, linoleic, palmitic, and stearic acids in Iberian pork [107], and the determination of different types of fats in raw beef (C_{14} – C_{18}) [91] have been reported. A method for the instantaneous classification of Iberian pork as a function of the animals' feeding regimen was based on the online analysis of polyunsaturated, monounsaturated, and saturated fatty acids in subcutaneous fat [108,109]. Other studies have been developed to determine the meat fat content and composition in pork fat [110], pork loin [90], and ground beef [91,111].

Some physicochemical and structural properties have been analyzed by NIR in fresh meat, such as water-holding capacity, pH, drip loss [89,94,106,112–115], textural properties, and sensorial tenderness in beef [115,116–118] and pork [114]; pigment content and color, expanding the NIR range to the visible range [88,93,117]. The next step in this research has been the application of predictions to the control of meat processing [94,114,119,120].

More complex prediction related to the quality and safety of fresh meat is used to distinguish online between pale, firm, and nonexudative (PFN) meat and PSE meat [121] (Figure 2.14).

Finally, NIR technology is also useful in predicting and controlling some meat treatments, such as the manufacturing of beef sausages [97], the effect of grinding on the color and chemical composition of pork sausages [101,122], textural and color changes in dry and cured ham [113], freeze-drying meat control [123], dry-cured pork meat control [124], and control of dehydration and heating through the cooking of beef meat [125].

2.2.5 Ultrasound

Ultrasound is energy generated by sound waves of frequencies ≥ 20 kHz. In practice, the frequency used in ultrasonic techniques varies from dozens of kilohertz to dozens of megahertz. Ultrasound

requires an elastic medium, and the wave propagation velocity depends on the medium, which is entirely in contrast to electromagnetic waves (e.g., NI), which require no medium and have almost constant velocity (speed of light). In the pulse-echo technique, a pulse of an appropriate frequency, duration, and amplitude is applied to a sample through a transducer. It propagates through the sample until it reaches a change of material, at which point it is partly reflected and partly transmitted; the echoes returned to the ultrasound transducer are measured as a function of time [126]. Ultrasound propagation (velocity and amplitude) is influenced by the characteristics of the medium [127]. For this reason, this attribute is used for material characterization and for food quality determination.

Ultrasound is a spectroscopic method which could be used for noninvasive, online measurement of meat quality under humid conditions. For instance, ultrasound equipment for online measurements of fat and lean meat content was developed [128]. Ultrasound could be used as a rapid technique for the characterization and classification of pig back fat from different origins in a nondestructive way [129]. Low-intensity ultrasound has been widely used to determine the physicochemical properties of foods [130]. Ultrasonic velocity measurements have been used to estimate the chemical composition of meat products [131,132]. Ultrasound has also been used to estimate the moisture content and textural characteristics of cured meat products [133]. In raw meat, the speed of sound measurements was used in the prediction of the IMF content of the *longissimus* muscle in beef [134]. These measurements were used to assess the percentage of lean meat in bull carcasses [135]. Several authors have used real time ultrasound to predict fat-free meat in swine [136,137] and to estimate the IMF content in the *longissimus* muscle in pig carcasses [138]. Real-time ultrasound has been used in the prediction of carcass yields of Iberian pigs fed on different feeding regimes (“montanera” or “cebo”) [139]. In other studies, ultrasound was used to predict pig carcass quality [140], to estimate beef carcass composition [141,142], and to study beef sensory attributes [143]. It was also used to evaluate the distribution of IMF in live beef animals [144] and in *longissimus dorsi* muscle of pigs [138].

2.3 Conclusions

Dielectric spectra can be used to describe physicochemical aspects, components interactions, and structural changes in foods. Owing to this fact, accurate measurements of these properties can provide scientists and engineers with valuable information for monitoring manufacture processes to improve food quality control. In this context, dielectric properties measurements, especially the dielectric spectra in a wide range of frequencies, appear to be a useful method to process quality control and to optimize some processes in the food industry. Measurements are set up in minutes and made in seconds, providing real time data. These measurements are nondestructive and very fast, and some of these techniques can even be noncontacting. These characteristics have made dielectric properties measurement even more attractive for the online control of food processes. Moreover, this quality control system can be considered a clean technology because it does not generate any residues or water consumption. More efforts must be made in this field to transfer these novel techniques to the food industry and to develop new methods to assess meat quality objectively.

In conclusion, online food evaluation is necessary to improve competitiveness and to respond to consumer quality demands. These novel techniques are still being researched, as they constitute important future trends for food evaluation.

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Chapter 3

Moisture and Water Activity

Young W. Park

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3.1 Introduction

Moisture content is one of the most important and widely used indices in processing and testing foods [1]. The terms “water content” and “moisture content” have been used interchangeably in literature to designate the amount of water present in foodstuffs and other substances [2,3]. Because dry matter content in food is inversely related to its moisture content, moisture content has great economic importance to the food processor and consumer. In meat, water or moisture is quantitatively the most important component of the product, constituting up to 75% by weight.

The amount of moisture is a measure of yield and quantity of food solids, and can be a direct index of economic value, stability, and quality of food products [1,2]. The abundance and chemical reactivity of moisture, and the determination of its quantity, are of great concern to many industries such as food, paper, and plastics, where acceptable levels of moisture vary between materials and in some cases, very small quantities of moisture can adversely affect the quality of product [4].

The amount of water in food is also directly related to its water-holding capacity (WHC) as well as water-binding capacity. WHC is an important quality parameter for the economic value of meat. It is the ability of meat to retain the tissue water present in its structure [5]. Since meat is sold by weight, drip is unsightly to the consumer, and excessive drip is a negative determinant of meat quality. WHC is also important with respect to the manufacturing properties of the meat together with water-binding capacity, which is the ability of meat to bind added water [5]. The desirability of meat with low water-holding and water-binding capacity is dependent on the purposes of both retail consumption and manufacturing.

Although the determination of moisture content in foods is highly important, the accurate analysis of moisture is frequently one of the most difficult tasks encountered by the food chemist. This is largely attributable to the difficulty of complete separation of all the water from a food sample without causing simultaneous decomposition of the product [2]. The production of water by decomposition and loss in weight would affect the accuracy of the determination [2,6]. The loss of volatile constituents from the food is another difficulty involved in moisture determination. The complexity of moisture assay will be dependent on the conditions of the food and the nature of other substances present [6,7].

Accurate, rapid, and simple methods of moisture assay applicable to all types of foods are continuously sought, although it may be doubtful that such a goal will ever be achieved [5]. However, an ideal method for moisture assay has been suggested [8]. The requirements are (a) to be rapid, (b) to be applicable to the broadest range of materials, (c) to be performable preferably even by nontechnical persons with brief training, (d) to use a readily available apparatus of low initial investment and low cost per test, (e) to have reasonable accuracy and good precision, and (f) to present no operational hazards. Analytical methods of moisture determination are usually selected for either rapidity or accuracy, even if both goals are simultaneously sought, especially in industry applications.

3.2 Properties of Water in a Food System

Water is a ubiquitous substance in nature and is unusually reactive due to its high polarity [9]. Physically, water can be present in three different forms as gas, liquid, and solid state. It exists in the gaseous state as monomolecular water vapor, in liquid state largely as dihydrol, in which two molecules of water are bound by hydrogen bond forces, and in several solid forms as ice varying in degree of association [10]. However, for moisture analysis, it is generally more important to recognize the different types of interactions of water within a food rather than the physical state of water [11].

Historically, water in a foodstuff exists in two forms, known as “free” and “bound” [6]. However, water can be classified in at least three forms [2,11–14]. The first form of water in foods exists as free water in the intergranular spaces and within the pores of the material. Such water serves as a dispersing medium for hydrophilic macromolecules such as proteins, gums, and phenolics to form molecular or colloidal solutions, and as a solvent for the crystalline compounds. The second form of water is adsorbed as a very thin, mono- or polymolecular layer on the internal or external surfaces of the solid components (i.e., starches, pectins, cellulose, and proteins) by molecular forces or capillary condensation. This water is closely associated with absorbing macromolecules by van der Waals forces or hydrogen bond formation. The third form of water is in chemical combination as water of hydration, so-called “bound water.” Carbohydrates such as dextrose, maltose, and lactose form stable monohydrates, and salts such as potassium tartrate also form hydrates. Water of hydration can be clearly observed from gels of proteins or polysaccharides in which the bound water is firmly held by hydrogen bonds [2,13–14].

Because of a variety of definitions, the concept of bound water is quite controversial, and terminating its use as a term has been suggested [3,11,15–17]. One alternative classification scheme involves three broad types of water—free, adsorbed, and chemically bonded [1,14,18]. This is why the state of water in colloidal systems and the nature of bound water are still not clear. It has been suggested that water found in biological material may exist as (a) occluded water, (b) capillary water, (c) osmotic water, (d) colloidal water bound by physical forces, and (e) chemically bound water [19].

Bound water has been defined by the majority of researchers as the form of water that remains unchanged when the food is subjected to a particular heat treatment [2]. A certain proportion of the total water present in the biocolloids, for example, is not separated readily by freezing (even at -230°C) or drying [2]. At -125°C , all the free water is usually frozen, and the remaining bound water is not frozen at considerably lower temperature [14,19]. Such bound-water concentration varies from one food to another. The ultimate accuracy of an analytical method for moisture determination is related to the bound water and not the free water. Part of the water in a sample during drying is retained for longer times at higher temperatures than the remainder. The range of bound water in foods is less than 0.5 to over 30% of the total water present, corresponding to 0.1–2.2 g/g total solids [14,19]. All water may be considered as bound water to a variable extent, except for surface water. Monolayer values are most commonly calculated using the Brunauer–Emmett–Teller (BET) or Guggenheim–Anderson–de Boer (GAB) equations for modeling moisture sorption isotherm data [20]. The most tightly bound water is the BET monolayer water [6]. For most foods, the BET monolayer values range from a few percent to approximately 12% (wet basis) of the food or food component [21].

Small changes in water content can exert a large influence on storage stability of low-moisture foods. Irreversible changes in texture of foods also occur during freezing and freezing storage. Thus, removal of free water rather than bound water from dried foods has been known to improve storage stability [22]. Likewise, decrease in free-water content of foods to be preserved by freezing, concentration, partial dehydration, or addition of sugar is believed to improve the storage stability of frozen foods and food products [14].

Researchers have shown that water displays abnormally high values of certain physical constants, including specific heat, specific gravity, heat of infusion, heat of evaporation, surface tension, and viscosity. These special constants may be derived from its remarkable and variable solvent power, high dielectric constant, dissolving and ionization ability, and its own molecular aggregation tendency [23]. The characteristics of water in chemical reactivity, volatility, solvent power, electrical properties (high dielectric constant, conductivity, and magnetic resonance absorption), thermal conductivity, and light scattering and absorption have been used in the determination of moisture content in foods and other materials [2,13,23].

3.3 Water in Meat and Other Foods

Water in meat is associated with muscle tissue, and proteins have a central role in the mechanism of water binding. The water content of meat is inversely related to fat content, whereas it is unaffected by protein content, except in young animals [5]. Muscle proteins impart a gel structure to the tissue in the living animal, and very little loss of water occurs from tissue cut immediately after slaughter. This is attributed to the water molecule behaving as a dipole and binding strongly to surfaces by a number of noncovalent forces [5].

Previously, up to 60% of water was thought to be bound by the myofibrils, but this figure was an overestimate, and about 10% would be more realistic. Approximately 85% of water is bound between the thick and thin myofibrils [5]. Because this binding is looser than in the living animal, some loss as drip from freshly cut surfaces is inevitable. The amount of drip loss is mainly a function of postmortem changes, which affect the pH of the meat and the changes in myofibrillar volume [5].

Drastic changes in WHC and tenderness in meat occur during heating of the meat products, including shrinkage and hardening of tissue and the release of juice, which are caused by changes in the meat proteins. This considerable decrease of WHC during heating is attributable to a tightening of the myofibrillar network by heat denaturation of the proteins [24]. Changes in WHC during heating are closely related to alterations in the tenderness and rigidity of tissue, and most decrease in WHC occurs at temperature between 30 and 50°C. The sarcomere length of the muscle fibers of raw meat is closely connected with the tenderness of cooked meat [24].

There are wide variations in moisture content of natural and processed foods of animal and plant origins (Table 3.1) [17]. First, with respect to the moisture content of meat and fish products, their moisture content depends primarily on the fat content and varies to a lesser degree with the age, source, and growth season of the animal. The range of moisture content in meat and fish is from 50 to 70%, whereas some organs may contain up to 80% water. Sausages have wide variations of water content. Poultry meats contain from 50% in geese to 75% in chicken, whereas fresh and dried eggs have approximately 74 and 5% water, respectively [1].

3.4 Water Activity

Although it is not perfect, a relationship exists between the water content of food and its perishability. Dehydration is performed for the purpose of reducing the water content of a food, which in turn increases the concentration of solutes and decreases the perishability of the food. However, various types of food with the same level of water content exhibit significant differences in stability and perishability [17].

Table 3.1 Water Contents of Various Foods

<i>Food</i>	<i>Water Content (%)</i>
Meat	
Pork, raw, composite of lean cuts	53–60
Beef, raw, retail cuts	50–70
Chicken, all classes, raw meat without skin	74
Fish, muscle proteins	65–81
Fruit	
Berries, cherries, pears	80–85
Apples, peaches, oranges, grapefruit	90–90
Rhubarb, strawberries, tomatoes	90–95
Vegetables	
Avocado, bananas, peas (green)	74–80
Beets, broccoli, carrots, potatoes	85–90
Asparagus, beans (green), cabbage, cauliflower, lettuce	90–95

Source: Fennema, O.R. in *Food Chemistry*, Marcel Dekker, New York, 1996, 17–94.

Water content alone is not a reliable indicator of perishability of food products, which is partially due to the differences in intensity of association of water with nonaqueous constituents. The term “water activity” (a_w) was developed to indicate the intensity with which water associates with various nonaqueous constituents [17]. Water engaged in strong association has less ability to support degradative activities than that in weaker association. The degradative activities of water include the growth of microorganisms and hydrolytic chemical reactions. a_w can predict food stability, safety, and other properties more reliably than water content can. Although a_w is not perfect, it correlates sufficiently well with rates of microbial growth and many degradative reactions as to be a useful indicator of product stability and microbial safety [17].

a_w can be defined as $a_w = f/f_0 = p/p_0$, where f is the fugacity of the solvent (fugacity means the escaping tendency of a solvent from solution), and f_0 is the fugacity of the pure solvent. p/p_0 is the term measured, and sometimes does not equal a_w [17]. Water activity is a property of solutions, and is the ratio of vapor pressure of the solution to the vapor pressure of pure water at the same temperature [25]. Water activity is related to relative humidity, and under equilibrium conditions, water activity equals RH/100. Quantitatively, a_w is a measure of unbound, free water in a system available to support biological and chemical reactions. a_w , not absolute water content, is what bacteria, enzymes, and chemical reactants encounter, affecting food materials at the microenvironmental level.

The effect of a_w on microorganism growth is very important in intermediate-moisture foods. At the usual temperatures permitting microbial growth, most bacteria require a water activity in the range of 0.9–1.00. The minimum a_w below which most important food bacteria will not grow is about 0.90, depending on the specific bacteria [25]. Some halophilic bacteria may grow at an a_w of 0.75, and certain osmophilic yeasts can grow even at lower a_w , but these microorganisms seldom cause food spoilage. Compared to most bacteria, molds are more resistant to dryness. Molds can grow well on foods having an a_w of about 0.80, and can show slow growth at room temperature

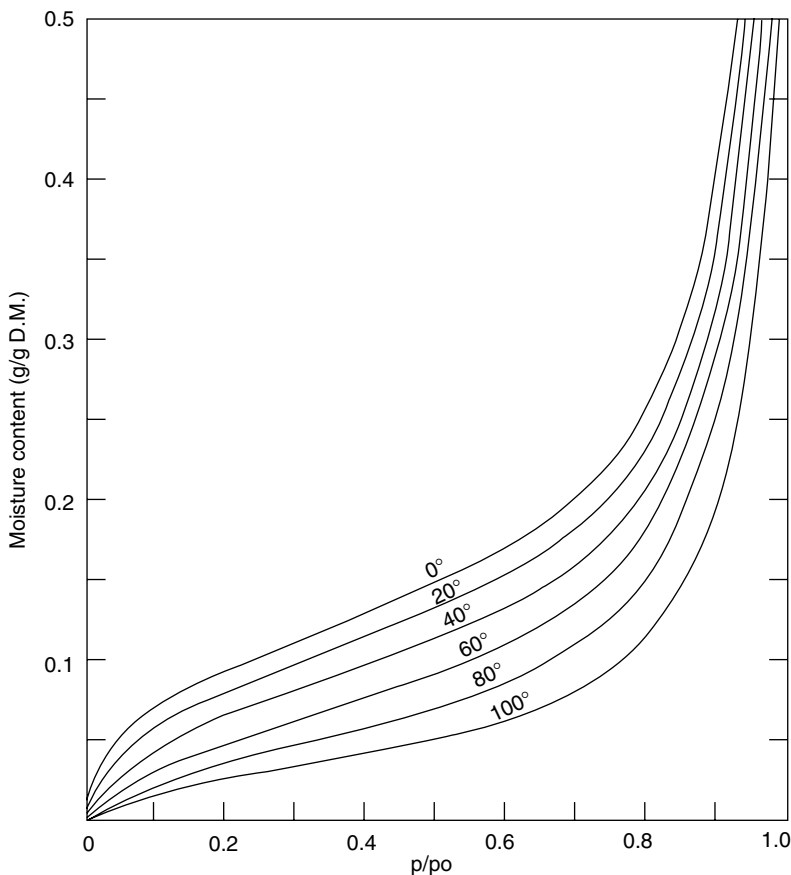


Figure 3.1 Moisture desorption isotherms for potatoes at various temperatures. (Görling, P., in *Fundamental Aspects of the Dehydration of Foodstuffs*, Society of Chemical Industry, London, 1958.)

for several months on some foods with an a_w as low as 0.70. Mold growth is completely inhibited at a_w below 0.65. However, such low a_w generally is not applicable in the fabrication of intermediate-moisture foods, many of which have below 20% moisture [25].

The humidity at which the product neither loses nor gains moisture is the equilibrium relative humidity (ERH) [2,25]. Diagrammatic plots of such data yield water sorption isotherms as shown in Figure 3.1 [17,26]. The ERH differ between foods. Below the atmospheric humidity level, food can be dried further, whereas above this humidity, it may pick up moisture from the atmosphere [2]. The ERH at different temperature can be measured by exposing the dried food sample to different levels of humidity in bell jars and weighing the sample after several hours of exposure [25]. Figure 3.1 illustrates that the product comes into equilibrium at 4% moisture at 100°C and 40% RH. Similarly, if the food product further dries to 2% moisture, the equilibrium is attained at 15% RH and 100°C [2,25]. Similar water sorption isotherms have been established for a wide variety of food products.

3.5 Mechanism of Drying Related to Moisture Determination

Removal of moisture from a meat sample is a critical aspect of the moisture determination. The rate of drying affects the quantification of moisture in the food. It is desirable to understand the mechanism of drying or moisture removal in the moisture determination of a sample. During the drying process, moisture loss from the sample occurs in two distinct stages or periods, which is detailed in the following.

In the initial stage, the drying rate remains constant, and equals that of evaporation from a free liquid surface. This stage of drying is controlled by surface evaporation, which is known as the constant-rate drying period [11,25,27,28]. This phase of constant rate continues as long as water reaches the surface of the material as fast as evaporation takes place [2,11,25,27]. After this stage, there is a sudden drop in the drying rate at the end of the constant-rate period, where the drying rate decreases dramatically, due to moisture diffusion being reduced by physical or chemical interactions within the food [11,25]. This sudden fall of drying rate is caused by the physicochemically bound water. This inflection point is frequently referred to as the critical moisture content [2,11,25].

The second stage of drying period, known as the falling-rate drying period, begins at the inflection point and extends to the final moisture content [25,27,28]. An example of a drying curve for carrots under unspecified drying conditions is shown in Figure 3.2 [25]. As shown, zero percent water is usually never reached under the typical operating conditions required to yield a high-quality dehydrated food product [25]. During moisture determination, however, quality is not the ultimate goal, and harsher operating conditions can be employed [11].

More extreme time–temperature combinations may be used for the analysis of moisture. However, care must be taken to avoid using higher temperatures causing product decomposition. Although decomposition of sugars can be most commonly illustrated [1,11,14,18], other products can also decompose when exposed to temperatures that are too high. For example, Figure 3.3 indicates decomposition of flour or other sugar-containing food products at temperatures above

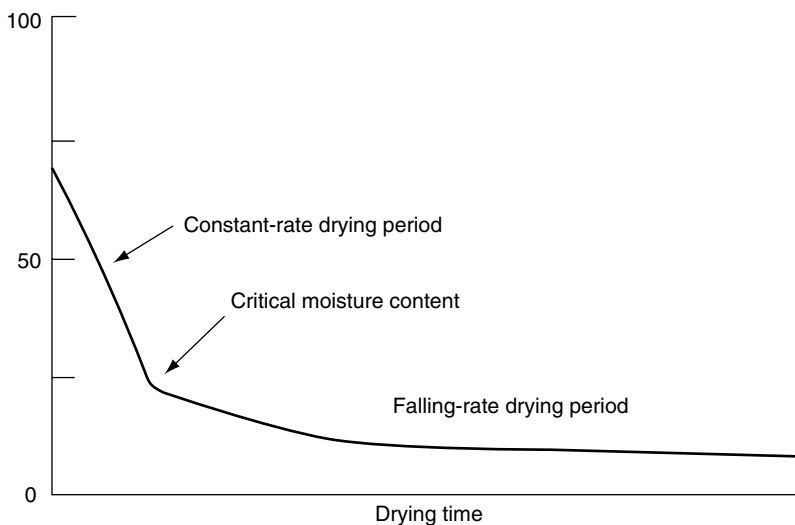


Figure 3.2 The phases of moisture removal in a food-drying process. (Charm, S.E, *The Fundamentals of Food Engineering*, AVI Publishing, Westport, CT, 1971.)

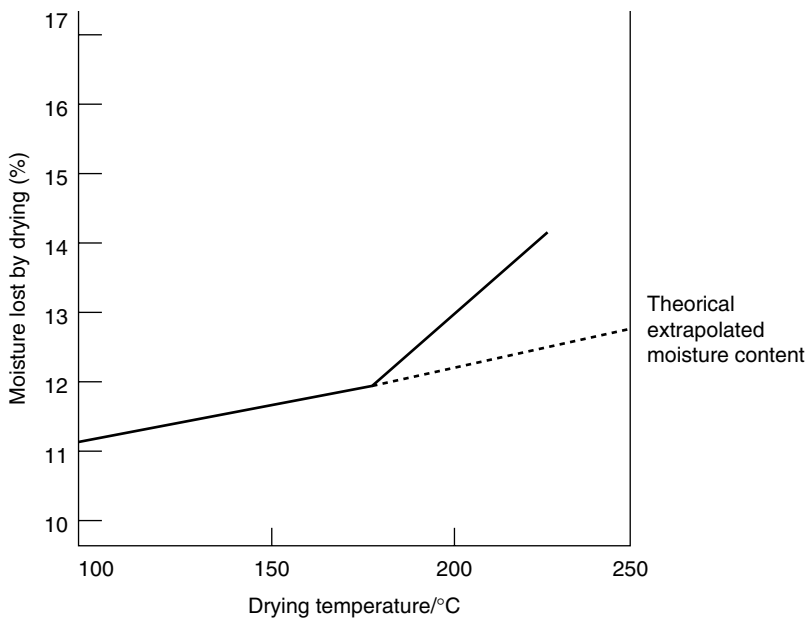


Figure 3.3 Effect of temperature on the moisture content. Determination of flour. (Park, Y.W. and L.N. Bell, *Handbook of Food Analysis*, Marcel Dekker, New York, 2002.)

180°C, as depicted by the discontinuity in the straight line [29]. Instead of using such high temperatures, the rate of moisture removal can be increased by drying under reduced pressure [29]. Researchers have shown that vacuum drying is particularly useful for foods that decompose at relatively low temperatures.

3.6 Sampling Methods for Moisture Determination

The accuracy of moisture determination of a meat sample is largely dependent on the method of selection and the handling of representative samples from larger batches [1,6,18,29]. Handling a sample during moisture determination is important because moisture is easily gained from or lost to the atmosphere due to water-activity gradients between the food and atmosphere [11]. Therefore, exposure to the atmosphere should be minimized for the bulk food, or samples taken from a bulk food, to avoid moisture exchange [1,11,18]. Once the samples are taken, they should be quickly placed in dry rigid plastic or glass containers with tight closures and clear labels, followed by storage at an appropriate temperature before chemical analysis [11]. When refrigerated samples are tested, the sample container should be allowed to warm up to room temperature before opening to prevent moisture from condensing on the cold food. In addition to avoiding sample exposure to atmosphere, it is also essential that a representative sample of the food must be prepared before moisture analysis. Unless the samples are representative of the sample population, no method of moisture determination is of any value. A single absolutely representative sample is difficult to obtain for laboratory analysis, since foodstuffs and food ingredients are relatively heterogeneous materials. Thus, sampling errors can sometimes be greater than the experimental error of analysis [6,7].

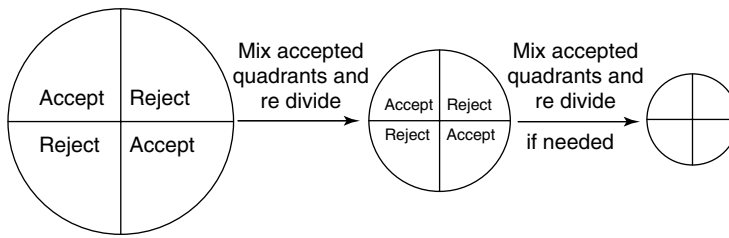


Figure 3.4 Quartering method of sample size reduction for homogeneous sampling. (Park, Y.W. and L.N. Bell, *Handbook of Food Analysis*, Marcel Dekker, New York, 2002.)

Finding a general sampling method applicable to all food types is difficult, but random sampling is the most recommended fundamental concept [6,7]. Random sampling is most appropriate for relatively homogenous food samples, whereas stratified random sampling is employed for heterogeneous food samples [7]. For the stratified random sampling, the sample population is subdivided into small groups that may be treated as homogenous [6,29]. Food samples should be as homogenous as possible to have precise analytical results for moisture content. The homogenization depends on the type of food sample. Reduction in the size of food particles and thorough mixing of samples can be efficiently accomplished using a number of mechanical devices. Blenders, mincers, graters, homogenizers, powder mills, and grinders are essential pieces of equipment for the homogenization of dry, moist, and wet samples [11].

Depending on the moisture determination technique, ground samples may be passed through a sieve of suitable mesh size (18–40 mesh) to obtain a uniform particle size distribution [1]. Variations in particle size can influence moisture values if too small a sample is analyzed [7]. The aliquot size of a powdered bulk sample can be reduced using the process known as quartering [7,29]. In quartering, the bulk food is formed into a uniform pile on a large sheet of glazed paper, glass, or the surface of a clean, laminated bench top. The pile is divided into four equal parts by separating quarter segments. Two quarters are rejected, and the other two quarters are thoroughly mixed. The process is repeated until a suitable sample size is obtained. The quartering method is depicted in Figure 3.4.

Meat, fish products, and some vegetables having high moisture content are best homogenized using a modern domestic food processor or blender followed by mixing. The minimum weight of the sample should be carefully considered, depending on the food type. The *Official Methods of Analysis of AOAC International* describes minimum weight considerations for specific food products [30]. Food-product conditions to be considered in sampling for moisture measurement are summarized in Table 3.1.

3.7 Methodologies of Moisture Determination

Many reviews on methodologies of moisture determination have been published [1,3,14,18,29]. Pande published one of the more extensive reviews, *Handbook of Moisture Determination and Control*, in four volumes [7]. Detailed methodologies for specific food products can be found in the *Official Methods of Analysis of AOAC International* [30].

Generally, analytical methods of moisture determination can be classified in two ways, as shown in Table 3.2. One way is by the four major analytical principles—drying, distillation, chemical, and physical methods [1]. The other is by direct and indirect procedures based on

Table 3.2 Factors Affecting Sample Preparation for Moisture Determination

1	Characteristics of food sample Solid versus liquid Homogeneous versus heterogeneous Type of water interactions within food
2	Particle size and shape of sample
3	Sample preparation Homogenization, blending, mixing, grating, milling, sieve size, heat from mechanical sampling device
4	Representative sampling Random sampling Stratified random sampling Quartering
5	Sample contamination Chemical Microbial (yeast, molds, bacteria) Atmospheric (moisture, dust)
6	Aging of sample Oxidation Decomposition Environmental relative humidity State of hysteresis Adsorption Desorption Equilibrium moisture content
7	Sample storage Storage period Storage temperature Time to be analyzed

Source: Park, Y.W. in *Handbook of Food Analysis*, Marcel Dekker, New York, 1996, 59–92; Park, Y.W. and L.N. Bell in *Handbook of Food Analysis*, Marcel Dekker, New York, 2002, 55–82.

the underlying scientific theory shown in Table 3.3 [6]. In direct methods, moisture analysis normally involves removing water from the solid-food samples by drying, distillation, extraction, or another method, and its quantity is measured by weighing, titration, and so forth [6]. However, for the indirect methods, moisture is not removed from the sample and quantified directly; instead, the properties of the food that depend on either the amount of water or number of hydrogen atoms are measured [6]. This indicates that indirect methods must be calibrated against standard moisture values that have been precisely determined using one or more of the direct methods. Therefore, the accuracy of indirect methods is dependent on the analytical values of direct measurements against which they are calibrated.

It is known that direct methods usually give accurate and even absolute values for moisture determination, although they are mostly tedious, manual, and time-consuming [6]. However, indirect methods are rapid, nondestructive, and offer the possibility of automation for continuous determination [6]. For the purpose of comparison, methodologies of moisture determination are classified into two categories, namely direct and indirect methods. In addition, Tables 3.4 and 3.5 show the advantages and disadvantages of each individual method under the two classifications.

Table 3.3 Classification of Analytical Methods for Moisture Determination

<i>Classification by Four Major Principles</i>	<i>Classification by Direct/ Indirect Procedures</i>
Drying methods	Direct methods
Oven drying	Gravimetric methods
Vacuum drying	Oven drying
Freeze drying (lyophilization)	Air oven
Chemical desiccation	Vacuum oven
Thermogravimetric analysis	Freeze drying
Distillation methods	Thermogravimetric analysis
Direct distillation	Chemical desiccation
Reflux distillation	Distillation methods
Chemical methods	Direct distillation
Karl Fischer titration	Reflux distillation
Generation of acetylene	Chemical titration method
Physical methods	Karl Fischer
IR absorption	Extraction method
NIR reflectance	GC
GC	Indirect methods
NMR	Spectroscopic methods
Refractometry	IR absorption
Neutron scattering	NIR reflectance
Electrical	NMR
Microwave absorption	Mass spectrometry
Dielectric capacitance	Electrical methods
Conductivity	Microwave absorption
Cryoscopic methods	Conductivity
	Dielectric capacitance
	Sonic and ultrasonic methods
	Neutron scattering
	Refractometry
	Cryoscopic methods

Source: Park, Y.W. in *Handbook of Food Analysis*, Marcel Dekker, New York, 1996, 59–92; Park, Y.W. and L.N. Bell in *Handbook of Food Analysis*, Marcel Dekker, New York, 2002, 55–82.

3.7.1 Direct Methods

3.7.1.1 Air-Oven Drying

Air-oven drying is very convenient, and is one of the most widely and commonly used methods for routine moisture determination in laboratories around the world [1,7]. Drying can be accomplished using either convection-type ovens or forced-draft ovens [1]. The ovens should be thermally regulated to $\pm 0.5^\circ\text{C}$ and have minimal temperature variations (less than $\pm 3^\circ\text{C}$) within the oven [1]. Forced-draft ovens offer a more consistent temperature throughout the oven than convection ovens [18]. Modern drying ovens are usually heated by electricity or infrared (IR) heaters and can be equipped with built-in balances for routine and fast analysis,

Table 3.4 Advantages and Disadvantages of Direct Methods for Moisture Determination

<i>Method</i>	<i>Advantages</i>	<i>Disadvantages</i>
Oven drying	Standard conventional method Convenient Relative speed and precision Accommodates large number of samples Attain the desired temperature more rapidly	Variations of temperature due to particle size, sample weight, position in the oven, etc. Difficult to remove all water Loss of volatile substances during drying Decomposition of sample (i.e., sugar)
Vacuum-oven drying	Lower heating temperatures possible Prevents sample decomposition Uniform heating and constant evaporation	Possible volatile loss Lower number of samples than drying oven Drying efficiency reduced for high-moisture foods
Freeze-drying	Excellent for sensitive, high-value liquid foods Preserves texture and appearance No foaming No case-hardening No oxidation No bacterial changes during drying	Expensive Long drying time Sample must be initially frozen Most applicable to high moisture foods
Distillation methods	Determines water directly rather than weight loss Apparatus is simple to handle Accuracy may be greater than oven-drying method Takes relatively short time (30 min to 1 h) to determine Prevents oxidation of sample Not affected by environmental humidity Suitable for samples containing volatile substances	Low precision of measuring device Organic solvents such as toluene pose a fire hazard Organic solvents may be toxic Can have higher results due to distillation of water-soluble components (e.g., glycerol and alcohol) Water droplets may adhere to internal surface of the apparatus, causing erroneous results Emulsions may form
Karl Fischer method	A standard method for moisture analysis The accuracy and precision are higher than with other methods	Chemicals of the highest purity must be used for preparing the reagent Titration endpoint may be difficult to determine visually

Table 3.4 (Continued)

<i>Method</i>	<i>Advantages</i>	<i>Disadvantages</i>
	Useful for determining water in fats and oils by preventing oxidation Once the apparatus is set up, determination takes a few minutes Automated equipment available	The reagent is unstable and needs standardization before use Titration apparatus must be protected from atmospheric moisture due to extreme sensitivity of reagent to moisture Ascorbic acid and other carbonyls can react with reagents, causing over-estimation of the moisture content
Chemical desiccation	Can serve as a reference standard for other methods Can be done at room temperature Good for measuring moisture in substances containing volatile compounds	Requires a long time to achieve constant dry weight Moisture equilibrium depends on strength of desiccant
Thermogravimetric analysis	More automated method than standard oven drying Weighing error is minimal because sample is not removed from oven Sample size is small	Excellent for research, but not practical Small sample may not be representative Sample may decompose or oxidize
GC	Analysis is rapid (takes 5–10 min per sample) Results similar to conventional methods	Unit cost per sample may be higher than drying oven Sample extraction required Requires expensive equipment

Source: Park, Y.W. in *Handbook of Food Analysis*, Marcel Dekker, New York, 1996, 59–92; Park, Y.W. and L.N. Bell in *Handbook of Food Analysis*, Marcel Dekker, New York, 2002, 55–82.

assuming the food is stable [6]. Because the principle of oven drying is based on weight loss, the sample needs to be thermally stable and should not contain significant amount of volatile compounds [6].

Operational procedures for the conventional method of moisture determination using a drying oven and analytical balance generally involve the following steps: sample preparation, weighing, drying, cooling, and reweighing. The general principles of the procedures are described as follows. These procedures have been basically adopted from officially accepted AOAC procedures [30] as well as other similar references [2,11].

3.7.1.1.1 Required Apparatus

1. Weighing dishes—nickel, stainless steel, aluminum, or porcelain. Metal dishes should not be used when the sample may be corrosive.
2. Analytical balance with 0.1 mg sensitivity.

Table 3.5 Advantages and Disadvantages of Indirect Methods for Moisture Determination

<i>Method</i>	<i>Advantages</i>	<i>Disadvantages</i>
Refractometry	Determination takes only 5–10 min (rapid) Does not require complex or expensive instrumentation Simple method Reasonable accuracy Excellent method for high-sugar products	Temperature sensitive Requires uniformity of fluid samples Solid samples (e.g., meat) require homogenization in an anhydrous solvent
IR Absorption	Can perform multicomponent analysis Most versatile and selective Nondestructive analysis	Accuracy depends on calibration against reference standard Temperature-dependent Dependent on homogenization efficiency of sample Absorption band of water is not specific
NIR reflectance spectroscopy	Rapid Precise Nondestructive No extraction required	Reflectance data are affected by sample particle size, shape, packing density, and homogeneity Interference between chemical groups (e.g., hydroxyl and amine) Temperature-dependent Accuracy depends on calibration of standard samples
Microwave absorption	Minimum sample preparation Nondestructive No extraction required More accurate than low-frequency resistance or capacitance meters	Equipment is expensive Possible leakage of microwave energy during measurement Has relatively low sensitivity and limited range for moisture determinations Depends on the fluctuation of the material density in the volume measured Results affected by factors such as particle size, temperature, soluble salt contents, polarization, and frequency of sample
Dielectric Capacitance	Has high sensitivity due to large dielectric constant of water Convenient to industrial operations with the continuous measurement system System can be modified to have universal applicability	Affected by texture of sample, packing, electrolytes, temperature, and moisture distribution Potential calibration difficulty beyond pH 2.7–6.7 Difficult to measure bound water at high frequencies
Conductivity	Measurement is instantaneous Nondestructive	Measures only free water Conversion charts are needed to obtain total moisture values

Table 3.5 (Continued)

<i>Method</i>	<i>Advantages</i>	<i>Disadvantages</i>
	Precise	Accuracy and precision are affected by temperature, electrolyte content, and contact between electrode and samples Difficult to maintain calibration of the equipment
Sonic and ultrasonic absorption	Bound water can be determined in aqueous solution of electrolytes and nonelectrolytes Nondestructive	Dependent on the type of medium for sound passes Appropriate standards required to get total moisture content
Mass spectroscopy	Can analyze simultaneously a large number of components from a complex matrix No electrical leakage problem due to low potentials applied to the beam tube	High variation between theoretical moisture values and hydrated substances Major instrumental problem is memory effect from the preceding sample
NMR Spectroscopy	Very rapid analysis Accurate Nondestructive	Cost of equipment is high Separate calibration curves are required for different substances Constant and correct sample weight required
	Applicable to many types of foods Can differentiate between free and bound water Particle size and packing of granular samples have no effect on signal absorption	Not applicable for foods having variable lipid contents
Neutron scattering method	Density and moisture measured simultaneously The absolute error is claimed to be less than $\pm 0.5\%$ Suitable for soil moisture assay	Applicable only to substances that are relatively proton-free Expensive

Source: Park, Y.W. in *Handbook of Food Analysis*, Marcel Dekker, New York, 1996, 59–92; Park, Y.W. and L.N. Bell in *Handbook of Food Analysis*, Marcel Dekker, New York, 2002, 55–82.

3. Desiccator—containing an efficient desiccant such as phosphorus pentoxide, calcium sulfate, or calcium chloride.
4. Atmospheric oven.
5. Blender—Oster, Waring, or equivalent for high-moisture samples.
6. Grinder and mill for low-moisture samples.
7. Spatula or plastic spoon.
8. Steam bath—it is used for predrying high-moisture samples such as dairy products.
9. Crucible tongs.
10. Thermometer (0–130°C).

Table 3.6 Atmospheric-Oven Temperatures and Time Settings for Oven Drying of Milk and Other Foods

<i>Product</i>	<i>Dry on Steam Bath</i>	<i>Oven Temperature (°C ± 2)</i>	<i>Time in Oven (h)</i>
Buttermilk (liquid)	X	100	3
Cheese (natural-type only)		105	16–18
Chocolate and cocoa		100	3
Cottage cheese		100	3
Cream (liquid and frozen)	X	100	3
Egg albumin (liquid)	X	130	0.75
Egg albumin (dried)	X	130	0.75
Ice cream and frozen desserts	X	100	3.5
Milk (whole, low fat, and skim)	X	100	3
Condensed skim		100	3
Evaporated milk		100	3
Nuts (almonds, peanuts, walnuts, etc.)		130	3

Note: X indicates that samples must be partially dried on steam bath before placing in oven.

Source: AOAC, Official Methods of Analysis of AOAC International, AOAC International, Arlington, VA, 1995.

3.7.1.1.2 Procedure for Oven Drying

1. Wash the empty dishes thoroughly, rinse, and dry in an oven for several hours at 100°C. Store in a clean desiccator at room temperature before use.
2. Mix the prepared sample thoroughly, and quickly weigh a 2–5-g sample into a preweighed dish using an analytical balance to the nearest 0.1 mg; the sample should be spread evenly across the bottom of the dish.
3. Place the dish without its cover on the metal shelf in the atmospheric oven, avoiding contact between the dish and the walls. Refer to Table 3.6 for steam-bath requirements, oven temperatures, and drying times for selected food products.
4. After a specified time in the oven, use tongs to place the cover onto the dish, remove the dish from the oven, and place it into the desiccator for at least 30 min to cool to room temperature.
5. Weigh the dish on the analytical balance and calculate moisture loss.

3.7.1.1.3 Calculations of Moisture Content

$$\text{Moisture (\%)} = \frac{(\text{loss of weight} \times 100)}{(\text{sample weight})}$$

$$\text{Solids (\%)} = 100 - \text{moisture (\%)}$$

It is important to carefully consider the length of time required in the oven when the drying method is used for moisture determination. Different oven temperatures and drying times for many food products have been determined and can be found in the *Official Methods of Analysis of AOAC International* [30]; some of these are summarized in Table 3.6. For products without an official method, samples are periodically weighed during the drying process until the change in mass is negligible. Typically, two successive weighings an hour apart should show a mass change of less than 2 mg/5 g sample [1]. Because many of these fluid products contain large amounts of sugar, the predrying temperatures of the steam bath should be kept below 70°C to prevent sample decomposition [14,18].

The accuracy of any particular drying procedure for the determination of moisture can be influenced by a number of factors. Erroneous results in moisture determination by oven drying may result from variations in sample weighings, oven conditions, drying conditions, and post-drying treatments. Sample weighing is influenced by adsorption of atmospheric vapor, length of weighing time, spillage, and balance accuracy. Oven conditions that influence the accuracy of moisture determination include temperature, air velocity, pressure, and relative humidity. Factors associated with the drying conditions are size and shape of the sample container, type and location of the heating element, drying time, scorching, loss of volatile compounds, and decomposition. Postdrying factors such as final temperature at weighing, desiccator efficiency, loss of dried sample, and balance buoyancy effect may also contribute to erroneous data. Advantages and disadvantages of air-oven drying methods, as well as those of other direct methods, are listed in Table 3.4.

3.7.1.2 Vacuum-Oven Drying

Since many drawbacks associated with air-oven drying can be overcome by vacuum-oven drying [6,7], this method is generally considered as the standard and most accurate drying method for moisture analysis in foods. Vacuum drying usually can heat foods up to 98–102°C, with low pressure of 25–100 mm Hg [1,14]. Lower temperatures (60–70°C) are used for high-sugar food products to prevent decomposition [1,7,14,18]. Moisture can be evaporated more quickly at the reduced pressure, and drying times can be dramatically reduced [1]. It may be impossible to obtain an absolute moisture content of the sample by drying methods, but vacuum drying can yield a close and reproducible estimate of the true moisture content of a food [1].

There are several types of vacuum ovens available. Laboratory type vacuum ovens can be connected to a vacuum line and electrically heated. These vacuum ovens are typically equipped with airtight front doors using vacuum grease on a rubber gasket. Although a vacuum of 100–600 mm Hg can be maintained inside the sample chamber [7], it is usually desirable to have pressures below 50 mm Hg, because the reduced pressure will increase the rate of drying [1]. Dry air is introduced into the vacuum oven during drying; without purging dry air into the oven, the vapor pressure of water inside the oven would reduce the usefulness of the vacuum oven, especially for high-moisture foods [1,18]. According to the AOAC procedures, moisture contents are usually determined by heating in a vacuum oven at 100°C for 2–6 h at a pressure of 25–100 mm Hg [30]; Table 3.7 lists some drying conditions for selected food products by vacuum oven and other direct methods for moisture determination of foods as recommended by AOAC. The advantages and disadvantages of the vacuum drying method are also described in Table 3.4.

The required apparatus and general procedure for vacuum-drying method are delineated as follows [2,11]:

Table 3.7 Moisture Content of Meat Samples Obtained by Conventional and GC Methods

Samples	Moisture (%) ^a	
	GC	Conventional
Fat	6.9	6.6 ^b
Pork jowl	27.2	27.1 ^b
Pork trim	36.6	37.2 ^b
Navels	38.2	38.0 ^b
Salami	40.5	39.3 ^b
Emulsion, frankfurter ^c	51.1	51.0 ^b
Cow meat	55.4	54.9 ^d
Bull meat	69.4	70.6 ^d
Cheek meat	71.4	69.9 ^d
Turkey	75.9	76.3 ^d

^a Mean of five determinations.

^b Toluene distillation.

^c Emulsion obtained immediately before extrusion.

^d Oven drying at 105°C for 24 h.

Source: Reineccius, G.A. and P.B. Addis, *J. Food Sci.*, 38, 355, 1973.

3.7.1.2.1 Required Apparatus

1. Vacuum oven—thermostatically controlled and connected with a vacuum pump capable of maintaining the pressure in the oven below 25 mm Hg. The oven should have a dry air inlet that passes through an indicating desiccant and a trap for releasing the vacuum.
2. Dishes—metal dishes with close-fitting lids and flat bottoms to provide maximum area of contact with the heating plate.
3. Other apparatus and equipment are the same as those for air-oven drying.

3.7.1.2.2 Procedure for Vacuum Drying

1. Metal sample dishes must be washed and dried in a laboratory oven. After cooling in a desiccator, weigh the dish to 0.1 mg.
2. Weigh the sample (3.0–5.0 g) into the preweighed dish using an analytical balance. Distribute the sample evenly over the bottom of the dish. Some samples require predrying as described for air-oven drying to prevent decomposition and splattering.
3. Put the sample dishes in the vacuum oven, partially uncover the dish, evacuate the oven, and dry the sample at an appropriate temperature and vacuum pressure. During drying, admit a slow current of air dried by passage through the indicating desiccant into the oven.
4. Turn off the vacuum pump after 5 h and slowly readmit dry air into the oven. Press the cover lightly onto the dish using tongs, transfer the dish to a desiccator to cool, and reweigh.
5. Dry for another hour to ensure that constant weight has been achieved.

3.7.1.2.3 Calculations of Moisture

$$\text{Moisture (\%)} = \frac{(\text{water volume} \times 100)}{(\text{sample weight})}$$

$$\text{Solids (\%)} = 100 - \text{moisture (\%)}$$

3.7.1.3 Freeze-Drying

There is no better method than freeze-drying for preserving freshness and textural quality of dried foods. Freeze-drying or lyophilization is especially suited for drying high-value liquid foods such as coffee and juices, as well as high-value solid foods such as strawberries, shrimp, diced chicken, sliced mushrooms, and even steaks and chops [25]. In recent years, the lyophilization process has evolved into a highly advanced drying technique. This drying method has the limitation that its cost may be two to five times greater per weight of water removed than other drying methods [25]. Much of the development work, therefore, has focused on optimizing both the lyophilization process and equipment to lower drying costs [25]. However, freeze-drying has many advantages, some of which are listed in Table 3.4.

The main principle of lyophilization is facilitating the sublimation of water from the sample under reduced pressure and temperature conditions. Sublimation is the direct conversion of ice to water vapor without melting into liquid water; thus, lyophilization preserves the physical structure of the food. Water evaporation from ice (sublimation) occurs at temperatures below 0°C and pressures below 4.6 mm Hg [31]. Under these conditions, water in the food remains frozen and water vapor leaves the food faster than water in the surrounding atmosphere reentering onto the food, which causes a net reduction in the moisture content of the frozen sample [25]. Heat is frequently applied to the frozen food to enhance the sublimation rate within the vacuum chamber of the dryer. The maximum drying rate occurs when the vacuum is maintained at 0.1–2 mm Hg and heat is added just short of melting the ice [25]. As freeze-drying progresses, moisture is initially removed from the surface and continues to recede toward the center of the frozen food until the final ice sublimates, leaving a moisture content of less than 5% [25]. Completion of drying times for freeze-drying may be 8 h or longer. The high cost of the equipment may limit the availability of this freeze-dry method for ordinary moisture analysis of food samples. However, this lyophilization method is desirable as a component of a standard reference method for moisture determination [1].

3.7.1.4 Distillation Methods

The property of “azeotropy” in water is utilized for this method, where water is simultaneously distilled with an immiscible liquid at a constant ratio. There are two main types of distillation methods for moisture determination—direct distillation and reflux distillation [1,14,18]. First, for the direct distillation method, a food is heated in a liquid (e.g., mineral oil), which is immiscible with water and has a high boiling point [1,14,18]. The water in the food distills directly from this liquid, condenses, and is collected in a graduated tube; the volume of the water removed is then measured.

Second, the reflux distillation procedure is more commonly used than the direct distillation method [1,6–7,14,18]. This procedure makes use of the azeotropic properties of solvent mixtures. Water and an immiscible solvent, such as toluene or xylene, distill off together during heating at a constant ratio and frequently at a temperature lower than the boiling point of either component [31]. As an example, the respective boiling points of water and toluene are 100 and 110.6°C, but the boiling point of the binary mixture is 85°C; the distillation ratio of the mixture is approximately 20% water and 80% toluene [31]. If water is denser than its co-distillate, as in the case with toluene, the water is again collected in a suitable measuring apparatus, where the water is separated and then the water volume is measured.

A rapid distillation can be achieved by distillation with a boiling liquid, which transfers heat effectively to the sample [1]. The lowered boiling point of the distillation mixture causes less decomposition of the food during heating [1,18]. This procedure also minimizes oxidative reactions [6–7,31]. This distillation method is especially suitable for samples having a high concentration of volatile compounds. The research data collected from azeotropic distillation have shown consistently the theoretical moisture content to within 0.1% [6]. A moisture value comparison between reflux distillation using toluene and oven-drying methods showed similar levels for a variety of products [31]. There are some potential difficulties for the reflux method, such as emulsion formation and suspended water droplets. Using clean glassware and allowing the apparatus to cool before reading the volume of collected water help with these two problems [18]. Table 3.4 delineates the advantages and disadvantages associated with the distillation procedures.

An apparatus of the reflux distillation system is shown in Figure 3.5. This system consists of a heating source under a round-bottom boiling flask, and the flask contains the food sample and the solvent (e.g., toluene). The round-bottom flask is connected to a Bidwell–Sterling receiver, which will collect and measure the distilled water in a side arm. A condenser is positioned directly above the side arm of the Bidwell–Sterling receiver. The apparatus, reagents, and procedure of the reflux distillation are outlined as follows [2,11].

3.7.1.4.1 Apparatus

1. Reflux distillation apparatus (see Figure 3.5)
2. Heating mantle

3.7.1.4.2 Reagents

1. Xylene or toluene

3.7.1.4.3 Procedure

1. Weigh a food sample containing 2–5 g water and place the sample in an appropriately sized round-bottom flask. This could be 10–15 g cheese or 40 g spice.
2. Add enough suitable solvent (e.g., toluene) to cover the food, usually 60–100 mL.
3. Assemble the reflux apparatus as shown in Figure 3.5.
4. Run cold water through the condenser and gradually heat the flask until refluxing starts.

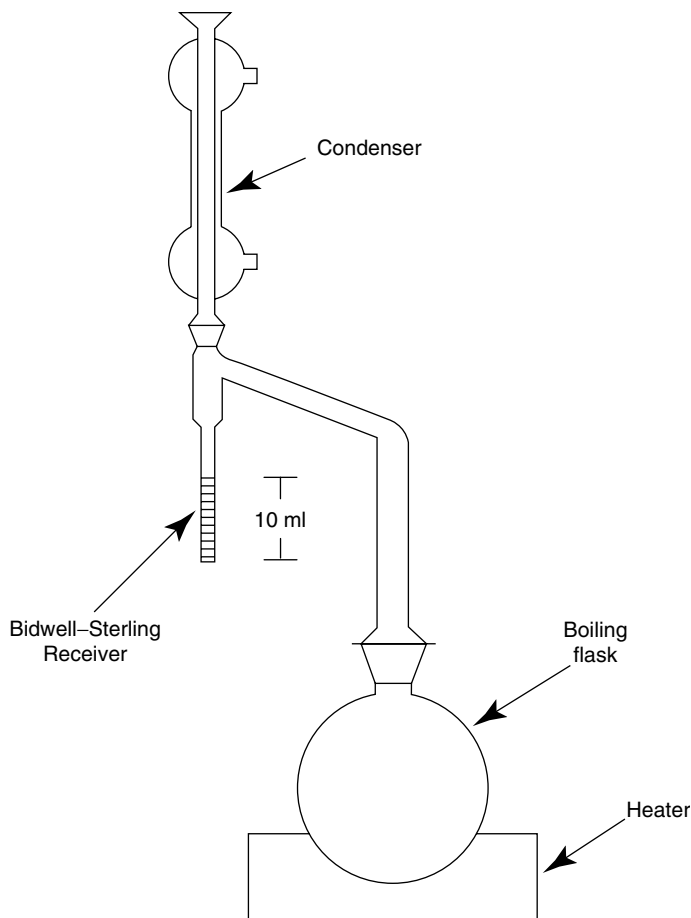


Figure 3.5 Apparatus for azeotropic distillation method with Bidwell-Sterling receiver. (Park, Y.W. and L.N. Bell, *Handbook of Food Analysis*, Marcel Dekker, New York, 2002.)

5. Adjust the heating to produce two drops of condensate per second. When the rate of water accumulation decreases, increase heat to yield four drops per second.
6. When no additional moisture is collected in the side arm, rinse the condenser with the solvent and continue heating a few more minutes. Total heat time is typically 1–1.5 h.
7. Turn off the heat and allow the apparatus to cool, especially the side arm.
8. Record the volume of water in the side arm.

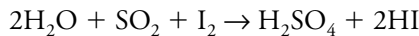
3.7.1.4.4 Calculations

$$\text{Moisture (\%)} = \frac{(\text{loss of weight} \times 100)}{(\text{sample weight})}$$

Detailed experimental protocols for specific food products can be found in the *Official Methods of Analysis of AOAC International* [30] as well as other sources [2,11].

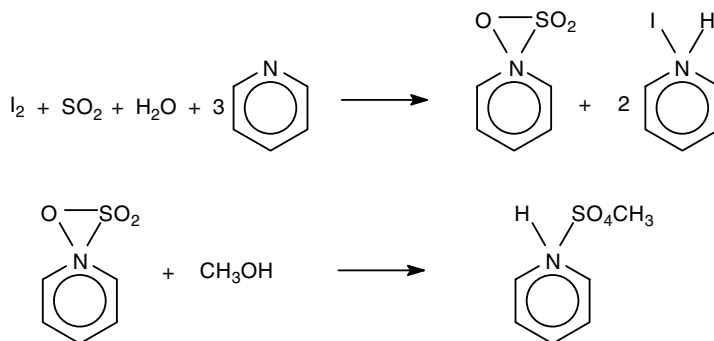
3.7.1.5 Karl Fischer Titration Method

The Karl Fischer method for moisture analysis is based on the reduction of iodine by sulfur dioxide in the presence of water, which was proposed by Bunsen in 1853. The main chemical principle of the method is



Fischer modified the conditions of the reaction, enabling quantification of moisture [32]. The Karl Fischer method has become a standard method for moisture determination of liquids and solids due to its selectivity, high precision, and speed [9]. It is especially applicable for measuring moisture in foods for which heating methods give erratic results [1,18]. Moisture assay using this chemical technique has been approved for dried vegetables, oils and fats, cacao products, liquid molasses, and sugar-rich foods [1,30]. This method has superior sensitivity compared to other methods, being able to quantify the amount of water to a few parts per million [6–7,18]. The accuracy and precision of the Karl Fischer method have been found to be higher than those of other methods [6]. A more detailed listing of the advantages and disadvantages of the Karl Fischer method is found in Table 3.4.

The titration reagent for the Karl Fischer method consists of a mixture of iodine, pyridine, sulfur dioxide, and methanol. The titration of water with this reagent follows the two-step reaction shown as follows [1,18,33].



The preceding reactions illustrate that titration of 1 mol water requires 1 mol iodine, 1 mol sulfur dioxide, 3 mol pyridine, and 1 mol methanol. The titration is performed either by volumetric titration, where the endpoint is indicated by the appearance of brown color from free iodine (detected either visually or by photometric determination), or by coulometric titration, where the endpoint is determined by a potentiometer [18]. Several diagrammatic representations of the Karl Fischer apparatus are shown by Pande [7].

The *Official Methods of Analysis of AOAC International* [30] shows the official Karl Fischer method for specific food products and typical use of automated equipment. The titration is automatically performed by these Karl Fischer instruments until the endpoint is reached. The moisture content is calculated from the amount of titrant consumed, which is often expressed in

milligrams. Milligrams of water can be converted into percent moisture using the initial sample mass. Liquid or solid food samples may be directly introduced into the reaction vessel if the water is easily accessible to the reagent. The water is frequently extracted into anhydrous methanol in solid foods where the water is not accessible. A known amount of the methanol/water solution is then injected into the reaction vessel. A methanol blank should also be prepared in a similar manner. The amount of water in this methanol blank would indicate whether any moisture from the atmosphere was introduced during sample preparation. The moisture in the blank sample should be subtracted from the moisture value of the food. The apparatus, reagents, and procedure of manual Karl Fischer method for a solid containing inaccessible water are described in the following.

3.7.1.5.1 Apparatus

1. Burette—automatic filling type, all glass, fully protected against moisture ingress
2. Titration vessel—having an agitation device such as magnetic stirrer slightly pressurized with dry inert gas (N_2 or CO_2) to exclude air
3. Electrometric apparatus and galvanometer—suitable for “dead stop” endpoint technique

3.7.1.5.2 Reagents

1. Methanol (anhydrous).
2. Karl Fischer reagent—to minimize loss of active reagent from side reactions, many laboratory suppliers provide the Karl Fischer reagent as two solutions: iodine in methanol and sulfur dioxide in pyridine. The solutions are mixed shortly before use.

3.7.1.5.3 Procedure

1. Weigh an amount of sample containing approximately 100 mg water into a predried 50-mL round-bottom flask.
2. Add 40 mL methanol into the flask, quickly place it on the heating range, and connect the reflux condenser
3. Boil the contents of the flask gently under reflux for 15 min.
4. Stop heating with the condenser attached, and let it drain for 15 min.
5. Remove and stopper the flask.
6. Pipette a 10-mL aliquot of the extract into the titration vessel, titrate with the Karl Fischer reagent to the “dead stop” endpoint, and record the volume of titrant used.
7. Run a blank flask without a sample following the same procedures described earlier.

3.7.1.5.4 Calculation

Using the preceding procedure, the following equation is used to calculate moisture content. If a known sample mass was introduced directly into the titration vessel rather than extracting with methanol, the dilution factor (i.e., 4) may be removed from the equation.

$$\text{Moisture (\%)} = \frac{([4 \times 0.1 \times F] \times \text{reagent used for sample [mL]} - \text{reagent used for blank [mL]})}{\text{sample weight}}$$

The standardization factor of the reagent, F , can be determined from titrating samples containing known amounts of water. It is measured in milligrams of water per milliliters of reagent [18].

Pyridine-free Karl Fischer reagents are available due to health concerns, and these reagents do not compromise the moisture determination. The replacement solvent systems actually can speed up the analysis and improve its precision [1]. As described earlier, the titration can be performed manually or using semiautomated equipment, but completely automated equipment is recommended, especially when Karl Fischer analyses are to be performed on a routine basis.

3.7.1.6 *Chemical Desiccation*

The chemical desiccation method is carried out by desiccation in an evacuated desiccator containing a substance that strongly absorbs moisture, usually for dried foods. The amount of water removed from the food depends on the strength of the desiccant employed [30]. Relative efficiencies of various desiccating agents were compared in several studies, as shown in Table 3.6 [31,34,35]. The most effective desiccating agents are phosphorus pentoxide, barium oxide, and magnesium perchlorate [30]. However, phosphorous pentoxide becomes explosive if it absorbs too much moisture [20]. Calcium sulfate (Drierite™) is a commonly used desiccant despite not being as effective.

Usually at room temperature, desiccation of the sample is achieved. With few exceptions, desiccation techniques are lengthy procedures, frequently requiring weeks and even months for the sample to achieve constant weight [1,6,20]. The equilibrium time depends strongly on the forces holding water in the sample relative to the desiccant. Slight heating may be used in conjunction with the desiccants to enhance moisture removal from the food. Although this method has some limitations, results obtained using chemical desiccation can serve as reference standards for calibrating moisture contents of more rapid procedures [30].

3.7.1.7 *Thermogravimetric Method*

The moisture of a food is removed by heating in thermogravimetric analysis (TGA), which resembles an automated version of the standard oven-drying method. The TGA instrumentation is equipped with a thermobalance, which automatically measures and records the weight loss of a food sample as a function of time and temperature while the sample is being heated [36]. For the procedure of TGA, a small amount of sample is loaded into the balance, which then heats under a controlled temperature program. Analytical data are recorded in the form of thermogravimetric curves, which plot the sample mass as a function of temperature. Moisture is continuously evaporated from the sample and the weight loss is recorded until the sample has reached a constant weight. Errors associated with sample weighing are minimized in TGA, because multiple sample transfers are not required for the analytical processes. However, care must be taken to prevent thermal decomposition of the sample to avoid erroneous results, as in other methods using heat for moisture determination.

Moisture assay using TGA has been shown to give results similar to other methods. The TGA method is advantageous in that it can be used to quantify chemically bonded water of hydration, as well as to analyze total moisture content.

3.7.1.8 *Gas Chromatography*

The gas chromatography (GC) method has versatile capability in analytical chemistry, which can be applied to moisture assay of foods [1,6]. The principle of the GC method for moisture

determination is as follows. A known amount of the food sample is initially homogenized, and water is extracted into an anhydrous solvent such as methanol, ethanol, or isopropanol. The extract is then analyzed using GC such that quantitative separation of the water–solvent mixture can occur. A Poropak Q column and a thermal conductivity detector have been used previously for moisture analysis of meat products [37]. The quantification of moisture content is carried out by determining the peak areas of water and solvent; these areas are then compared to the areas of solutions containing known amounts of water (i.e., a standard curve). The moisture values determined by GC compared with values determined by distillation or oven-drying methods have shown that the GC values were not different from those obtained by the more conventional methods [33]. Table 3.7 illustrates the moisture contents of meat samples obtained by conventional and GC methods. The GC analysis is rapid, but requires specialized and expensive equipment.

3.7.1.9 Application of Direct Methods in Moisture Determination

As mentioned earlier, various direct methods have been discussed for moisture analysis. The most widely used method is air-oven drying, which is usually conducted for the least temperature-sensitive foods. A complete moisture removal is assumed at some appropriate time/temperature combination. Vacuum-oven drying speeds up moisture removal and is especially useful for foods susceptible to decomposition (e.g., sugar-containing products). Since moisture can be removed more rapidly at lower pressures, the vacuum-oven method uses pressures lower than that of the atmosphere to reduce the boiling point of water while preventing product decomposition. Distillation methods give comparable moisture values to those from the oven methods. Distillation methods are carried out at atmospheric pressure, and often completed in a shorter time than the oven-drying methods. The Karl Fischer titration method is rapid, although it remains primarily a laboratory technique. This method also requires a considerable degree of skill for the performing analyst. Vacuum desiccation requires too much time to be considered as a method for product quality control. GC, although able to give rapid results comparable to other methods, requires a large initial capital investment. For the analysis of different food samples, an official method for moisture determination can be selected, depending on the types of foods analyzed [30]. The official methods for moisture determination of various foods recognized by the Association of Official Analytical Chemists International are described in Table 3.8 [30].

3.7.2 Indirect Methods

As mentioned earlier, a variety of indirect methods for moisture determination also exist. These methods measure a property of water that is dependent on its content. Thus, moisture contents can be calculated using appropriate calibration curves.

3.7.2.1 Refractometry

Refractometry is an optical method measuring the refractive index of a solution, which can be used for determining its moisture content. A schematic presentation of the refraction, or bending, of light as it passes through two different sucrose solutions is shown in Figure 3.6. The more

Table 3.8 Comparison of Direct Methods for Moisture Determination of Foods as Recommended by AOAC International

Moisture-Assaying Method	Temperature (°C)	Pressure (mm Hg)	Sample Weight (g)	Time Required (h)	Food Products
Air-oven drying	100–102	760	2	16–18	Meat products
	100	760	5	3	Cane and beet sugar
	130	760	2	1–2	Flour
	100	760	2	Until constant weight	Cacao products
Vacuum-oven drying	60–70	50–100	2–5	2–6	Dried fruits, honey, syrup
	98–100	25	2–5	5	Pasta products, coffee, wheat flour
	100	100	2–3	4–5	Dried milk, cheese, nuts, tea
	75	100	5	5	Fats and oils
Distillation	129–134	760	10–15	1–2	Cheese (amyl alcohol and xylene)
	85	760	40	1–2	Spices (toluene)
Karl Fischer method	—	—	5–15	A few minutes (if apparatus is set up)	Cacao products
			2–3		Dried vegetables
			5–25		Fats and oils
			0.5–1		Molasses

Source: AOAC, *Official Methods of Analysis of AOAC International*, AOAC International, Arlington, VA, 1995.

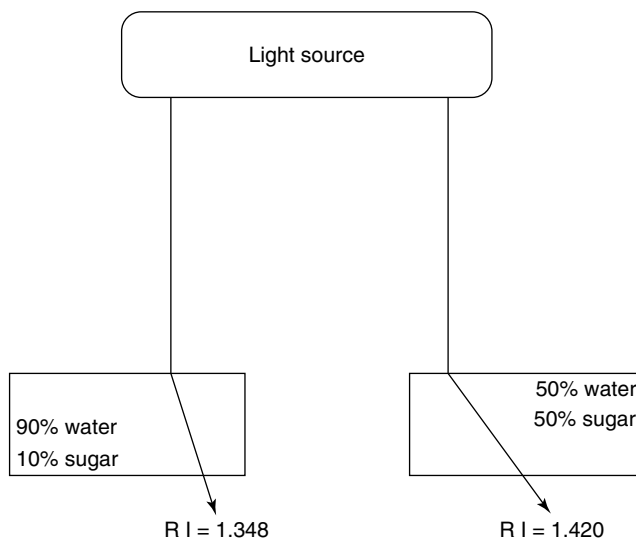


Figure 3.6 Schematic moisture determination by refractometry. (Park, Y.W. and L.N. Bell, *Handbook of Food Analysis*, Marcel Dekker, New York, 2002.)

refraction, the higher the concentration in the solution. The moisture content can be rapidly determined by measuring the refractive index of a solution or slurry, using an appropriate calibration curve [1,6,18,38].

The sample can be homogenized with an anhydrous solvent (e.g., isopropanol) for solid or semisolid foods, and then the refractive index of the solution is measured using a refractometer. A calibration curve is produced by measuring the refractive index of solutions containing the same solvent with known amounts of added water. The moisture content of the sample is calculated using the calibration curve, and the mass of food is homogenized in the solvent. Because the refractive index measurement is temperature-sensitive, a uniform sample is required, and strict temperature control is necessary.

3.7.2.2 *Infrared Absorption Spectroscopy*

Many reports have shown that IR spectroscopy can be used for moisture determination [1,6–7,18]. The IR spectrum of a chemical compound has been described as one of its most characteristic physical properties [7]. Since IR has these properties, it is one of the most versatile methods for measuring the moisture content of a large variety of solid, liquid, or gas substances by employing specific wavelengths at which maximum absorption is expected to occur. For water, the spectral region of interest is 700–2400 nm; absorption bands occurring at 1450 and 1940 nm are frequently used [1,6–7,18]. Determination of the moisture content of a sample can be performed by comparing the band intensity with that of the same band for standard concentrations of water.

The basic concept of this methodology is that an IR beam passes through an optical filter, which consequently transmits energy at a specific wavelength through the sample cell and then to a detector [39]. Ideally, the wavelength used is that of maximum absorption for the compound being measured. The IR technique must be calibrated using standards of known concentration. Then the absorption values of the sample can be compared with those of the standards for moisture determination. An appropriate calibration technique [18,30] is required for each chemical compound.

3.7.2.3 *Near Infrared–Reflectance Spectroscopy*

Near infrared (NIR)–reflectance spectroscopy technology has been developed recently to use its high resolving power of reflectance spectra in the NIR range (800–2500 nm) as an analytical tool for components analysis. The mid-IR range (2,500–24,000 nm) has high resolution in the absorption spectrum and can absorb IR radiation effectively from many compounds, but resolution of the reflectance spectrum is poor [40].

Since this methodology developed in early 1970s, NIR–reflectance spectroscopy has assumed immense economic importance as a rapid, integrated multicomponent testing method for a wide range of products. The NIR technique has been widely used to predict the composition (i.e., moisture, oil, and protein) of grains and oil seeds as well as other foods [41]. The primary advantage of the method is the speed of analysis. The accuracy of the NIR reflectance method depends on the calibration curve, derived from wet chemical analysis of the standard samples. If the calibration sample set does not adequately represent the range of the unknown samples, then the analysis will be error-prone. Linear calibration curves (correlation coefficients >0.98) have been established between the moisture values of raw pork and beef determined by the oven-drying method and by the NIR–reflectance spectroscopic method [41].

The NIR spectrophotometer can generate the reflectance spectra with a monochromator operated in single- or double-beam mode, which then can be downloaded into a computer [41,42].

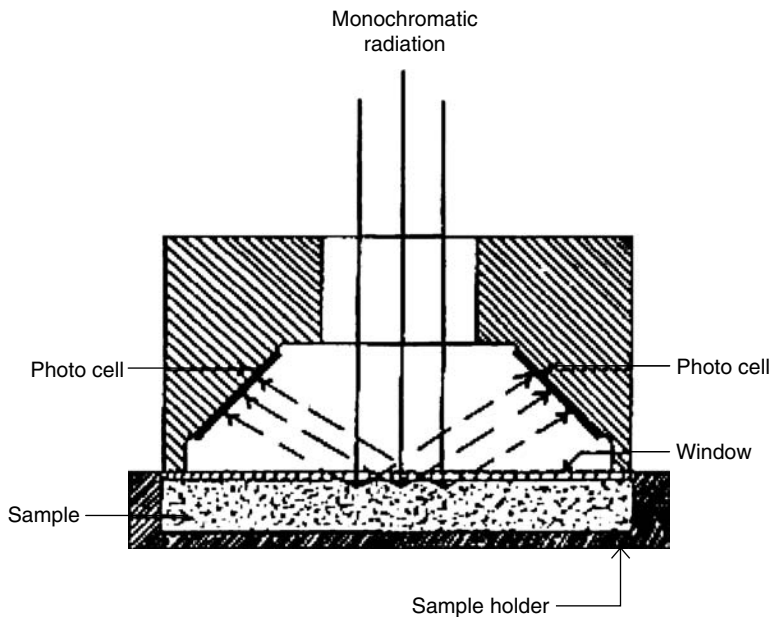


Figure 3.7 Large, solid detector of NIR equipment. (Park, Y.W., *Handbook of Food Analysis*, Marcel Dekker, New York, 1996; Ruan, R.R. and P.L. Chen, *Foods and Biological Materials: A Nuclear Magnetic Approach*, Technomic Publishing, Lancaster, PA, 1998.)

Ground samples are packed into a sample holder maintaining direct contact with a concentric IR-transmitting quartz window. The reflected radiation signals of the diffused spectra from the glass window are collected with four lead sulfide detectors equally spaced around the incident beam. Figure 3.7 depicts the NIR radiation being reflected from a food sample to surrounding detectors. The signals from the detectors are amplified with a logarithmic-response amplifier, digitized, and fed into a computer. The wavelength range from approximately 1100 to 2500 nm is scanned every 2 nm (or 0.5 nm) along the width of the reflectance curve [41,42]. Both the IR reflectance (R) curve and the $\log(1/R)$ curve can be recorded as the second derivative of the original curves to help evaluate overlapping absorption bands [41,42].

Since the NIR method is not a direct method for moisture determination, the reflectance data cannot be used directly for quantitative analysis. Moisture contents of standard samples are inputted in the computer along with the NIR data, which analyzes them with a stepwise multiple linear-regression method to develop prediction equations by a regression analysis of NIR spectral data against chemical data [41,42]. For moisture assay of the raw-meat samples, this spectral data consisted of taking the ratio of the second derivative of the $\log(1/R)$ data at two different wavelengths [41], showing the complexity of the analysis.

NIR instruments are commercially manufactured on the basis of three geometries according to the method of collecting the reflectance. These are integrating sphere, large solid-angle detector, and small detector. Each of these types has advantages and disadvantages, as discussed by Norris [41]. The large solid-angle detector was used to collect the moisture data on pork and beef, as mentioned earlier [41].

3.7.2.4 Microwave Absorption Method

One of the distinct properties of water is that it absorbs several thousand times more microwave energy than a similar amount of a dry substance [6]. For example, at frequencies between 1 and 30 GHz, the loss tangent of water is 0.15–1.2, whereas for dry materials the loss tangent is 0.001–0.05 [30]. Because of these differences, the absorption of microwaves can be used to determine the water content of a variety of food products. The absorption of microwave energy at 2450 MHz increases linearly with increasing moisture content [30]. This led to the development of the microwave moisture meter almost 40 years ago.

The microwave absorption method consists of a constant source of microwave radiation, a waveguide, a detector, a microwave attenuator and amplifier, and an indicating meter [30]. The sample is placed between a microwave transmitter and receiver. The attenuation of the sample is the difference in attenuation readings between the transmitter and the receiver, which is dependent on moisture content and is therefore used to construct a calibration curve [30]. Various factors can affect the accuracy of the microwave measurement, including leakage of microwave energy, sample temperature, particle size, polarization of different material, and the presence of soluble salts [30]. The microwave absorption device was used to determine the moisture content of cakes, and was found to give results similar to that from a drying oven [43]. The microwave absorption method has not evolved into a commonly used analytical technique for moisture determination, even if the positive results have been obtained.

3.7.2.5 Dielectric Capacitance

Another distinct property of water is its dielectric constant (DC). Its DC at 20°C is about 80, whereas those of fatty acids and sucrose are both about 3 [31]. There is a positive correlation between moisture content and DC, whereby a 1% increase in moisture content of a substance will theoretically increase its DC by approximately 0.8 [6]. In addition, the DC of water-containing substances increases almost linearly up to approximately 30% moisture content [6,19,30]. Instruments utilizing dielectric measurements for moisture determination were developed based on this principle and applied most commonly to cereals [1].

The major component of the dielectric instrument is a capacitance cell, which consists of two metal plates spaced apart, with equal but opposite charges [1]. These charges reverse at fixed frequencies to yield an alternating current [1]. The principle of moisture measurement by dielectric instrument is as follows. A sample placed between the two plates will become polarized and change the capacitance of the plates. This capacitance change, affected by moisture content, is measured by the instrument. On calibration with standards of known moisture content, moisture contents of food samples can be determined. Analytical results using a capacitance meter are influenced by moisture distribution, presence of electrolytes, temperature, and sample density [6,18]. Table 3.5 describes the advantages and disadvantages of the DC method and other indirect methods.

3.7.2.6 Conductivity Method

The principle of the conductivity method is that conductivity and resistance are measured in an electrical circuit containing a food sample [1]. A distinct relationship exists between the moisture content of materials and their electrical properties [6,32]. As the moisture content of a food

sample increases, electrical resistance of the sample decreases, and its conductivity increases [6,18]. Measuring resistance appears to be most useful [18]. Frequently, the logarithm of resistance is plotted as a function of either moisture content or humidity. The logarithm of resistance is basically linear as a function of humidity, although showing curvature as a function of moisture content [30].

Several conductivity instruments are widely used by the industry for rapid routine moisture determinations by measuring either resistance or conductance of foods [1]. The accuracy of this method being less than $\pm 0.5\%$ will depend on the proper calibration [30]. An official AOAC method describes a schematic of the electric circuit used to measure the conductivity of raisins and prunes [32]. To determine moisture by a conductivity meter, the food is placed between two electrodes and the current flowing through the sample is measured by the change of electrical resistance [30]. Conductance readings are converted into moisture contents using a table that corrects for sample temperature [32]. Conductivity methods measure only the free water in the sample; the amount of bound water needs to be added to more closely approximate the total moisture content [6]. Conversion charts may be required due to the variation of bound water among samples. The accuracy and precision of the conductivity method are affected by moisture distribution, temperature, and electrolyte content as well as the quality of contact between the electrodes and samples, as in the conditions of the dielectric capacitance method [6,30].

3.7.2.7 *Sonic and Ultrasonic Absorption*

The degree of absorption of sound energy would depend on the type of medium through which it is transmitted [30]. Thus, as moisture content of the medium changes, so too does the amount of sonic and ultrasonic absorption [6]. Using this principle, ultrasonic velocity measurements have been developed for the determination of moisture content [30]. In addition to absorption of sound energy, the high frequency of ultrasonic waves also enables their reflection and refraction [6].

In this ultrasonic absorption method, the food sample is positioned between an energy generator and microphone for analysis [6]. The energy output of the sample is amplified, yielding a voltmeter reading. Voltmeter readings are converted into moisture contents using an appropriate standard curve. Ultrasonic methods have been used in laboratory settings to determine the composition, including moisture content, of chicken [44] and dry fermented sausages [45]. This ultrasonic method was shown to be rapid and nondestructive, and the analytical results were similar to other standard methods [44].

3.7.2.8 *Cryoscopic Methods*

The cryoscopic method is another indirect method for analyzing moisture, which utilizes the colligative property known as freezing-point depression [1,18]. The freezing point of water decreases as the concentration of dissolved solutes increases. Therefore, the freezing point is inversely correlated with the amount of water present for liquids containing a constant type of solute [18]. The most common use of this method is to measure water added to fluid milk [1,18], although it could be applied to other foods.

3.7.2.9 *Other Indirect Instrumental Methods*

There are several other indirect methods that can be applied for determination of moisture in foods, but require substantial instrumentation. These methods include nuclear magnetic resonance

(NMR) [1,6,30,46], mass spectrometry [30], and neutron scattering [30]. These techniques are less frequently employed methods; therefore, they are briefly introduced in this section, and the details can be found in other sources [30].

NMR is a fast and nondestructive method of moisture determination [1,6,30,46]. This technique utilizes the nuclear properties of its protons, instead of utilizing properties of water [6,30,46]. The challenge is to differentiate the proton NMR signal of water from the other hydrogen-containing substances in the food. The accuracy of NMR techniques is approximately 0.2% [6]. Modern NMR techniques have been described for determination of moisture contents [47]. Table 3.5 lists the advantages and disadvantages of NMR methods.

Mass spectrometry is another indirect method for moisture determination [30]. Although mass spectrometry has been widely utilized for identification of unknown substances in food samples [1], the quantification of water is more problematic [18,33]. The greatest problem lies in the “memory effect” or carry-over effect from the already analyzed sample, which influences the results of the next sample [9,30]. Some success has been obtained by reacting cryogenically concentrated moisture with calcium carbide to yield acetylene, which is then quantified [9]. However, mass spectrometry remains an uncommon method for moisture determination due to its uncertainty.

Neutron and γ -ray scattering occur when energized neutrons interact with nuclei. Hydrogen atoms are the most effective at scattering neutrons; this forms the basis of a determination of moisture using neutron-scattering methods [9,30]. As with the NMR method discussed earlier, neutrons will scatter from any hydrogen nucleus, not just those associated with water. Thus, neutron-scattering methods are most appropriate for samples low in nonaqueous protons, such as inorganic substances [9,30]. The application of the neutron-scattering method to moisture determination in foods is virtually nonexistent, because food is primarily composed of proton-rich organic material.

3.7.2.10 Summary of Indirect Methods

The indirect methods are generally faster than the direct methods for moisture determination. When done properly, the indirect methods can be as accurate and precise as the more standard methods. However, the accuracy and precision of the indirect methods depend on careful preparation and analysis of known standards to establish reliable calibration curves. Although most indirect methods require a large capital investment in equipment, the potential application for rapid on-line quality control might make the investment worthwhile. Nevertheless, preparation of the standards and accurate calibration curves must be verified by a specific direct method to establish a reliable indirect method of instrumentation that can achieve accurate and precise predicted values.

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Chapter 4

Ingredients: Meat, Fat, and Salt

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4.1 Introduction

There is a great variability in processed meat and poultry products, most of which include meat, fat, and salt as the basic ingredients in their formulation. Meat is the main source of their nutritional value, supplying protein and also vitamins (B group) and minerals (Fe, P, Mg). Fat is an important ingredient from the sensorial point of view, because it contributes in a decisive way to properties such as texture and juiciness, and also plays an important role in aroma, both directly and as a consequence of compounds derived from lipolysis and oxidation reactions. Fat is also an important ingredient from the nutritional point of view. Its fatty acid profile and the cholesterol content make fat an important ingredient that should be well characterized. Salt is necessary to guarantee the stability of meat products, being also clearly involved in the functionality of proteins, as well as in the final taste of the product.

The objective of this chapter is to describe the main parameters to be taken into account in relation to the technological quality of these ingredients when used in processed meat and poultry products. The main methodologies used for their determination are briefly described.

4.2 Analysis of the Technological Quality of Meat

The final quality of meat processed products depends mainly on the quality of the meat used in their production. Technological meat quality depends on multiple factors, such as genetic background, differences in breeds or rearing systems, transport and slaughter conditions, individual stress reactivity,¹ and postmortem manipulation of carcasses. These factors affect ante- and postmortem muscle metabolism, amount and type of collagen (connective tissue), intramuscular fat content, and the color and texture of fresh meat. The analysis of pH, color, and meat texture gives rise to its categorization as normal; pallid, soft, and exudative (PSE); or dark, firm, and dry (DFD) meat. Moisture and water-holding capacity (WHC) are important properties (see Chapter 3) that determine the sensory quality of the final product and also the process yield after treatments such as curing and cooking. The amount and type of connective tissue and intramuscular fat, especially in products such as ham and

cured ham,^{2,3} are also related to the technological quality of meat. Proteolytic processes (Chapter 12) are also involved in the development of textural and sensorial characteristics of raw meat.⁴

Most of the current techniques to determine meat quality parameters are extremely time-consuming, expensive, and destructive; therefore, the development of fast and nondestructive methods are of interest for on-line prediction of the technological and sensory qualities of meat.⁵

Table 4.1 summarizes the main parameters in the analysis of meat as an ingredient in processed products.

4.2.1 Carcass Classification

There are different criteria to classify the carcass, depending on the meat species. However, as a consequence of the current nutritional recommendations, the lean content of carcasses can be considered one of the main criteria to be taken into account when estimating its quality and that of the meat products produced from it.

Several on-line instruments allow the classification of carcasses according to their lean meat content: noninvasive methods such as rulers, calipers, and ultrasound instruments; and invasive methods based on probes or metal tubes, which are inserted into the carcass, giving a reflection curve that indicates the fat and muscle thickness.⁶ Information about on-line instruments to be used in pigs for this purpose is gathered on www.eupigclass.net.

4.2.2 pH

pH is one of the most important and common parameters to be measured in meat to determine its suitability for processed food products, because the rate and extent of postmortem pH decline largely govern meat quality attributes. It is known that, as a consequence of normal metabolism during postmortem processes, pH falls normally from 7.0–7.2 to 5.5–6.5. However, abnormal processes might take place in some cases, giving rise to an excessively rapid decline of pH (PSE meat) or to a high final pH (DFD meat). Two other categories, reddish, soft, and exudative³⁵ and pale, firm, and nonexudative Nam et al.³⁶ have been recognized recently as major quality defects in Canada.³⁷

Final pH is related to the potential water-binding capacity of meat, although the addition of salt and additives such as phosphates can also significantly affect it.

4.2.2.1 Electrode

The measurement of pH is usually done with a voltmeter equipped with a glass electrode. The electrode is introduced into the meat, obtaining the result in a short period of time.

It is a fast, easy, and nondestructive method.

4.2.2.2 Nuclear Magnetic Resonance

Monin⁷ in a review of the methods for predicting the quality of whole meat, pointed out that techniques relying on local electrical stimulation for 1–3 min followed by pH measurement have been developed,^{38,39} but none had been put into practice. These authors also presented the possibility of using nuclear magnetic resonance (NMR) as an easier alternative to determine the pH in comparison to chemical techniques. This technique evaluates the changes in muscle energy through the

Table 4.1 Analysis of Meat as an Ingredient of Processed Products

<i>Properties</i>	<i>Parameters</i>	<i>Methodology</i>	<i>Characteristics</i>	<i>Reference</i>
Carcass quality	Fat and lean content	Ruler/hand calipers/ultrasound	Probes	6
	pH	Electrode (voltmeter)	Quick Easy	7
Protein-binding capacity	Water/protein and Feder number Cooking loss Napole Yield Thawing loss Hydroxyproline Fiber optic probe	31P NMR (measure of changes in muscle energetics)	Nondestructive Useful only in laboratory applications Expensive	8–11
		Chemical analysis		12
		Weight measures	Simple Time-consuming Destructive	13–16
		Colorimetry	Official method	17
		UV fluorescence	On-line Variability depending on pH and myoglobin concentration	18
Collagen, texture, lipid content	Image analysis Curie point pyrolysis-mass spectrometry	Uses microscopic images of tissues		19
		Thermal fragmentation of compounds and detection of produced ions	Gives information on several different parameters simultaneously Expensive Destructive	20

Marbling (intramuscular fat content)	Total fat	Solvent extraction	Official method	21
	Computer image analysis CIE L* a* b*	Images obtained at different wavelengths Spectrophotometer and colorimeter	Expensive Time-consuming Application on-line Quick Nondestructive	22-25 26
Color	Computer vision	Analysis of .jpegimages	Quick Supplies different types of information	27
	Visual evaluation	Assessment by trained panel	Time-consuming	60
	Shear test	Specific texture analyzer	Largely influenced by cooking procedures	28
	Tensile method	A load deformation curve to complete rupture is obtained	In raw and cooked meat	29
	TPA	A plunger is driven twice into the meat and force- deformation curves are recorded	Quite well related to test panel	30,31
Color, texture	NIR spectroscopy	Measurement of acoustic parameters related to physical properties of matrix	Rapid Nondestructive Can easily estimate sensory properties and other composition characteristics Needs further development	5,32,33 34

measurement of phosphorylated compounds such as adenosine triphosphate, creatine phosphate, sugar phosphates, and inorganic phosphate (Pi). The pH can be evaluated from the position of the Pi resonance (^{31}P NMR), enabling the investigation of pH heterogeneity within muscle tissue and even the prediction of the ultimate quality (normal, PSE, DFD) from excised muscle samples measured at 30 min postmortem.

This technique is quite expensive and only useful for laboratory applications.^{8–11}

4.2.3 Analysis of Water/Protein Ratio and Processing Yield

Muscle contains about 75% water. Distribution of the water is 60% bound to myofibrils, 25% in sarcoplasm, and 15% in connective tissues and extracellular space. The ability of meat to retain water is an important property in most processed meat products. Moisture losses can significantly affect the sensory quality of the end product, and also the weight, and therefore the price. This property has especial relevance in cooked products, in which the heat treatment gives rise to the denaturation of proteins and therefore to the loss of their capacity to bind water molecules.

4.2.3.1 Water/Protein

The water/protein ratio is an interesting index used in the control of meat and meat products. It is based on protein capacity to bind water molecules. In some countries, such as in France, a factor known as Feder number is used for control purposes¹²:

$$\text{Feder number} = \text{water (\%)/organic nonfat (\%)}$$

where organic nonfat (%) = 100 – fat (%) + ash (%) + water (%).

4.2.3.2 Cooking Loss, Napole Yield, and Thawing Loss

Cooking loss and Napole yield are two important technological quality attributes. They are related to the WHC of meat during storage and processing, and could have important economic consequences.¹³ In the case of frozen meat, the influence of freezing procedure and freezer storage on the processed meat quality attributes can also be measured by the thawing loss.^{14,15}

Procedure for the determination of cooking loss. Samples (approximately 1 cm³) are weighted before (w1) and after (w2) they are cooked (water bath 10 min, 85°C). The cooking loss is calculated according to the following equation:

$$\text{Cooking loss} = 100\% \left(\frac{w1 - w2}{w1} \right)$$

Procedure for the determination of Napole yield.^{13,40} Samples (approximately 1 cm³) are weighed (w1) and placed for 24 h in 2 mL of a 13.6% NaCl solution containing nitrite (0.6% nitrite in NaCl) at 4°C. Subsequently, the sample is exposed to heat treatment in a water bath at 85°C for 10 min, equivalent to a core temperature of 75°C. Finally, the sample is lightly dabbed and weighed (w2). Napole yield is determined according to the following equation:

$$\text{Napole yield} = 100\% \left(1 - \frac{w1 - w2}{w1} \right)$$

*Procedure for the determination of thawing loss.*¹⁶ Frozen samples are weighted (w_1) then thawed over a period of 16 h at 5°C and weighed again (w_2). Thawing loss is determined according to the following equation:

$$\text{Thawing loss} = 100\% \left(\frac{w_1 - w_2}{w_2} \right)$$

These techniques are simple but time-consuming and destructive.

4.2.4 *Connective Tissue*

Meat pieces used for some processed meats are usually rich in connective tissue. As is widely known, these tissues do not have the high-quality protein (biological value) found in the rest of the muscle; neither is the technological quality as good as the muscle tissues. Meats with a high proportion of connective tissues are harder and also have less water-binding capacity.

Connective tissues are mainly formed from fibers of collagen and small amounts of elastin. In general, it is assumed that the amount of connective tissue is approximately the same as the amount of collagen. The collagen of young animals is partially cross-linked, and flexible but relatively inelastic, but when the animal grows it becomes more inflexible and toughness increases. Total collagen content has seemed to be the best predictor of tenderness among muscles.⁴¹ The most common method to determine collagen content is the hydroxyproline (HyP) determination, as this is a amino acid characteristic of collagen (collagenous connective tissue contains 12.5% HyP).

4.2.4.1 *Determination of Hydroxyproline*

The most common methodology is a colorimetric method.¹⁷

Briefly, a meat sample (4 g) is hydrolyzed in H_2SO_4 at 105°C, filtered, and diluted (final dilution should be in the range 0.5–2.4 $\mu\text{g}/\text{mL}$). Then HyP is oxidized with chloramine-T to pyrrole. The red–purple color that develops after addition of 4-dimethylaminobenzaldehyde is measured photometrically at 560 nm.

The total content of HyP can be transformed to the collagen amount using the following equation:

$$\text{Total collagen (\%)} = \text{HyP (\%)} \times 8$$

In Germany, the connective-tissue-protein-free meat-protein (bindesgewebeeisweiß-frei-Fleischeisweiß) factor is determined from the amount of HyP through the following transformations¹²:

$$\text{Collagen} = N (\%) \times 5.55$$

$$\text{Dry connective tissue} = \text{HyP (\%)} \times 8$$

$$N \text{ due to connective tissue} = \text{HyP (\%)} \times 8/5.55 = 1.42 \times \text{HyP (\%)}$$

$$\text{Muscle protein} = 6.25 (N [\%] - 1.42 \times \text{HyP} [\%])$$

4.2.4.2 *Ultraviolet Fiber Optic Probe Measurements of Connective Tissue*

Several works have demonstrated that it is possible to detect connective tissues due to their auto-fluorescent properties using a probe with a single optical fiber to detect the ultraviolet (UV) fluorescence of collagen and elastin.^{18,42–48} Measurements are correlated with taste panel scores for chewiness, and have been used also for studying in detail the characteristics of the different types of connective tissue.

The probe detects collagen and elastin fluorescence through a single optical fiber as it penetrates the meat.¹⁸ The data for each transection are divided by the minimum value in the transection, to scale the data to a common baseline equivalent to a window of lean meat without any connective tissue fluorescence. The window of minimum fluorescence (devoid of connective tissue) has a low level of UV reflectance at wavelengths where the cutoff filters for excitation and emission overlap. This provides a partial correction for moderate drifting in UV source intensity. Height, width, area, and smooth intervals of the resulting peaks provide information on the characteristics of the connective tissue of meat.

The limit of the determination of connective tissue by this method is that the measures can be affected by variations in pH and myoglobin concentrations. However, the probes cause very little damage to the carcass, and are fast enough to be used at typical line speeds.

4.2.4.3 *Determination of Connective Tissue by Magnetic Resonance Microscopy*

Studies cited by Monin⁷ indicate the possibility of the analysis of connective tissue using magnetic resonance microscopy. Research by Barra et al.⁴⁹ showed that two-dimensional images have been obtained with a spatial resolution of 50 μm , which allows the visualization of the perimysium.

Bonny et al.⁵⁰ concluded that this nondestructive and noninvasive method can be successfully used for characterizing the muscle connective tissue structure and not only for studying the relationships between connective tissue distribution and meat quality, but also for making *in vivo* experiments to provide insight into the morphology and development of connective tissue as a function of age, breed, and rearing techniques.

4.2.4.4 *Determination of Intramuscular Connective Tissue by Image Analysis Application*

Del Moral et al.¹⁹ have developed a method to permit the automatic, accurate, objective, and reliable quantification of intramuscular connective tissue and fiber retraction in muscle. It is based on digital analysis of microscopic images of meat tissue. It can analyze 20 images per minute, and offers more precise measurements compared with conventional morphometric methods.

4.2.4.5 *Curie Point Pyrolysis-Mass Spectrometry*

The Curie point pyrolysis–mass spectrometry method has been evaluated by Sebastian et al.²⁰ as a potential methodology to predict the content of collagen and lipids and also the texture of meat. The authors observed that with the application of this technique, the mean prediction error was 10% for lipids, 11% for collagen, and 12% for texture.

This technique allows fingerprinting the overall composition of the matrix of products. It detects positive ions produced by electron impact fragmentation of the thermal fragmentation products of the studied samples.

Briefly, the method consists of applying high temperature (530°C) to get a balanced fragmentation of glucidic, lipid, and protein fractions. The pyrolysate then enters an expansion chamber heated to 160°C, where it diffuses through a molecular beam tube (170°C) of the ionization chamber of the mass spectrometer (180°C). Nonionized molecules are retained on a cold trap cooled by liquid nitrogen. The mass spectrometer scans the ionized pyrolysate 65 times during the pyrolysis. Data are collected as a range of atomic mass units and spectral information on ion counts for the individual masses scanned.

An interesting advantage of this method is that it can give information about protein quality, lipid content, and texture at the same time. Therefore, it can predict the quality of the processed products. However, it is expensive, time-consuming, destructive, and has not been sufficiently investigated.

4.2.5 Intramuscular Fat Content

The amount of intramuscular fat content is an important parameter related to the sensory quality of final meat products. It can be determined by different methods.

4.2.5.1 Quantitative Extraction

The most traditional technique is the quantitative determination of fat through a solvent extraction with petroleum ether.⁵¹ The soluble material is extracted from dried test samples of meat by a two-step treatment with petroleum ether solvent (Soxhlet extraction procedure). Another, less frequently used official method to determine fat in meat is by rapid microwave-solvent extraction using CH_2Cl_2 as solvent, requiring a microprocessor for digital readout,⁵² and by rapid specific gravity using C_2Cl_2 .²¹

4.2.5.2 Systems Based on Computer-Image Analysis

Research has investigated the ability of different instrumental visions to assess the marbling in beef and pork meat.^{22,53–56}

Faucitano²² developed a technique for a quantitative description of marbling fat by means of computer image analysis, observing that the obtained results were significantly correlated with intramuscular fat content and also with tenderness.

These results agreed with those previously obtained by Albrecht et al.²³ and Gerrard et al.,²⁴ applying the same technique for the marbling analysis in beef.

One of the most recent methods is the one developed by Qiao et al.²⁵ using hyperspectral imaging techniques that provide not only spatial information, as do regular imaging systems, but also spectral information for each pixel within an image. This technique can be used to obtain information about molecular composition and also for quality assessment of different types of food. It has been successfully used to inspect the contamination of chicken carcasses^{57,58} and also to estimate marbling scores of pork by image texture indices extracted from a digitalized meat marbling standard.²⁵

Samples cut into 1-cm-thick chops are transported by a conveyor into the field of view of a line scan spectrograph. Images are acquired at different wavelengths, and they are appropriately processed. The advantage of this method is that it gives objective measurements, whereas the disadvantages are that it is expensive and time-consuming, and has to be applied in the laboratory, not on-line.

For the analysis of the lipid composition and oxidation status of this fraction of meat see Section 4.3 of this chapter.

4.2.6 *Color and Texture Properties of Meat*

The color and texture of final meat products can be greatly influenced by the quality of these properties in the meat used as an ingredient. It has been shown that properties such as water-binding capacity, intramuscular fat, and connective tissue are related to these two properties, and their analysis can be used to predict them. They can also be directly measured in fresh meat.

4.2.6.1 *Instrumental Measurement of Color*

4.2.6.1.1 *Pigment Content and Spectrophotometric Measurements*

Early methods of evaluating the color of meat products in an objective way involved the determination of pigment content using extraction techniques that do not prevent the conversion of one myoglobin form to another and provide no reliable information on pigment form stability.^{59–62} These methods involve the use of chemical reagents and are more time-consuming than making physical measurements such as spectral reflectance curves by spectrophotometers, which are usually determined in the visible wavelength region between 400 and 700 nm, at intervals of 10 nm. The recommended parameters are a light source of D65 and standard observer at 10°. The aperture should be as large as possible as supplied for the instrument (within the limitations of the sample to be measured). The measures are done through an overwrap film that normally covers the sample, which should be at least 1 cm thick to be opaque. From these spectral curves, it is possible to calculate the tristimulus values defined in 1931 (<http://www.cie.co.at/>) by the International Commission on Illumination (CIE) (X,Y,Z). Several mathematical transformations of these values can be used to obtain the three color coordinates that describe a color in different color spaces systems. One of these coordinates concerns the parameter lightness (L^* in the CIE $L^*a^*b^*$ system, L in the Hunter Lab system, or Y in the xyY system⁶³) and the two others concern chromaticity (a^*,b^* in the CIE $L^*a^*b^*$ system; a,b in the Hunter Lab system; and xy in the xyY system). In the CIE $L^*a^*b^*$ system, which is the most popular numerical color space system in the food industry,²⁶ a^* measures the red and green characteristics, whereas b^* measures the yellow and blue characteristics. L^* has been successfully used for assessing the quality of the raw material before production of hams, confirming a significant relationship between visually assessed meat and this parameter.⁶⁴ Other useful parameters that are obtained from combinations of $L^*a^*b^*$ are ΔE , which measures total color change by accounting for combined changes in L^* , a^* , and b^* , ΔC Chroma, which measures bone marrow discoloration $(a^2 + b^2)^{1/2}$, and Hue ΔH ($\arctg b/a$). Chromometers can be also used to obtain the chromatic values directly without reflectance spectra.

Advantages. Nondestructive. Repeated measurements over time can be made on the same sample. Easy to use and rapid.

Disadvantages. In some species/muscles, differences of considerable magnitude exist between lateral and medial sites on the cross-section of the muscle. To overcome this problem, several measurements should be made. Location within a muscle should also be considered when making instrumental color measurements.

4.2.6.1.2 Computer Vision

Computer vision based on analysis of digital camera images has distinct advantages over traditional color evaluation.²⁷ O'Sullivan et al.³⁰ noted several benefits associated with digital-camera-derived jpeg (Joint Photographic Experts Group) images, including (a) as opposed to a colorimeter, only a single digital observation is needed for a representative assessment of color, (b) digital images can account for surface variation in myoglobin redox state, and (c) digital image data can be converted to numerous color measurement systems (Hunter, CIE, XYZ, etc.).

4.2.6.1.3 Visual Color

Visual determinations are the gold standard for assessing treatment effects and estimating consumer perception. To perform this type of measurement, a trained panel and standardized conditions are crucial. To maximize appearance yet to minimize photooxidation, recommended lighting is 1614 lux (150 fc) of fluorescent lighting, which should have a color temperature of 3000–3500 K (lamps such as deluxe warm white, natural, deluxe cool white, SP 3000, SP 3500). Cool white and lamps giving unreal pink, blue, or green tints should be avoided. Detailed descriptions of visual color scales used for different purposes can be found in the *Guidelines for Meat Color Evaluation*, published by the American Meat Science Association in 1991.⁵⁹

4.2.6.2 Instrumental Measure of Texture

Methods for the assessment of meat tenderness are extremely variable in terms of approach and usefulness. Those proposed by a group of experts at the 41st International Congress of Meat Science and Technology are the Warner Bratzler shear test, the tensile test method, and the texture profile analysis (TPA).³⁹

The shear test is most useful when the influence of connective tissue is low and variations in the myofibrillar component are to be measured. The parameters to be measured in cooked meat from the force deformation curve are the peak force (the maximum recorded Warner Bratzler peak force) and the total energy.

The tensile method is best suited for structural investigations and can be carried out on raw or cooked meat; the parameter to be measured is breaking strength.²⁹ The TPA resembles the process of mastication and ease of the first bite between the teeth.³¹ The following parameters should be recorded: hardness (N), maximum force required to compress the sample; springiness (m), ability of the sample to recover its original form after deforming force was removed; adhesiveness ($N \times s$), area under the abscissa after the first compression; cohesiveness, extent to which the sample could be deformed before rupture; and chewiness (J), work required to masticate the sample before swallowing. These measurements are made instrumentally by texture analyzers.⁶⁵

4.2.6.3 *Near Infrared Spectroscopy and Ultrasonic Methods to Predict Texture and Color*

Hildrum et al.³² showed that sensory hardness and tenderness can be predicted by near infrared (NIR) spectroscopy, which is a rapid and nondestructive technique when applied on meat cuts. The efficacy of this technique for predicting toughness was confirmed by Byrne et al.,³³ who did not find so predictive results for sensory tenderness and texture.

Leroy et al.⁵ tried to predict technological and organoleptic properties of beef cuts by NIR reflectance and transmission spectra. NIR analyses were performed in reflectance and transmission modes with a Fourier transform spectrometer. Spectral acquisition in reflectance mode was performed with a fiber-optic probe in the 4000–12000 cm^{-1} range at 16 cm^{-1} . NIR transmission analyses were made with the Bag SamplIR accessory, obtaining spectra in the same range (between 4000 and 12000 cm^{-1}). The NIR spectra collected on fresh meat showed good potential to predict CIE L^* and b^* parameters in reflectance mode. For the parameters of WHC and tenderness, the accuracy of the predictive models seemed to be weak.

The ultrasonic method is based on the measurement of acoustic parameters that are closely related to physical properties of the propagating medium. The propagation of ultrasound is closely dependent on its mechanical properties. In the case of meat, the distribution and the amount of both fat and collagen in the muscle play an important role in the constitution and organization of the connective tissue, and consequently in their propagating properties.

Ultrasound has been used to estimate beef carcass composition,^{66,67} to study sensory attributes,⁶⁸ to evaluate the marbling pattern or distribution of animal fat in live beef animals,⁶⁹ and to evaluate hardness and compression work.⁷⁰

This method is recognized as accurate, rapid, nondestructive, noninvasive, relatively inexpensive, and suitable for on-line applications. However, further development of the technique is still needed before it can be concluded that it has potential for predicting some meat quality traits.³⁴

4.3 **Analysis of the Technological Quality of Fat**

The amount and nature of the fat used as an ingredient are determining factors in the final quality of processed meat and poultry. Fat composition (fatty acid profile and lipid fractions distribution) and its susceptibility to oxidation and lipolytic processes significantly affect the texture and the flavor of the final products.⁷¹ Table 4.2 summarizes the main parameters dealing with the analysis of fat as an ingredient of processed products.

4.3.1 **Fatty Acid Profile**

The fatty acid profile of both meat and fat is clearly involved in the nutritional quality of meat and meat products, a topic that will be discussed in Part 2 of this book. However, its role in fat quality and implications for meat products production are also worth mentioning. Effectively, the fatty acid profile of the fat used in processed meats has great influence in their organoleptic properties, especially texture and flavor, and also in their susceptibility to oxidation. A high unsaturation index in meat and meat products may affect their oxidative stability, because the unsaturated fatty acids are more prone to oxidation.^{86–90} Different methods have long been used for its evaluation.

Table 4.2 Analysis of Fat as an Ingredient of Processed Products

<i>Properties</i>	<i>Parameters</i>	<i>Methodology</i>	<i>Characteristics</i>	<i>Reference</i>
Fatty acid profile		Lipid extraction–derivatization and GC–FID detection	Detailed description of the content of each fatty acid	72–74
Lipid fractions	Neutral lipids, free fatty acids, phospholipids	TLC Minicolumns SPE	Quantified by densitometry Further evaluation of each fraction by GC or HPLC	75 76,77
Cholesterol		HPLC–UV–Vis detection GC–FID detection	Simultaneous detection of tocopherols and carotenes	78,79 80,81
Fat firmness	Melting point	Capillary method		82–85
Oxidation status	thiobarbituric acid test (TBA) peroxides, hexanal, anisidine, etc.	Described in detail in Chapter 10		

4.3.1.1 Gas Chromatography

Gas chromatography (GC) has been the most common approach used to determine the fatty acid profiles in different types of meat matrix.^{72–74} To analyze these lipidic components, it is necessary to transform them into low–molecular-weight, less polar, more volatile analytes, normally methyl esters. In addition, it may be advisable to mask other polar functional groups in a similar manner, or to prepare specific derivatives as an aid to identification.

In the early GC studies, a variety of support materials for packed columns, liquid phases of different polarities and detector systems, mostly thermal conductivity, and later flame ionization detectors (FID) were available for analytical work, whereas the more reliable analysis of minor fatty acid isomers did not become possible until the introduction of capillary columns. Polyethylene glycol–type stationary phases have proved effective for the separation of the most important saturated and polyunsaturated fatty acids, generally with few problems from coelutions.

The whole procedure includes the following steps: Lipid extraction, derivatization, chromatographic development, and quantification.

Lipid extraction. The selection of solvents, the mode of extraction of lipids, and in some cases, the purification and separation of lipid classes by solid phase extraction (SPE) have been extensively studied. Most lipid analysts use chloroform-methanol (2:1 by volume), with the endogenous water in the tissue as a ternary component of the system, following the widely used method of Folch et al.⁹¹ and that of Bligh and Dyer.⁹²

Derivatization. Methyl ester derivatives of fatty acids (FAME) are the most common derivatives formed for GC fatty acid analysis. They can be prepared either by acid-catalyzed esterification and transesterification, by base-catalyzed transesterification, or by using diazomethane.

Chromatographic development. Chromatographic conditions are largely conditioned by the length and internal diameter of the column, the carrier gas flow, the resolution required, the size of sample, and time of analysis, and consequently should be adapted in each case.

Quantification. Quantification of FAME by GC with FID has been effectively and widely performed, whereas detection with mass spectrometry (MS) has been traditionally used only for qualitative analysis. In some instances, the methyl ester derivatives alone give adequate mass spectra for identification purposes. More often, it is preferable to prepare pyrrolidides or picolinyl esters, because these give characteristic fragmentations that permit the location of many functional groups, including double bonds and methyl branches, in aliphatic chains. Sometimes it is necessary to prepare derivatives of other functional groups to facilitate chromatography and to ensure that interpretable mass spectra are obtained. Nevertheless, according to Dodds et al.,⁹³ the quantitative performance of GC-MS can be satisfactorily compared with that of GC-FID.

4.3.1.2 *Reversed-Phase High-Performance Liquid Chromatography*

Before GC was developed, liquid–liquid partition chromatography was the most useful technique for separating individual fatty acids from natural mixtures. The most widely used stationary phase consists of octadecylsilyl (C18 or octadecylsilyl [ODS]) groups, linked to a silanol surface by covalent bonds, although C₈ phases are increasingly being found to have utility. Unsaturated fatty acids are eluted appreciably ahead of the saturated fatty acids of the same chain length, each double bond reducing the retention time by the equivalent of about two carbon atoms.⁹⁴

4.3.2 *Lipid Fractions*

Not only fatty acid profile but also the total distribution of lipids in the different fractions (triglycerides, monoglycerides, diglycerides, free fatty acids, and phospholipids) determine the extent and intensity of lipolysis and oxidation, affecting the properties of the final meat products. Lipolysis and its repercussions on the amounts of different lipid fractions are extensively treated in Chapter 13 of this book.

The separation of the different lipid classes can be performed by thin-layer chromatography (TLC) on silica gel G-60 plates developed with petroleum ether/diethyl ether/acetic acid (80/20/1) (v/v/v), using triolein, diolein, monoolein, oleic acid, and cholesterol as reference standards. A spray of a 0.05% FeCl₃ · 6H₂O solution in a mixture of water/acetic acid/sulfuric acid (90/5/5)(v/v/v), followed by heating in an oven at 120°C for 30 min, is used to visualize all lipid fractions. Lipid classes can be then quantified by densitometry at 390 nm using calibration curves for the standards employed in TLC analysis.⁷⁵

Different SPE methods have also been proposed and widely used for analysis of lipid fractions (neutral lipids [NL], free fatty acids, and phospholipids [PL]) in muscle foods.^{76,95} After a comparison of both methods, Ruiz et al.⁹⁶ concluded that the method of Pinkart et al.⁷⁶ was more convenient, as no coelutions were observed. Briefly, intramuscular fat is dissolved in hexane/chloroform/methanol (95/3/2) and added to a previously activated aminopropyl minicolumn. NL are eluted with chloroform and FFA with diethyl ether/acetic acid (98/2). PL are eluted in two different fractions (the first one with methanol/chloroform [6/1] and the second one with sodium acetate in methanol/chloroform [6/1]).

García-Regueiro et al.⁷⁷ developed a method for the determination of neutral lipids by capillary GC and high-performance liquid chromatography (HPLC) using prior separation of

neutral lipids, employing minicolumns of aminopropyl and silica stationary phases, into three fractions—triglyceride, cholesteryl esters and cholesterol, and mono- and diglycerides.

Information on the exact composition of a triacylglycerides (TAG) mixture is crucial for understanding the behavior of the fat during processing. The full characterization of a TAG mixture requires the separation of all individual TAG, followed by quantification of the separated species. For that purpose, combination of different analytical methods is needed. The combination of chromatography and mass spectrometry is a powerful tool, giving detailed information on different parameters. Also, HPLC coupled with an evaporative light scattering detector has been successfully used by Tejada et al.⁹⁷

Comprehensive chromatography is a new approach toward chromatography that allows a sample to be separated according to two independent axes. In the case of the application into TAG separation, the most promising combinations are (a) comprehensively coupled silver phase liquid chromatography \times carbon number GC, or (b) coupled silver phase \times FAME GC.⁹⁸ Using the new comprehensive setups, three-dimensional graphs are obtained that represent the separation of triglycerides according to two independent parameters: carbon number versus number of double bonds, or fatty acid composition versus number of double bonds. The information content of such graphs by far exceeds that of the current generation of analytical methods.

Among the advantages of comprehensive chromatographic methods⁹⁹ are (a) the enhancing resolving power, (b) the possibility of two-dimensional chromatogram formation of chemically similar compound patterns, and (c) the enhanced sensitivity. However, some drawbacks can also be mentioned: (a) higher costs of a GC \times GC instrument and gases, (b) difficult quantitation, and (c) method optimization is much more complex compared to conventional GC and is normally a question of compromise.

4.3.3 Cholesterol

The amount of cholesterol in fat and lean meat is basically of interest from a nutritional point of view. However, a brief consideration about the methodologies used for its analysis is included in this chapter.

The most usual method is CG including direct saponification of the food sample and extraction of the unsaponifiable matter with an organic solvent like hexane, rather than the initial lipid extraction usually performed using a chloroform–methanol mixture or diethylether as solvents, followed by saponification of the polar fraction.⁸⁰ A derivatization for obtaining the trimethylsilyl ethers can be carried out before the injection in the GC and quantification using cholestane or dihydrocholesterol as internal standards.⁸¹ HPLC methodologies with different detectors are also set up and validated for cholesterol quantification purposes.^{78,79}

Also, cholesterol can be oxidized to cholesterol oxidation product (COPs). The methodology for their determination by GC-MS analysis can be found in different papers.^{100–102} It consists basically of a process that includes saponification (KOH in methanol, preferably at room temperature), extraction with diethylether, purification with silica cartridges, and derivatization of COPs to obtain the trimethylsilyl ethers for further chromatographic analysis. HPLC analysis of this type of compound has also been performed.^{103,104}

4.3.4 Fat Firmness

Fat tissue firmness is a fatty acid–related technological meat quality parameter, as well as shelf-life and flavor, which are the scope of other chapters in this book. In pork, beef, and lamb, the melting point of lipid and the firmness/hardness of carcass fat are closely related to the concentration of stearic acid

(18:0).¹⁰⁵ In the 18C fatty acid series, stearic acid (18:0) melts at 69.6°C, oleic acid (18:1) at 13.4°C, 18:2 at -5°C, and 18:3 at -11°C. Thus, as unsaturation increases, melting point declines, having a significant effect from the technological point of view. For instance, in pork and beef frankfurters, the final target comminution temperature is about 16–18°C, but it is only 10–12°C for poultry meat products, due to the greater unsaturation of poultry lipid and its lower melting point.¹⁰⁶

The melting point of fat is measured by the capillary tube method described both by an Association of Official Analytical Chemists (AOAC) official method⁸² and a British Standards Institution method.⁸³ Fat is introduced in a thin-wall capillary tube and, after overnight refrigeration, it is subjected to a slow increase in temperature. The temperature at which the fat becomes transparent in the capillary tubes is recorded as the melting point.^{84,85}

4.3.5 Oxidation Status

Lipid oxidation is one of the primary causes of deterioration in processed meats, especially in poultry products, generating undesirable odors and flavors, and limiting shelf-life and commercial stability.¹⁰⁷ Also, health problems are related to lipid oxidation products, which are related with cronical diseases^{108,109} and with direct toxicological effects, as in the case of cholesterol oxidation products.^{110–112} The different technological and culinary steps applied to the processed meats (mincing, heating, storage, cooking) can increase the lipid hydrolysis and oxidation.^{72,113–115} Therefore, control of the susceptibility of fat to these effects is needed.

The most common techniques used to determined the oxidation status of fat as an ingredient are peroxide value, anisidine values, TBA, malondialdehyde (MDA), hexanal, or phospholipids derived from lipid oxidation.¹¹⁶ The oxidation status of fat is extensively reviewed in Chapter 10.

4.4 Analysis of Salt

Salt (sodium chloride) is an important ingredient in meat- and poultry-derived products. It affects the sensory quality of the final products and also their stability. Most of these products are characterized by a typical salty taste requiring a minimal salt concentration. Although there have been many attempts to substitute the sodium chloride with other compounds, only a partial substitution has been successfully achieved. Salt is also involved in the textural properties of products, increasing meat and fat binding and WHC. Salt effectively restricts microbial growth, contributing in an efficient way, together with other factors such as acidity, preservatives, and heat treatments, to the stability of the products.

The salt used in processed meats is usually high-purity sodium chloride (more than 99%). The control of this component is only relevant concerning its final amount in the ready-to-eat product. There are several ways to obtain the total amount of salt; some of them determine the amount of chlorides and others determine the amount of sodium (Table 4.3).

Table 4.3 Analysis of Salt

<i>Properties</i>	<i>Parameters</i>	<i>Methodology</i>	<i>Characteristics</i>	<i>Reference</i>
NaCl amount	Chloride Sodium chloride Sodium	Volumetric method Ion selective methods Flame photometric methods	Official method	117

4.4.1 Volumetric Method

The volumetric method is the AOAC reference method for determining the salt content in meat.¹¹⁷

It is based on the precipitation of AgCl, adding AgNO₃ in the presence of nitric acid, and the further determination of excess Ag with NH₄SCN, employing ferric ammonium sulfate solution as an indicator. The indicator reacts with an excess of thiocyanate, forming a salmon-colored complex that indicates the endpoint of the titration.

4.4.2 Ion Selective Electrodes

Sodium can be measured with a selective ion electrode. Micro ion electrodes are available for NMR tubes.

4.4.3 Flame Photometric Method

A more precise technique is the flame photometric method. Meat has to be transformed into ash, which is then dissolved with acid. The flame photometer is set up with the appropriate standards, and then the sodium content of the sample solution is calculated by simple proportion.

The amount of salt can be determined with the respective transformations from the known amounts of chlorines or sodium.

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Chapter 5

Additives: Preservatives

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5.1 Introduction

Processed meats and poultry are extremely perishable products, and one of the principal agents of their spoilage is microorganisms (bacteria, yeasts, and molds). Microorganisms cause nutritional and sensory deterioration of meat products, producing loss of quality and limiting shelf life. Besides the economic implications of meat spoilage (deterioration of raw materials and processed products before they can be sold, loss of brand image, etc.), microorganisms can also be responsible for human illness.

A variety of preservation procedures have been tried to limit the speed and extent of such processes and their consequences. Traditionally, much use has been made of physical, chemical, and microbial methods of preservation. Preserving processed meats by chemical means is based, among other possibilities, on the use of additives known as preservatives. Preservatives are chemical

Table 5.1 Preservatives Permitted in the EU for Use in Meat Products

<i>E No.</i>	<i>Formula</i>	<i>Name</i>	<i>E No.</i>	<i>Formula</i>	<i>Name</i>
E-200	C ₆ H ₈ O ₂	Sorbic acid ^a	E-220	SO ₂	Sulphur dioxide ^b
E-202	C ₆ H ₇ O ₂ K	Potassium sorbate ^a	E-221	Na ₂ SO ₃	Sodium sulfite ^b
E-203	(C ₆ H ₇ O ₂) ₂ Ca	Calcium sorbate ^a	E-222	NaHSO ₃	Sodium hydrogen sulfite ^b
E-210	C ₇ H ₆ O ₂	Benzoic acid ^c	E-223	Na ₂ S ₂ O ₅	Sodium metabisulfite ^b
E-211	C ₇ H ₅ O ₂ Na	Sodium benzoate ^c	E-224	K ₂ S ₂ O ₅	Potassium metabisulfite ^b
E-212	C ₇ H ₅ O ₂ K	Potassium benzoate ^c	E-226	CaSO ₃	Calcium sulfite ^b
E-213	(C ₇ H ₅ O ₂) ₂ Ca	Calcium benzoate ^c	E-227	Ca(HSO ₃) ₂	Calcium hydrogen sulfite ^b
E-214	C ₉ H ₁₀ O ₃	Ethyl- <i>p</i> -hydroxybenzoate ^d	E-228	KHSO ₃	Potassium hydrogen sulfite ^b
E-215	C ₉ H ₁₀ O ₃ Na	Sodium ethyl- <i>p</i> -hydroxybenzoate ^d	E-249	KNO ₂	Potassium nitrite
E-218	C ₈ H ₈ O ₃	Methyl- <i>p</i> -hydroxybenzoate ^d	E-250	NaNO ₂	Sodium nitrite
E-219	C ₈ H ₈ O ₃ Na	Sodium methyl- <i>p</i> -hydroxybenzoate ^d	E-251	NaNO ₃	Sodium nitrate
			E-252	KNO ₃	Potassium nitrate

^a Abbreviation for this group: Sa.

^b Abbreviation for this group: SO₂. An SO₂ content of not more than 10 mg/kg is not considered to be present.

^c Abbreviation for this group: Ba.

^d Abbreviation for this group: PHB.

Note: The European Food Safety Authority² has recommended withdrawal of approval for propyl paraben; propyl-*p*-hydroxybenzoate (E-216), and sodium propyl-*p*-hydroxybenzoate (E-217).

E number (E No.) is used to classify food additive and signifies approval of an additive by the European Union.

Source: Directive No. 95/2/EC of the European Parliament and of the Council of February 20, 1995 on food additives other than colors and sweeteners; Directive 2006/52/EC of the European Parliament and of the Council of 5 July amending Directive 95/2/EC on food additives other than colors and sweeteners and Directive 94/35/EC on sweeteners for use in foodstuffs.

compounds that, when added to foods, inhibit, retard, or prevent the activity and growth of spoilage and pathogenic microorganisms. Their chief purposes are to extend the shelf life of foodstuffs by protecting them against deterioration caused by microorganisms, and to enhance their safety. Control and regulation of the use of preservatives is essential both to ensure their effectiveness and because in inappropriate amounts and conditions these additives can have adverse health effects. The use of preservatives is therefore subject to strict legal regulation to protect consumers, who are increasingly aware of aspects of food that affect health, most especially the presence of additives, and among these preservatives. Legislative requirements relating to the use of preservatives in meat products are regulated by the European Union (EU) through various European Community Directives^{1,2} (Tables 5.1 and 5.2).

In view of the importance of preservatives in terms of the law and food safety, it is essential to have accurate analytical methods. A variety of published analytical methods are available in the literature, mostly cited in reviews relating to their application to food analysis.³⁻⁵ However, depending on the preservative, such reviews are not generally concerned specifically with analytical methods for processed meats and poultry. This chapter considers the published methodology available for determining permitted preservatives designed for use in meat matrices.

Table 5.2 Meat Products and Levels of Conditionally Permitted Preservatives in the EU

<i>Meat Products</i>	<i>Preservative^a</i>	<i>Maximum Level (mg/kg)</i>	<i>Maximum Residual Level (mg/kg)</i>
Surface treatment of dried meat products	Sa + Ba + PHB	<i>Quantum satis</i>	—
Jelly coating of meat products (cooked, cured, or dried); <i>pâté</i>	Sa + PHB	1000	—
Surface treatment of dried, cured sausages	E-235	1 mg/dm ² surface (not present at a depth of 5 mm)	—
Fresh “longaniza” and fresh “botifarra” sausage	SO ₂	450	—
Burger meat with a minimum vegetable and cereal content of 4%	SO ₂	450	—
Breakfast sausages	SO ₂	450	—
Meat products	E-249 and E-250	150	—
Sterilized meat products ($F_0 > 3$)	E-249 and E-250	100	—
Traditional immersion-cured meat products	E-249 and E-250		50–175
	E-251 and E-252	0–300	10–250
Other traditionally cured meat products	E-249 and E-250	0–180	0–50
	E-251 and E-252	250–300	10–250
Non-heat-treated meat products	E-251 and E-252	150	—

^a For abbreviations see Table 5.1. Sa + Ba + PHB: Sa, Ba, and PHB used separately or in combination. For these preservatives the indicated maximum use levels refer to ready-to-eat foodstuffs prepared following manufacturers' instructions.

Note: For E-249, E-250, E-251, and E-252, maximum use levels refer to the maximum amount that may be added during manufacture.

Source: Directive No. 95/2/EC of the European Parliament and of the Council of February 20, 1995 on food additives other than colors and sweeteners; Directive 2006/52/EC of the European Parliament and of the Council of 5 July amending Directive 95/2/EC on food additives other than colors and sweeteners and Directive 94/35/EC on sweeteners for use in foodstuffs.

5.2 General Considerations Regarding Analytical Methods

There are many methods available for the analysis of preservatives in foods. Although they vary according to the preservative, in many cases the methodologies are not specific to meat matrices; this means that further development to adapt them to processed meat and poultry analysis is required. In general, the choice of an analytical method must take into account the means available, the selectivity and sensitivity necessary to achieve the required level of detection in a complex matrix, and the possibility of high throughput analysis. Versatility and minimal requirements for sample preparation and handling are also very useful. Several preservatives are frequently added simultaneously, and therefore the preferred methods will be those that allow for the analysis of several preservatives in a single operation, especially all the compounds in the same family. Quantitative analysis may be the ultimate objective in most cases, but there are many occasions

when it may be enough to use a qualitative method that checks for the absence of the additive (e.g., control of raw materials or verification of labeling in consumer products).

Generally speaking, there are two distinct stages in the methods used to determine preservatives: (a) extraction of the preservative(s), frequently followed by a cleanup procedure to eliminate interferences; and (b) separation, identification, and quantification of the preservative(s). Preservative extraction in meat products can be complex due to diversity of properties and of modes of interaction between functional groups and components of food matrix and solvent systems. A variety of different separation methodologies and detection systems have been used for determination of preservatives.

5.3 Sorbates, Benzoates, and *p*-Hydroxybenzoate Esters

Sorbic acid, benzoic acid, and the methyl and ethyl esters of 4-hydroxybenzoic acid (parabens) or their salts are organic acids widely used as preservatives, and present the antimicrobial activity typical of undissociated acids. They act as effective antimicrobial agents (e.g., mold and yeast growth inhibitors) in meat. They are allowed in the surface treatment and jelly coating of many processed meats, used singly or in combination (sorbates, benzoates, and parabens in concentrations ranging from 1000 mg/kg to *quantum satis*), and in *pâtés* (sorbates and parabens used singly or in combination), in the latter case up to a maximum level of 1000 mg/kg (Table 5.2). The acceptable daily intake (ADI) is 25 mg/kg for sorbic acid⁶ and 5 mg/kg body weight for benzoic acid.⁷ The European Food Safety Authority has established a full-group ADI of 10 mg/kg body weight for the sum of methyl and ethyl-*p*-hydroxybenzoic acid and esters and their sodium salts.²

5.3.1 Analytical Methods

Methods that have been reported for the determination of organic preservatives in foods include spectrophotometry, thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and others.^{4,5,8} Most published studies on methodology for the determination of these preservatives have been conducted on beverages and dairy products; there are very few references to their specific application in meat products.

Extraction procedure. Sorbates, benzoates, and parabens show moderate reactivity and can easily be isolated from food and beverage matrices.⁵ However, depending on the type of food matrix and the determination methodology, efficient sample cleanup procedures are essential to eliminate various interferences in the matrix (e.g., proteins, fats, and polysaccharides). Generally speaking, solid, complex matrices (such as meat products) require more cleanup. Specific extraction methods for analysis of these preservatives in food matrices apply some of the following procedures: direct extraction of an acidified sample by an organic solvent, solid-phase extraction (SPE), extraction as an ion pair, and steam distillation.^{5,8-11}

Spectrophotometric methods. Sorbic acid, benzoic acid, and parabens present strong ultraviolet (UV) absorption, so UV detection is the method most commonly used. However, the sensitivity of detection differs as a result of the considerable differences between them in maximum absorbances.⁵ Benzoate and sorbate determination in ground beef¹² includes extraction with water, which is mixed with HCl and petroleum ether followed by

Table 5.3 Examples of Official and Internationally Recommended Methods for Determination of Preservatives in Meat Products

<i>Preservative</i>	<i>Methods</i>	<i>Matrix</i>	<i>Applicable to Determination of (mg/kg)</i>	<i>Reference</i>
Sa ^a + Ba ^a	GC	Food	—	14
Ba	TLC	Food	—	15
Sa + Ba + sulfites	Spectrophotometry	Ground beef	—	12
Sulfurous acid (free)	Titrimetric	Meats	—	16
Sulfurous acid	Color	Food	Qualitative	17
Sulfites	Color	Meats	Qualitative	18
Sulfurous acid (total)	Modified Monier-Williams	Food	—	19
Sulfite (total)	Optimized Monier-Williams	Foodstuffs	≥10	20,21
Sulfites	Enzymatic	Foodstuffs	—	22
Sulfites (total)	DPP	Food	≥10	23
Sulfite (total)	FIA	Foods and beverages	≥5	24
Sulfites	Ion-exclusion chromatography	Foods and beverages	≥10	25
Nitrites	Colorimetry	Cured meat	—	26
Nitrates and nitrites	Xylenol	Meat	—	27
Nitrate and nitrite	Spectrophotometric	Meat products	—	28
Nitrate and nitrite	Ion-exchange chromatography	Meat products	Nitrite >40	29

^a For meanings of these abbreviations see Table 5.1.

measurement of absorbance at 250 nm for sorbates and 225 nm for benzoates (Table 5.3). Because this type of aqueous extraction or steam distillation gives relatively poor recoveries (sorbic acid) when applied to raw beef, Campos et al.¹³ described an improved procedure for sorbic extraction based on disintegration and dispersion of raw beef with sand prior to steam distillation and determination by UV absorption.

Thin-layer chromatographic methods. TLC and high-performance thin-layer chromatography (HPTLC) have been used in qualitative and quantitative determination of preservatives. Qualitative determination of benzoates and hydroxybenzoates in foods (including seafoods) based on TLC separation was developed by Pinella et al.³⁰ These authors also described the quantitative determination of benzoic acid by steam distillation, extraction (ethanol) after the TLC separation, and determination in the UV region (310–205 nm). A similar procedure is reported as an Association of Official Analytical Chemists (AOAC) method¹⁵ for benzoic acid determination in food (Table 5.3). Quantitative (TLC and HPTLC) methods for determination of mixtures of benzoic acid and sorbic acid without an extraction or cleanup step,³¹ or previously isolated and concentrated by SPE,³² have been applied in beverages.

Gas chromatographic methods. GC, with or without derivatization, has been widely employed for the determination of sorbates, benzoates, and parabens in foods and beverages.^{4,5,33} GC methods are sensitive, specific, and accurate, but may require lengthy extraction prior to GC analysis. Sample pretreatment prior to GC analysis involves organic solvent extraction, precipitation of protein, liquid–liquid extraction, SPE,^{33,34} headspace solid-phase microextraction,³⁵ or stir-bar sorptive extraction.³⁶ An AOAC GC method¹⁴ (Table 5.3) for

determination of benzoic acid and sorbic acid in foods involves extraction with ether and successive partitionings into aqueous NaOH and CH₂Cl₂, derivatization to a trimethylsilyl ester, and flame ionization detection (FID). A GC-mass spectrometric technique has been reported for the simultaneous determination of sorbic acid, benzoic acid, and parabens in foods.³⁷ A new application of pyrolytic methylation has been developed to determine benzoic acid in soft drinks by GC without any pretreatment and using a special pyrolyzer. Samples containing benzoic acid could be accurately determined by direct-injection GC on the medium polar stationary phase column.³⁸

Specific GC determination of benzoates in meat products has been reported.^{39,40} Simultaneous GC determination of preservatives (sorbic acid, benzoic acid, and their esters) in fatty foods (*pâté*) without derivatization has been described.⁴¹ Sample pretreatment includes solvent extraction and SPE. GC-FID for quantification and mass spectrometry has been used in the conventional electron impact mode for identification. Sorbates, benzoates, and parabens were simultaneously analyzed in cured meat products by GC-FID.⁴²

High performance liquid chromatographic methods. Determination of preservatives by HPLC offers high specificity with minimal preparation and does not require derivatization. Numerous HPLC methods for simultaneous determination of benzoic acid, sorbic acid, and parabens have been reported as applicable to selected foodstuffs.^{4,5,43} In general, the extraction system of the applicable procedures varies according to the complexity and composition of the foodstuffs, and may include clarification/purification, which is essential for eliminating high-molecular-mass matrix interferences (e.g., proteins, fats, and polysaccharides). Sample pretreatment prior to HPLC analysis involves solvent extraction, filtration, centrifugation, and SPE. Separation is done essentially by reversed-phase HPLC with UV detection at the wavelengths of maximum absorption of the compounds. Different eluents have been used, including phosphate buffer, methanol, tetrahydrofuran, acetate buffer, and acetonitrile.^{4,43-46}

Nowadays HPLC is the most common analytical procedure for the detection and quantification of these preservatives in foods, although there are very few published analytical methods that are specifically applicable to meat systems. Ali⁴⁷ reported that benzoic acid, sorbic acid, and parabens were extracted from meat with 70% ethanol. After filtration, extracts were analyzed using reversed-phase liquid chromatography. An analytical procedure has been developed for the analysis of benzoic acid; *p*-hydroxybenzoic acid; and methyl-, ethyl-, propyl-, isopropyl-, and butyl esters of *p*-hydroxybenzoic acid by micellar liquid chromatography in food samples, including chicken spread.⁴⁸

Capillary electrophoretic methods. CE has recently been employed as an efficient tool for preservative determination in food due to its many advantages, which include high separation efficiency, excellent resolution, and short analysis time. Various CE methods, such as capillary zone electrophoresis, micellar electrokinetic chromatography (MEKC), and microemulsion electrokinetic chromatography (MEEKC), have been reported for determination of preservatives in foods. These methods have generally been used on carbohydrate-rich matrices (soft drinks, wine, jam, soy sauce, etc.). In most cases, real samples cannot be injected directly into CE systems, and an extraction cleanup process is necessary. This stage can be even more necessary in complex matrices (protein, fat, etc.), like meat derivatives, for which no published methods have been found.

Kuo and Hsieh⁹ described a CE method for the simultaneous separation of nine preservatives, including benzoic acid, sorbic acid, *p*-hydroxybenzoic acid, and six alkyl esters of

p-hydroxybenzoic acid in plum preserves, bean curd, and soy sauce. MEKC has been successfully used to simultaneously analyze *p*-hydroxybenzoic acid methyl ester, *p*-hydroxybenzoic acid ethyl ester, benzoic acid, and sorbic acid.⁴⁹ Huang et al.¹¹ used the MEEKC method to separate parabens (methyl, ethyl, propyl, and butyl), sorbic acid, and benzoic acid in various food products (soft drinks, soy sauces, and wines). The separation and detection of benzoate and sorbate in soft drinks by both conventional CE and microchip electrophoresis with capacitively coupled contactless conductivity detection has been reported.⁵⁰ Capillary electrochromatography, a hybrid separation technique that combines the features of HPLC and CE, has been used to analyze sorbic acid, benzoic acid, and parabens in some products (cold syrups, lotions, soy sauces, and wines).¹⁰

Enzymatic determination. A method for the determination of sorbic acid based on spectrophotometric measurement of sorbyl coenzyme A at 300 nm has been reported.⁵¹ The method has been tested for various food matrices (wine, alcoholic and nonalcoholic beverages, fruit preserves, and tomato ketchup).

5.4 Sulfites

Sulfur dioxide and sulfites comprise the group of compounds known collectively as sulfites (Table 5.1). These sulfiting agents, or S(IV) oxoanion compounds, are considered relatively strong preservatives, because of their strong antimicrobial activity. Moreover, even a small amount of sulfite in meat imparts a bright red color. These compounds are not permitted for use in meat in the United States. In the European Community, the maximum permitted amount of added sulfiting agents in the various different meat products is 450 mg/kg, expressed as SO₂ (Table 5.2). Sulfites have been associated with allergic reactions and food intolerance symptoms. They are known to degrade thiamine (vitamin B₁), of which meat is a good source. The ADI for sulfite (expressed as SO₂) is 0.7 mg/kg body weight.⁵²

When added to a food matrix, some of the sulfiting agents bind to different components of food. The portion of sulfiting agent that does not combine with the food is called free sulfite. Bound sulfite can be categorized as reversibly or irreversibly bound sulfite. The relative presence of each one varies according to the reactivity of sulfur dioxide in meat systems; this in turn is associated with factors involved with composition or with processing and storage conditions. For instance, following incorporation of additives to meat products, there can be irreversible losses of as much as 50%, depending on these factors.^{53,54} Cooking meat products also causes sulfite reduction.⁵⁵ Then again, sulfite ions may cleave disulfide bonds in meat proteins.⁵⁴ The analytical determination of sulfite, then, does not reflect the preservatives that were initially added.

5.4.1 Analytical Methods

Many analytical methods have been reported for sulfite determination in foods and beverages. These methods include titrimetry, spectrophotometry, enzymatic analysis, chromatography, flow injection analysis (FIA), and electroanalysis.^{5,56,57} However, not all of them are equally suitable for the determination of sulfites in solid, complex protein matrices such as processed meats, where sulfite-binding problems may arise from interaction with other food components or entrapment within food particles.^{56,58}

Extraction procedure. Analytical determination requires some means of removing and recovering the sulfur dioxide (free and reversibly bound) and then quantifying the level found. Sample preparation and analysis should be as rapid as possible to avoid loss of labile forms of sulfite. Numerous procedures utilize the Monier-Williams distillation process for sulfite separation by means of distillation from a suspension or solution of the food in hot HCl. In some cases, Monier-Williams distillation has been used as a preparatory tool for obtaining free and bound sulfite fractions to accommodate more selective quantitation techniques, whereas in others extraction has been used instead of distillation.⁵⁶ Other procedures that do not utilize distillation or vapor phase transfer require the conversion of sulfite to a stabilized sulfite derivative compound to take advantage of some property that serves for quantitation.

Titrimetry (Monier-Williams) method. This procedure, derived from the classic studies of Monier-Williams, measures free sulfite plus the reproducible portion of bound sulfite. It is based on acid distillation followed by vapor phase transfer of the SO₂, facilitated by a carrier gas stream, to an oxide-trapping solution. The sulfur dioxide is oxidized to sulfuric acid for quantitation by titration or determination of sulfite by precipitation with barium. The sulfuric acid is stoichiometrically related to the sulfur dioxide distilled from the test solution (modified Monier-Williams method)¹⁹ (Table 5.3). An optimized Monier-Williams method²⁰ for the analysis of sulfites in foods has been collaboratively tested and accepted as an official method (Table 5.3). Very few foods not treated with sulfite give a false positive even at levels below 10 ppm; *Allium* and *Brassica* vegetables and isolated soy protein are important exceptions to this rule. As a comparative procedure, this methodology has been assayed in different muscle foods including beef, pork, and chicken meat products.^{53,55} Monier-Williams methods are the ones most commonly employed by food control laboratories for meat product analysis (Table 5.4). A method based on distilling of sample in an acidic medium followed by iodometric titration has also been also assayed (Table 5.4), although its use is limited to products with high levels of sulfite.⁵⁶

Spectrophotometric methods. Various spectrophotometric procedures have been reported for determination of sulfiting agents in foods. An AOAC method¹² (Table 5.3) for sulfite

Table 5.4 Examples of the Analytical Methods Used for Meat Product Preservative Determination in Evaluation of Proficiency Testing of Analytical Laboratories (Interlaboratory Comparisons)

Analytical Methods	Sulfite ^a in Pork Sausage (Number of Laboratories Participating: 75)		Nitrite ^b in Gammon Steak (Number of Laboratories Participating: 101)	
	Used by Laboratories (%)		Analytical Methods	Used by Laboratories (%)
Modified Monier-Williams	43.9		Colorimetry	52.2
Optimized Monier-Williams	28.1		IC	21.7
Distillation and titration with iodine	15.8		HPLC	18.5
Spectrophotometry	8.8		FIA	4.4
IC	1.8		Enzymatic	2.2
Others	1.8		CE	1.1

^a Food Analysis Performance Assessment Scheme (FAPAS), Proficiency Test 2046. August–October 2006.

^b FAPAS, Proficiency Test 1547. January–February 2007.

determination in ground beef is based on a colorimetric reaction with *p*-rosaniline after reaction with mercuric extractant. Another method often used for determination of sulfur dioxide in meat products is based on distillation/spectrophotometric analysis using 5,5'-di-thiobis-(2-nitrobenzoic acid) (DTNB). It has been used in fresh sausages,⁵³ and comminuted pork meat.⁵⁴ Determination of sulfites has been performed in commercial sausages by direct extraction and spectrophotometric methods based on a step reaction using the reagent DTNB.⁵⁸

Differential pulse polarographic method. A method based on differential pulse polarography (DPP) and applicable to the determination of sulfites (total) in foods has been proposed²³ (Table 5.3). The method, based on a collaborative study,⁵⁹ was tested on a number of muscle foods including shrimp. It measures SO₂, which is purged with N₂ from acidified test suspension, collected in electrolyte-trapping solution, and then determined by DPP. Modified Monier-Williams distillation followed by DPP has also been used to determine sulfiting agents in foods.⁶⁰

Chromatographic methods. Anion exclusion chromatography has proven a useful technique for determining sulfites in foods. An electrochemical detection system is the most commonly used,^{55,61-63} although conductivity detection^{64,65} and direct UV detection have been also reported.⁶⁶ In the AOAC chromatographic method²⁵ (Table 5.3), SO₂ is released by direct alkali extraction, followed by anion exclusion chromatographic separation and electrochemical (amperometric) detection.^{62,67} The method was tested on different food matrices, including a muscle food (dehydrated seafood). Improvements in amperometric detection of sulfite in food matrices have been reported.⁶⁸

Although chromatographic methods may be appropriate for use on meat products, very little has been published in the literature in that respect. Free and total sulfite have been determined in fresh sausages by HPLC;⁶³ the method includes extraction of both free and total sulfite by dissolution of the sample in a suitable solvent and determination by HPLC (anion exclusion column) using electrochemical detection. This procedure was also used to determine sulfite content in fresh and cooked (beef, pork, and chicken) burgers.⁵⁵

Enzymatic methods. Enzymatic methods have been developed for sulfite analysis in food. In these procedures, sulfite is usually oxidized to sulfate; this is catalyzed by sulfite oxidase to release hydrogen peroxide, and the hydrogen peroxide is measured by linking it to the oxidation of reduced nicotinamide adenine dinucleotide (NADH) in the presence of NADH peroxidase. Hydrogen peroxide was measured by spectrophotometry.^{22,56,69} Various sulfite oxidase biosensors have also been reported^{70,71} (Table 5.3). Enzymatic methods have been used to determine SO₂ in muscle foods (shrimp).⁷²

FIA. This method offers the advantages of simplicity and precision with a high analytical sampling rate, while requiring only low-cost equipment, reducing the need for large volumes of toxic reagents, and requiring little analysis time. Several FIA methods have been used for sulfite determination in food and beverages,^{4,72,73} but few published reports can be found dealing with their application to muscle foods, and most of these refer to shrimp.^{72,74} With the support of an interlaboratory study,⁷⁴ an AOAC method²⁴ has been proposed for FIA sulfite determination (Table 5.3) based on sulfite reaction with malachite green. A test solution is made to react with NaOH to release aldehyde-bound sulfite; then, the test stream is acidified to produce SO₂ gas, which diffuses across a Teflon membrane in the gas diffusion cell into a flowing stream of malachite green, which is discolored. With this procedure it is possible to assay samples containing ingredients from liliaceae (garlic, onions, leeks) for which the Monier-Williams reference method is not suitable.

Capillary electrophoresis. Sulfite content in foods and beverages can be determined by CE. The sulfite is converted to sulfur dioxide and finally to sulfate by Monier-Williams distillation. The sulfate is then determined by CE. The results for sulfite content of seafood agree very well with those determined by titrimetry.⁷⁵

5.5 Nitrite and Nitrate

Sodium and potassium nitrates and sodium and potassium nitrites (Table 5.1) are used in meat curing because they stabilize red meat color, inhibit some spoilage and food poisoning anaerobic microorganisms, delay the development of oxidative rancidity, and contribute to flavor development. Depending on the type of processed meat, processing conditions, presence of sodium ascorbate, and other factors, the added nitrite reacts with many components in the matrix (myoglobin, nonheme proteins, lipids), so that the analytical detection of the nitrite or nitrate content does not reflect the preservative initially added. Analytical methods therefore usually determine the residual nitrate/nitrite, which can reach only about 10–20% of the original nitrite amount added.^{76,77} To detect bad practice and use of high nitrite levels, it is more effective to control nitrite at input.⁷⁷ Nitrite levels in meat products are important because nitrite can react with secondary amines to form nitrosamines, which are recognized as having carcinogenic effects. In the EU, potassium and sodium nitrite and nitrate are authorized for use in different meat products, and maximum ingoing amounts (150 mg/kg for nitrite and 300 mg/kg for nitrate) are established for all products, as well as maximum residual levels for some of them (Table 5.2). Current regulations on use of nitrite and nitrate in the United States vary depending on the curing method used and the product that is cured. For comminuted products, the maximum ingoing nitrite and nitrate limits are 156 mg/kg and 1718 mg/kg, respectively. For immersion-cured and massaged or pumped products, those limits are 200 mg/kg and 700 mg/kg, respectively. In dry-cured products nitrite is limited to 625 ppm and nitrate to 2187 ppm.⁷⁸ The ADI for nitrites, as nitrite ion, is 0.07 mg/kg body weight, and 3.7 mg/kg for nitrate, as nitrate ion.^{79,80}

5.5.1 Analytical Methods

Several methods have been reported for quantitative determination of nitrate and nitrite in foods, including spectrophotometry, chromatography, electrochemical detection (potentiometry, amperometry, polarography), CE, and others.^{4,5,81,82} However, not all of them are equally suitable for use on highly heterogeneous solid matrices like processed meats and poultry.

Extraction procedure. Nitrite and nitrate determination requires an extraction stage, generally involving dispersion in hot water. Meat products contain various compounds (ascorbic acid, fat, protein, sodium chloride, etc.) that can interfere in nitrite and nitrate measurement, and so a number of procedures have been tried to clean up the extracts prior to determination. These include clarification stages (fat and protein precipitation, filtration, etc.) using different compounds or solvents (Carrez or borax reagents, zinc sulfate or potassium ferrocyanide, acetonitrile, and others) or pretreatment cartridges to remove sample matrix interferences.^{3,83} Another possibility is to separate the fat by centrifugation and rapid cooling followed by in-line dialysis to remove protein and remaining fat. Obviously the choice of a specific

procedure to clean up an extract prior to determination will depend on the analytical method used.

Spectrometric methods. Of the methods available for quantitative determination of residual nitrite in meat products, the most commonly used are based on colorimetric determination using Griess diazotization, which involves the formation of azo dye produced by coupling a diazonium salt with an aromatic amine or phenol. The diazo compound is formed when nitrite (aqueous extract from meat) reacts with sulfanilamide and the coupling agent *N*-(1-naphthyl) ethylenediamine-2HCl.^{3,4,81} The color that develops is measured spectrophotometrically (540 nm). The same reaction can be used to determine nitrate. To do this, the relatively inert nitrate is reduced to nitrite, which can then be determined by Griess diazotization. Nitrate can be reduced by chemical and enzymatic procedures. A variety of agents have been investigated for chemical reduction,⁸¹ the most common arrangement being a spongy cadmium column, which can achieve efficiencies of nitrate-to-nitrite conversion approaching 100%. Enzymatic reduction of nitrate to nitrite has been accomplished with nicotinamide adenosine dinucleotide phosphate in the presence of the enzyme nitrate reductase.²⁸ Spectrophotometric methods involving the reduction of nitrate to nitrite and subsequent colorimetric determination of nitrite with a diazo coupling reaction have been adopted^{26,28} for meat products (Table 5.3). Nitrite and nitrate have also been determined in meat products using *m*-xylenol²⁷ (Table 5.3).

Other colorimetric reactions have been used to determine nitrites and nitrates in meat products. A number of these are based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by sodium sulfide, which is oxidized by the addition of nitrite, causing a reduction in the intensity of the blue color and a reduction in the absorbance measured at 814 nm;⁸⁴ others are based on the catalytic effect of nitrite on the oxidation of methyl red by bromate, and the absorbance is measured at 520 nm.⁸⁵ A spectrofluorimetric method has been developed for nitrite determination in meat systems.⁸⁶

Enzymatic methods. Procedures based on enzymatic reduction coupled with spectrophotometric detection can be used to determine nitrite and nitrate in meat samples. Nitrite is measured enzymatically through its reaction with nitrite reductase coupled with NADH, and the reaction is measured spectrophotometrically.⁸⁷

FIA. Several FIA-based methodologies for the determination of nitrite and nitrate in meat products have been reported. Most of the FIA methods that are used to simultaneously determine nitrates and nitrites in meat products are based on a diazotization/coupling reaction.⁸⁸⁻⁹¹ There have also been other applications of FIA, based on the reduction of nitrite and nitrate to nitric oxide followed by reaction with iron (II) and thiocyanate in an acid medium to form FeSCNNO⁺ chromophore, which is measured at 460 nm;⁸² based on the reaction of nitrite with safranin to form diazonium salt, which absorbs at 520 nm;^{92,93} based on the catalytic effect of nitrite on the oxidation of gallocyenin by bromate and the decrease in absorbance of the system at 530 nm;⁹⁴ or based on gas phase molecular absorption UV (205 nm) spectrophotometry.⁹⁵ Spectrophotometric determination of nitrite and nitrate in cured meat has been reported using sequential injection analysis, a feasible and mechanically simpler alternative to FIA.⁹⁶

An FIA method with flame atomic absorption spectrometry (FAAS) detection has been used to determine nitrite and nitrate in meat products. It is based on the oxidation of nitrite to nitrate using a manganese (IV) dioxide oxidant microcolumn, where the flow of the

sample through the microcolumn reduces the MnO_2 solid-phase reagent to Mn(II) , which is measured by FAAS.⁹⁷

Chromatographic methods. Ion chromatography (IC) and HPLC methods for the detection of nitrite and nitrate have been developed in pursuit of procedures that are faster, more accurate, and more sensitive than spectrophotometric methods.⁹⁸ Derivatization protocols are essential for GC, whereas it is relatively easy to insert the sample in most HPLC and IC systems.⁸¹ However, in the case of meat matrices, some form of cleaning up of the extracts is required to avoid interference, which makes such methods less attractive.

Separation techniques based on ion-exchange chromatography have been used to evaluate residual nitrite and nitrate in various meat products using detection systems based on conductivity,^{99,100} UV absorption,^{98,101} and bulk acoustics.¹⁰² Determination of nitrite in cured meats by ion exclusion chromatography with electrochemical detection has been reported.¹⁰³

Bianchi et al.⁹⁹ reported determination of nitrates in 76 different pork meat products using IC with a conductivity detector coupled to an anion micromembrane suppressor. Commercial samples of ham and salami have been analyzed by IC with UV absorbance (225 nm) detection.⁹⁸ Nitrite and nitrates have been determined in muscle tissue (beef, pork, horse, and chicken) and dry-cured meat by anion-exchange chromatography/conductivity and mass spectrometry detection.¹⁰⁰

IC has been used in a collaborative study to devise an alternative chromatographic method for determining residual nitrite and nitrate in meat products.¹⁰¹ This method, which has been accepted by the European Committee for Standardization²⁹ (Table 5.3), is based on extraction of nitrite and nitrate from the sample with hot water followed by treatment in an aqueous solution with acetonitrile to remove any interfering substances. The nitrite and nitrate contents of the solution are then determined by ion-exchange chromatography separation and UV detection at 205 nm.

HPLC techniques have also been reported for determination of nitrate and nitrite in processed meats.^{104–109} These analytical procedures vary in terms of the extraction conditions and the need to limit interference by means of protein precipitation or sample processing steps using reversed-phase or ion-exchange pretreatment cartridges.⁹⁸ Similarly, there are varying conditions of separation (ion-exchange or ion-pair reversed phase) and anion detection by UV absorption, conductivity, indirect photometry, fluorometry, chemiluminescence, or electrochemical detection.¹⁰⁹

Capillary electrophoretic methods. CE is a powerful separation technique for determination of nitrite and nitrates.⁸¹ These methods has been used for simultaneous analysis of nitrite and nitrite in meat products using UV detection.^{110,111}

Electrochemical methods. Various electrochemical detection techniques based on amperometric,^{112,113} voltametric,^{114–116} or potentiometric^{117,118} procedures have been used in determining nitrites and nitrates in food samples. However, only a few were used in processed meat and poultry analysis.^{112,114,117}

5.6 Concluding Remarks

Preservatives in meat products need to be quantified to assure quality and compliance with legal regulations and to minimize the health risk to consumers. There are numerous methods for determining preservatives in muscle foods, but in some cases such procedures have not been assayed in highly heterogeneous solid matrices like meat products. Because of the complexity of the additive/matrix,

these methodologies generally require further development for use in processed meat and poultry analysis. Generally speaking, to be suitable for use in laboratories (both official and private) for food analysis, a procedure must meet standards of sensitivity, versatility, effectiveness, rapidity, and cost. The number of such criteria helps to explain the diversity of analytical methodologies normally chosen by food analysis laboratories to determine preservatives in meat products (Table 5.4).

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Chapter 6

Additives: Smoke Flavorings

Dietrich Meier

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6.1 Introduction

Additives used in meat and meat products should be harmless to health. They serve solely to influence odor and flavor, color and consistency, or shelf life. Without additives it would be impossible to distribute many foods, as they either could not be made at all or would spoil too quickly. Additives are grouped into those that are intended to be eaten along with the food and those that are components of coverings on the surface of the food but are usually not eaten [1].

Apart from special additives in meat such as bactericide [2], curing agent [3], and tenderizer [4], the application of smoke is the most frequently used additive for meat. Therefore, the analysis of smoke components will be the focus of this chapter. Smoking, together with drying and salting, is perhaps the oldest process to preserve foodstuffs. It has also been called man's first spice [5]. The smoke components effects are germicidal and desiccating. They coagulate the proteins and thus work to preserve. Moreover, they add aroma and color to the food, making it more attractive for the consumer. In the course of the past 50 years, preservation has become less important. Currently smoking of fish and meat is mostly done to enhance flavor [6].

In principle, smoke used for meat is the result of the pyrolytic degradation of wood, which is basically composed of two types of polymers: (1) polysaccharides (cellulose and hemicelluloses) and (2) lignin. Each of these polymers gives a characteristic spectrum of pyrolytic products. The degradation products of polysaccharides are mainly furans, acids, alcohols, anhydrosugars, esters, and aldehydes, and are predominantly responsible for the staining and bactericidal effects of smoke, whereas the phenolic lignin degradation products such as guaiacol, syringol, and derivatives are generally responsible for the typical smoky flavor.

The most typical woods used in smoke generation are beach (*Fagus sylvatica*, a common wood in Europe), hickory (*Carya ovata*, a common wood in United States and Canada), oak (*Quercus* spp.), and maple (*Acer* spp.). Some other species such as cherry (*Prunus* spp.), apple (*Malus* spp.), mesquite (*Prosopis glandulosa*), and pine (*Pinus* spp.) are used in minor amounts. The list indicates that—with the exception of pinewood—smoke generation is based on hardwood pyrolysis. They contain much less extractives than softwood, whose pyrolysis products might add undesirable smell to the smoke flavor. However, more important is the different lignin structure. Hardwood lignins are polymers derived from a mixture of coniferyl alcohol (Figure 6.1) and sinapyl alcohol (Figure 6.2), whereas softwood contains only moieties of the coniferyl-type. Furthermore, the

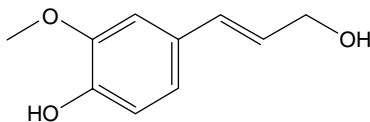


Figure 6.1 Lignin precursor coniferylalcohol.

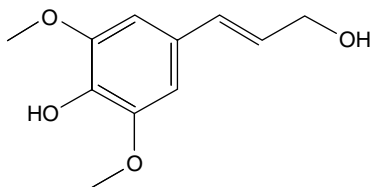


Figure 6.2 Lignin precursor sinapylalcohol.

superior sensory properties can be attributed to the phenolic sinapyl derivatives, which provide the typical smoke aroma.

Regardless of the process of smoke generation, the basic smoke components are chemically very similar. They differ mainly in their amount and their ratio due to the different technologies of smoke generation. Processed meat can either be smoked directly with the help of different types of smoke generators or by applying liquid smoke flavorings, which are becoming more prevalent [7–11].

6.1.1 Smoking with Smoke Generators

Technical smoke generators for direct smoking mainly work according to three operation principles:

- Glowing
- Friction
- Superheated steam

Thus, the aroma can be controlled via temperature and type of wood. Furthermore, not only the pyrolysis temperature but also the temperature of the vapors in contact with the meat is important, as the final chemical composition and the amount of toxic polycyclic aromatic hydrocarbons (PAHs) are determined by temperature.

The higher the temperature, the shorter is the exposition time of the food in the smoke. The preserving effect is improved by leaving the foodstuffs for a longer time at low smoke temperatures, as cold smoke penetrates more easily and more deeply into meat and fish.

With respect to temperature control, three smoking technologies are known [6]:

- Cold smoking (smoke temperature in the range of 15–25°C), used for crude sausage, crude ham, and salami
- Warm smoking (smoke temperature in the range of 25–50°C), used for frankfurters
- Hot smoking (smoke temperature in the range of 50–85°C), applied for cooked ham, eel, mackerel, and halibut

6.1.2 Smoking with Liquid Smoke

6.1.2.1 Development of Liquid Smoke Flavor

The history of liquid smoke flavors (LSFs), as they are used today, starts in the early 1970s, although early treatments of meat with LSFs goes back to 1811 [12]. LSFs were first applied in the United States and Eastern Europe. Their application to meat products is through dipping, spraying, or treatment with aerosols, similar to the treatment in traditional smokehouses. The aerosol technology was first applied by Hickory Specialities in 1969, and was the breakthrough for producing LSFs [12]. The liquid smoke technology has made such progress that it has been applied in many countries throughout the world. This is due to several reasons: ease of application, speed, uniformity of the product, reproducibility of physical and chemical properties, and cleanliness of application [13]. In addition to these advantages, and the more efficient use of resources involved, another important reason for using smoke flavorings instead of smoke directly is the fact that the amount of (known) toxic compounds found in smoke can be controlled before being

added to the food [14]. A decrease of the content of certain PAHs has been observed [15,16]; according to Pszczola, PAHs are virtually absent [5].

It is important to note that LSFs not only can replace traditional smoking, but can be added to various other foodstuffs such as soups, sauces, savories, cheese, and spices.

6.1.2.2 Manufacturing of Liquid Smoke Flavor

Liquid smoke flavorings are solutions obtained from pyrolysis of wood. Wood is thermally degraded in the absence of oxygen, and the vapors are condensed either in water or vegetable oils. On a global basis, there are more than half a dozen of producers. Production plants operate pyrolysis reactors in either continuous or batch mode. The exact process conditions are corporate secrets. The volatile smoke constituents are continuously removed from the hot reaction zone and condensed in special equipment. The raw products are divided into different classes according to their solubility in water (Figure 6.3); water-soluble condensates are called “primary smoke condensates.” The water-insoluble tarry phase is cleaned mostly by extraction and called “primary tar fraction.” Both fractions are refined through further process steps such as extraction, distillation, and concentration by evaporation, absorption, or membrane filtration. During condensation other water-insoluble oily products are formed that are not utilized.

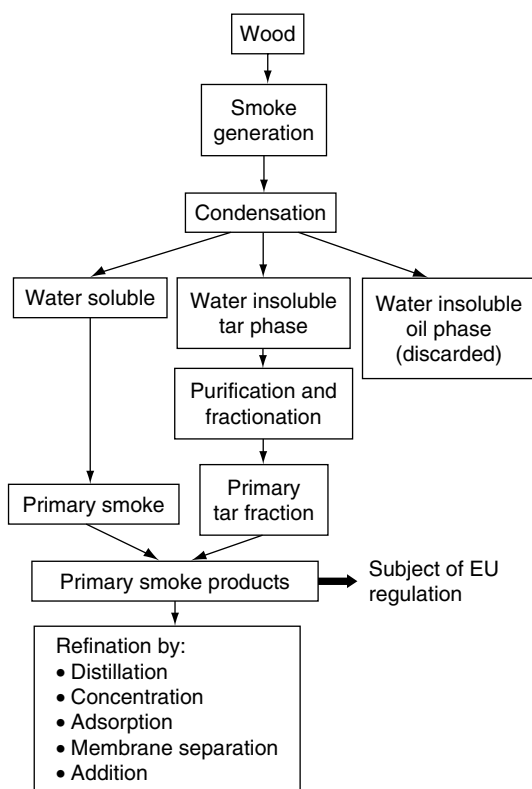


Figure 6.3 Simplified diagram of the manufacture process for LSFs. The primary smoke condensates are the subject of E.U. regulation 2065/2003. (From Meier, D., *Fleischwirtschaft International*, 4, 37–40, 205. With permission.)

The application of liquid smoke is more economic than the operation of smoke generators. Liquid smoke is not only cheaper but also can be standardized and adopted to the needs of customers. Thus, products can be manufactured with constant quality, taste, and uniform appearance.

Regulations for the use of LSFs in different countries vary considerably. For example, in Germany “freshly generated smoke” is classified as a food additive. For toxicological reasons, only solid food-stuffs are allowed to be smoked. Also in Scandinavia a special regulation exists for smoke aromas. All other countries in and outside of the European Union (E.U.) allow the unrestricted use of liquid smoke, as long as the maximum content (10 µg/kg) of 3,4-benzo[*a*]pyrene is not exceeded [6].

As more liquid smoke aroma penetrated the German market, the legal situation became complicated, as many exceptions had to be put into force. This unsatisfactory situation is now resolved by new E.U. regulation 2065/2003, which directs the authorization and characterization of all smoke aromas. For the first time, the Council of Europe and the European Scientific Committee have considered sanitary and toxicological aspects.

6.1.2.3 Legal Marketing Aspects of Liquid Smoke Flavor

There is a document available to give guidance to petitioners and other interested parties wishing to introduce smoke-flavoring primary products according to the European Parliament and Council Regulation 2065/2003 of November 10, 2003 on smoke flavorings used or intended for use in or on foods (*Official Journal of the European Union* L 309, November 26, 2003, p. 1). It gives guidance on the administrative and technical data required, on the range of toxicological tests generally required for smoke flavoring primary products, and on the format for formal submissions (hereafter referred to as “dossiers”) to the competent authority of a member state for further transmission to the European Food Safety Authority.

As stated in Regulation 2065/2003, the use of a primary product in or on foods shall only be authorized if it is sufficiently demonstrated that it does not present risks to human health. A list of primary products authorized to the exclusion of all others in the community for use in or on food and for the production of derived smoke flavorings shall therefore be established after the authority has issued an opinion on each primary product. Following the establishment of this list, all new applications need a favorable opinion by the authority for inclusion in the list.

According to the E.U. regulation, the primary product should be chemically characterized as far as it is necessary to describe and define its identity. For this purpose the following data should be provided:

- Information on the identity (name and Chemical Abstract Service [CAS] number) and the concentration of the major chemical constituents of the primary product
- Information on the concentration in the primary product of the PAHs listed in Annex 2
- Information considered adequate to characterize and to recognize the primary product (e.g., gas chromatograms, liquid chromatograms, mass spectra, and infrared spectra)
- The solvent-free fraction (% m/m = weight %) in the primary product and how it is determined
- The volatile fraction (% m/m = weight %) in the primary product and how it is determined
- Information on the fraction of unidentified constituents. (e.g., solid contents and proportions of acids, phenols, and carbonyls)
- Any other information on chemical composition considered to be relevant for evaluation of the primary product

The state of the art and development of legislation in Europe for PAH analysis is summarized by Wenzl et al. [17].

6.2 Direct Sampling of Components from Smoked Meat

6.2.1 Sampling of Smoke Flavorings

6.2.1.1 Headspace-Gas Chromatography

The headspace (HS) is the gas space in a chromatography vial above the sample. HS analysis is therefore the analysis of the components present in that gas. Headspace-gas chromatography (HS-GC) is used for the analysis of volatile and semivolatile organics in solid, liquid, and gas samples. Common applications include alcohol in blood, monomers in polymers and plastic, flavor compounds in beverages and food products, and fragrances in perfumes and cosmetics.

It is best suited for the analysis of the very light volatiles in samples that can be efficiently partitioned into the HS gas volume from the liquid or solid matrix sample. Higher boiling volatiles and semivolatiles are not detectable with this technique, due to their low partition in the gas HS volume. HS analysis also lends itself to automation for quality control or sample screening. This is made possible by modern instrumentation that can reproducibly prepare samples in an efficient manner.

Complex sample matrices, which would otherwise require sample extraction or preparation, or be difficult to analyze directly, are ideal candidates for HS-GC, because they can be placed directly in a vial with little or no preparation.

An HS sample is normally prepared in a vial containing the sample, the dilution solvent, a matrix modifier, and the HS. Volatile components from complex sample mixtures can be extracted from nonvolatile sample components and isolated in the HS or gas portion of a sample vial. A sample of the gas in the HS is injected into a GC system for separation of all the volatile components. Once the sample phase is introduced into the vial and the vial is sealed, volatile components diffuse into the gas phase until the HS has reached a state of equilibrium. The sample is then taken from the HS.

Direct HS-GC of volatiles from smoked meat is not very common. Wittkowski et al. [18] described a method using this technique combined with GC and flame ionization detector (FID). Their procedure is as follows: The solid sample was placed in a 250-mL Erlenmeyer flask, and after conditioning the sample for several minutes at room temperature, 5 mL of the HS volume was injected with a special syringe into the GC. To prevent peak broadening, the first section of the GC column was immersed into a Dewar flask filled with liquid nitrogen.

Hierro et al. [19] used HS in combination with GC and mass spectrometry (GC-MS) for the analysis of volatile components from salted and smoked dried meats. In general, 110 volatile compounds were identified and quantified. The HS-GC method, first described by Elmore et al. [20], is as follows.

6.2.1.1.1 Sampling Technique

Twenty-five grams of meat sample were introduced into a glass flask and equilibrated for 30 min at 30°C. Volatiles were extracted at 30°C by a nitrogen flow of 40 mL/min for 1 h and adsorbed on a steel trap (105 mm length, 3 mm inner diameter) containing 85 mg of Tenax TA. A standard of 131 ng of 1,2-dichlorobenzene in 1 mL of methanol was added to the trap at the end of the collection, and excess solvent and any water retained on the trap were removed by purging the trap with nitrogen at 40 mL/min for 5 min.

6.2.1.1.2 Separation and Detection of Volatiles

Analyses were performed on a Hewlett-Packard 5972 mass spectrometer fitted with a HP5890 Series II gas chromatograph and a G1034 Chemstation (Hewlett-Packard, Palo Alto, CA). A CHIS injection port (Scientific Glass Engineering Ltd., Staffordshire, U.K.) was used to thermally desorb the volatiles from the Tenax trap onto the front of a CP-Sil 8 CB low bleed/mass spectrometry (MS) fused silica capillary column (60 m, 0.25 mm inner diameter, 0.25 μm film thickness, Chrompack, Middelburg, the Netherlands). During a desorption period of 5 min, volatile compounds were cryofocused by immersing 15 cm of column adjacent to the heater in a solid CO_2 bath while the oven was held at 40°C. The bath was then removed and chromatography achieved by holding at 40°C for 2 min followed by a programmed rise to 280°C at 4°C/min and holding for 5 min. A series of *n*-alkanes (C6–C22) (Sigma-Aldrich Inc., St. Louis, Missouri) was analyzed under the same conditions to obtain linear retention index values for the aroma components. The mass spectrometer was operated in electron impact (EI) mode with an electron energy of 70 eV and an emission current of 50 mA. Approximate quantities of the volatiles were estimated by comparing their peak areas with those of the 1,2-dichlorobenzene internal standard (IS), obtained from the total ion chromatograms, using a response factor of 1.

6.2.1.2 Solid-Phase Micro Extraction

Solid-phase micro extraction (SPME) is an innovative, solvent-free technology that is fast, economical, and versatile. SPME is a fiber coated with a liquid (polymer), a solid (sorbent), or a combination of both. The fiber coating removes the compounds from the sample by absorption in the case of liquid coatings or adsorption in the case of solid coatings. The SPME fiber is then inserted directly into the hot injector of a gas chromatograph for desorption and analysis. SPME has gained widespread acceptance as the technique of preference for many applications, including flavors and fragrances, forensics and toxicology, environmental and biological matrices, and product testing.

6.2.1.2.1 Sampling Technique

In principle the meat sample is placed in a vial and sealed with a septum. The piercing needle of the SPME device (Supelco Inc., Bellefonte, PA) is drilled through the septum, and the needle is inserted into the vial. The needle tip is adjusted in such a way that the fiber does not have contact with the meat when the plunger is extended to expose the SPME fiber into the HS. Now sampling time starts, and after a certain time the fiber is retracted into the needle, the manifold is removed from the vial, and the needle is introduced into the hot injector of a gas chromatograph to desorb the volatiles.

Several papers have been published using SPME for volatile constituents of cooked meat [21–23] or contaminants, and even for the analysis of PAHs [24–28]. Volatiles from wood pyrolysis liquids (bio-oils) have been analyzed by Meier using SPME-GC-MS [29]. Although the sampling technique is rather simple, there is no information in the literature on the use of SPME for the analysis of smoke aroma components. More general information, with a practical SPME applications guide, is available from Supelco (www.sigma-aldrich.com/supelco).

6.2.2 Sampling of Polycyclic Aromatic Hydrocarbons

PAHs are inevitably formed during the smoke generation process, regardless of the pyrolysis technology. Therefore, their determination is of paramount importance to fulfill the legislative requirements.

The major problems associated with the sampling of PAHs in food are

- Only trace amounts available
- Coextraction of PAH-like materials

Chen [30] and Šimko [31] give a comprehensive overview. From the analytical point of view, meat is regarded as a problematic matrix, and the sampling procedure is an important step in the entire analysis sequence. Šimko compiled a comprehensive list of methods used for extracting PAHs from meat products. Sample preparation includes three basic steps: saponification, extraction, and cleanup. Typical procedures are as follows:

- Saponification with mixture of ethanol, water, and KOH, extraction with cyclohexane, preseparation by solid-phase extraction (SPE) on isolute aminopropyl and C18 columns
- Saponification in methanolic KOH, liquid–liquid extraction (methanol–water–cyclohexane and N, N-Dimethylformamide [DMF]–water–cyclohexane), precleaning on silica gel, and gel permeation chromatography (GPC) on Sephadex LH 20
- Saponification with a mixture of methanol, water, and KOH, partition with DMF, precleaning on Kieselgel 60

Recently, Jira [32] presented a practical method described in detail for the extraction of PAHs from smoked meat products. The procedure is as follows:

1. *Accelerated solvent extraction (ASE)*. About 6–8 g of the homogenized meat product is levigated with the same amount of the drying material polyacrylic acid and partial sodium salt-graft-polyethylene oxide. The resulting material is placed into a 33-mL cell with micro glass fiber filters at the outlet of the extraction cell. Then 500 μ L of the 13C-PAH standard mixture is added as IS. The extraction was performed with an ASE 200 (Dionex, Sunnyvale, CA) using *n*-hexane at 100°C and 100 bar with a static time of 10 min. The flush volume was 60%, and the purge time was 120 s. After two static cycles, the solvent is evaporated with a nitrogen steam, with the sample placed in a water bath at 40°C.
2. *GPC*. The evaporated ASE-extract is dissolved in 4.5 mL cyclohexane/ethylacetate (50:50 v/v) and filtered through a polytetrafluoroethylene (PTFE) filter with a pore size of 1 μ m. The GPC column is filled with Bio-Beads S-X3 (filling height 42 cm). Samples are eluted at a flow rate of 5 mL/min, with the same solvent used for sample dissolution. Dump time was 0–36 min; collection time was 36–65 min. Solvent is removed with a rotary evaporator, and the residual solvent is removed in a nitrogen stream.
3. *Column chromatography with silica gel*. The dried GPC eluate is dissolved in 1 mL *n*-hexane. The silica gel column (7 mm inner diameter) is filled with 2.5 g silica gel (15% deactivated with water). Samples are eluted with 30 mL *n*-hexane/dichloromethane (80:20 v/v). Solvent is removed with a rotary evaporator, and the eluate is dried in a nitrogen stream.
4. *SPE*. For complex matrices such as liquid smoke, the dried eluate from the silica gel column is dissolved in 1 mL *n*-hexane and applied onto a conditioned cyano (CN) SPE cartridge (500/1000 mg bed material). The SPE cartridge is first rinsed with 2 mL *n*-hexane, and the eluate is discarded. The PAH fraction is eluted with 5 mL acetonitrile/toluene (75:25 v/v). Solvent is removed with a rotary evaporator, and the eluate is dried in a nitrogen stream. The sample is now ready for GC-MS analysis.

6.3 Separation and Detection of Smoke Components

6.3.1 Polycyclic Aromatic Hydrocarbons

Separation of the PAHs can be accomplished by GC separation followed by detection with a mass spectrometer or after separation with high-performance liquid chromatography (HPLC) and detection with a fluorescence detector (FLD).

6.3.1.1 Gas Chromatography

Several methods for PAH analysis in smoke or smoke flavorings have been described in the literature [15,16,33–36]. However, none of these methods have been validated or cover all 15 priority E.U.-PAHs [10]. In the E.U., the maximum allowed concentration of benzo[*a*]pyrene (BaP) in food treated with smoke flavors was set to 0.03 µg/kg in 1998, and in November 2003, a new regulation was adopted by the European Council and Parliament (No. 2065/2003) to control smoke flavors at the point of production [37]. The regulation defines the aqueous part as primary smoke condensate (PSC) and the purified tar extract of condensed wood smoke as primary tar extract. Both fractions are considered to be primary products to be used for the manufacture of derived smoke flavors. For these primary products, the European regulation established maximum permitted concentrations of 10 µg/kg for BaP and 20 µg/kg for benzo[*a*]anthracene. Furthermore, the regulation determines that only validated analytical methods can be used for the verification of these limits. To support the implementation of legislation, the method described here was developed and validated in a single-laboratory approach according to the IUPAC harmonized guideline [38] to enable the quantification of the E.U.-PAHs in PSC.

The method for sample preparation recently described by Simon et al. [37] is described in the following section. Further details on the instrumentation GC-MS conditions and data evaluation can be found in Section 6.3.1.1.1 [37].

6.3.1.1.1 Sample Preparation

Ten grams of the liquid sample and 100 L of IS solution (50 g/L of each labeled standard) in 2-propanol were combined in a 250-mL round-bottom flask. To this mixture, 3.2 g solid potassium hydroxide and 32 mL methanol were added. The solution was refluxed for 30 min. The sample was extracted three times with 25 mL cyclohexane by liquid/liquid partitioning. The organic phases were combined in a round-bottom flask and dried with anhydrous sodium sulfate. After evaporation of the solvent under reduced pressure, the sample was reconstituted in 1 mL cyclohexane. For removal of residual interferences, the extract was passed through a silica gel SPE tube (Supelclean, 3 mL, 0.5 g) and eluted with 7 mL cyclohexane. After evaporation of the eluate and reconstitution of the residue with 1 mL *n*-hexane, 1 µL of the solution was analyzed by GC-MS.

A drawback of the GC-MS method is its incapacity to resolve the two isomers benzo[*j*]fluoranthene and benzo[*k*]fluoranthene.

A further detailed description is given by Jira [32], who suggested the following procedure for GC-MS analysis:

1. *Preparation for GC-MS analysis.* The dried eluate of the silica gel column (or optional, the eluate of the CN-SPE cartridge) is dissolved in 500 µL of the deuterated PAH standard mixture in isooctane and transferred to a 1-mL tapered vial. The sample is then carefully concentrated to a volume of approximately 50 µL in a nitrogen stream.

2. *GC.* The GC is as follows: capillary column dimensions are 60 m × 0.25 mm inner diameter, 0.25 μm film thickness; film is cross-linked 5% phenyl–methyl–siloxane. Helium is used as carrier gas; injection temperature is 300°C, and injection volume is 1 μL (splitless). The following oven program is used: isothermal at 80°C for 1 min, rising by 20°C/min to 100°C, by 8°C/min to 130°C, and by 5°C/min to 320°C, then isothermal at 320°C for 10 min.
3. *MS.* The MS analysis is performed with a high-resolution mass spectrometer working in the EI positive mode, using an electron energy of 35 eV. Transfer line and ion source temperature are kept at 280 and 250°C, respectively. Selected ion monitoring is used for acquisition of spectral data.

6.3.1.2 *High-Performance Liquid Chromatography*

The determination of the most toxic PAH BaP in liquid smoke by liquid chromatography was demonstrated in 1992 [39]. Owing to the incapacity of the GC-MS method to resolve the two isomeric PAHs benzo[*j*]fluoranthene and benzo[*k*]fluoranthene and the need of having a second validated method in case of legal conflicts, a second method based on HPLC with ultraviolet adsorption and fluorescence detection for the separation and determination of the 15 priority E.U.-PAHs was developed at the Joint Research Centre of the European Commission [40].

6.3.1.2.1 *Sample Preparation for High-Performance Liquid Chromatography*

Ten grams of the liquid smoke condensate were refluxed for 30 min with alkaline methanol (3.2 g of potassium hydroxide in 32 mL of methanol) to saponify interfering compounds and ionize weak acids, for example, phenol. The analytes were extracted from the methanolic solution three times with 25 mL of cyclohexane each. The organic phases were pooled, and the aqueous phase was discarded. The organic phase was dried with sufficient anhydrous sodium sulfate. The organic phase was removed by rotary evaporation under reduced pressure ($T = 40$, $p = 100$ mbar) to dryness. The sample was reconstituted with 500 μL of cyclohexane and transferred onto a silica cartridge activated with 2 mL of cyclohexane. The flask was rinsed with a second 500 μL of fresh cyclohexane, which was also transferred onto the cartridge. The first milliliter of the eluate was discarded. The analytes were eluted with 7 mL of cyclohexane. The eluate was now collected, and the solvent was removed under reduced pressure as mentioned earlier. The nearly colorless sample was redissolved in 1 mL acetonitrile by vortexing for 1 min and transferred to a capped amber 2-mL autosampler vial.

6.3.1.2.2 *Analysis and Instrument Conditions*

A 20-μL aliquot was injected into an HPLC (1100 series, Agilent, Waldbronn, Germany) system equipped with autosampler, quaternary pump, thermostated column oven ($T = 40$) and FLD (G1321A). For the separation a Pinnacle II reversed-phase column for PAHs, 250 × 2.1 mm, 5-μm particle size (Restek GmbH, Bad Homburg, Germany) was used. The flow of the aqueous mobile phase (acetonitrile/water) was set to 0.3 mL/min. The gradient program for the mobile phase started with 80% acetonitrile (0 min) changing linearly to 85% (30 min) and 100% (40 min). After 60 min, the mobile phase was reset to the initial composition within 10 min and allowed to equilibrate for another 10 min. Total runtime of one analysis was 80 min. The analytes were detected and quantified by monitoring the UV absorbance at 375 nm and the fluorescence

emissions simultaneously at 370, 420, 470, and 500 nm with one common excitation wavelength of 270 nm. Details on data evaluation can be found in Ref. 40.

6.3.2 *Aroma Constituents from Traditional Smoking*

The analysis of smoke components is a very difficult task due to the complexity of the pyrolysis products [12,41]. Smoke composition can be studied either using condensed smoke from traditional smoke generators or by analyzing commercial, modern LSFs.

6.3.2.1 *Traditional Smoking*

Basically, saw dust from beech wood is used as feedstock, whereas in the United States hickory wood is preferred. Alder or oak may be added to beech to get a darker color. Moreover, juniper berries, herbs, pine needles, and fir cones may be used to enhance the smell and taste. Probably, each smoke generation company has its own recipe and individual taste.

Analysis of smoke preparations from traditional smoking processes were already described by Fujimaki et al. [42] and Kim et al. [43] in the early 1970s. A review of main smoke components and the chemistry of smoked foods was presented by Gilbert and Knowles [44]. Very detailed studies on the phenolic fraction of preparative smoke samples obtained from a laboratory smoke generator were presented by Tóth et al. [45–47].

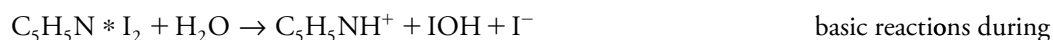
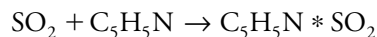
6.3.3 *Aroma Constituents from Liquid Smoke Flavorings*

6.3.3.1 *Determination of Major Chemical Parameters in Liquid Smoke Flavorings*

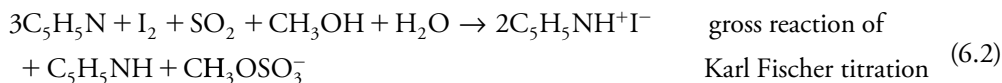
Major chemical parameters are often needed for the description of typical chemical and physical properties. These data are useful for an application for product authorization in the E.U. and for the technical data sheet of a liquid smoke product. There are no public detailed data available, as most of these methods described in the following sections are so-called “house methods.” Therefore, only the basic principles are explained.

6.3.3.1.1 *Water*

Water in LSFs should not be determined by distillation, as many other components form an azeotropic mixture with water. It is recommended to determine the water content in LSFs by Karl-Fischer titration. There are ready-to-use solutions, such as Hydranal Composite 2 from Riedel de Haën. The solution for the titration consists of iodine, sulfur dioxide, pyridine, and methanol in the ratio 1:3:10:50. The detection is based on the oxidation of sulfur dioxide with iodine. During the reaction, water is consumed. The following chemical reactions are involved:



Accordingly, the gross reaction is as follows:



The endpoint of the titration is determined potentiometrically by dead-stop indication.

6.3.3.1.2 pH

The pH can simply be determined with a standard pH meter.

6.3.3.1.3 Acids

Acids are measured as titratable acidity calculated as acetic acid. Liquid smoke contains a wide variety of organic acids. The total content of organic acids can be determined potentiometrically by titration with sodium hydroxide to pH 7. Generally, a standardized 0.1 M NaOH solution is used. The consumption of NaOH in 1 mL is equivalent to the acid number, and the reporting is as acetic acid.

6.3.3.1.4 Phenols

There are several colorimetric methods for the determination of phenols:

1. *Gibbs method.* The sample is mixed with Gibbs reagent (2,6-dichloro-*p*-benzoquinone 4-chloroimine). The reagent adds to the *para* position of phenol derivatives to give indophenol, a blue color.
2. *Modified Gibbs method.* Phenols are calculated as 2,6-dimethoxyphenol. It is determined by means of 2,6 dibromoquinone-4-chlorimide. The reagent reacts with phenols to produce a magenta color. The extinction is measured at 590–610 nm.
3. *Emerson's method.* Phenols also react with 4-aminoantipyrine (Emerson's reagent) in alkaline solutions to give a red color. Potassium ferricyanide is used as alkaline oxidant.

6.3.3.1.5 Carbonyls

A widely used method for total carbonyl determination is the hydroxylamine hydrochloride method, which was used as early as 1895 and has been improved by using pyridine as the oximation catalyst for quantitative determination of pure compounds. Hydrochloric acid is titrated potentiometrically with NaOH solution to pH 2.90. The total carbonyl content is reported as 2-butanone.

The methods for overall characterization are not very specific and thus give only approximate results of the sample composition. Hence, the results can only be used for comparing different samples.

6.3.3.1.6 Staining Index

The color-forming potential of carbonyl compounds from liquid smoke solutions with selected amino acids can be determined with a colorimetric procedure [48]. A common amino acid is glycine, which gives in an acidic environment a typical color that is determined at 440 nm.

6.3.3.2 Determination of Single Smoke Constituents

6.3.3.2.1 Direct Determination by Gas Chromatography (“Dilute and Shoot”)

For the determination of single constituents, high-resolution capillary gas chromatography is necessary. Based on the legislative requirements of the E.U., a practical method is helpful to determine as many smoke components as possible. A reliable method, which is already used in LSF analysis, is based on the “dilute and shoot” principle [6]. In this method, the complete liquid smoke product is dissolved with acetone containing fluoranthene as IS. The solution is injected into a GC system equipped with both a mass spectrometer and a flame ionization detector. Example chromatograms of three different LSF formulations are shown in Figure 6.4

Special attention should be paid to the selection of the capillary column. The liquid smoke components have a wide range of boiling points and a wide range of different polarities ranging from strong and weak acids (acetic acid and phenols, respectively) to neutral compounds such as alcohols, aldehydes, lactones, and anhydrosugars.

A suitable film phase for the “dilute and shoot” method is 14% cyano-propyl-phenyl–86% dimethylpolysiloxane (GC phase number 1701). This semipolar film has been successfully used to separate both carbohydrate-derived [49] and lignin-derived pyrolysis products [50,51].

The column end is connected to a T-piece which splits the column flow in a ratio of 1:1. One part goes into the MS, the other part into the FID, so that the generated mass spectra can be used for identification and the FID signal for quantitation. A collection of mass spectra from wood carbohydrates and lignin can be found in Refs 52–55.

The sample preparation is as follows:

LSF is dissolved with acetone containing IS fluoranthene (200 µg/mL). The concentration of the LSF, based on the organic part, should be approximately 5%. If necessary, filter the solution through a 0.45-µm filter.

The GC conditions are as follows:

- *Injection.* Split injection, split ratio 30:1; temperature 250°C, constant flow 2 mL, 226 kPa helium pressure
- *Oven.* 45°C constant for 4 min, heating rate 3°C/min to 280°C, hold for 20 min
- *Detector.* FID, 280°C, column: 60 m × 0.25 mm, 0.25 µm film thickness, phase composition: 14% cyanopropyl-phenyl–86% dimethylpolysiloxane (1701)

The MS conditions are as follows:

- Ionization with EI at 70 eV
- Ion source temperature 230°C
- Scan range 33–400

Table 6.1 shows the complete list of constituents, which can be determined by the “dilute and shoot” method. For quantification, the components should be calibrated according to the IS method. For this purpose, the response factor of each compound versus the detector response of the IS has to be determined. In this case, fluoranthene is used as IS. The relative response factor (RRF) is calculated as follows:

$$\text{RRF} = \frac{\text{amount}_{\text{sample}}}{\text{area}_{\text{sample}}} \times \frac{\text{area}_{\text{IS}}}{\text{amount}_{\text{IS}}} \quad (6.3)$$

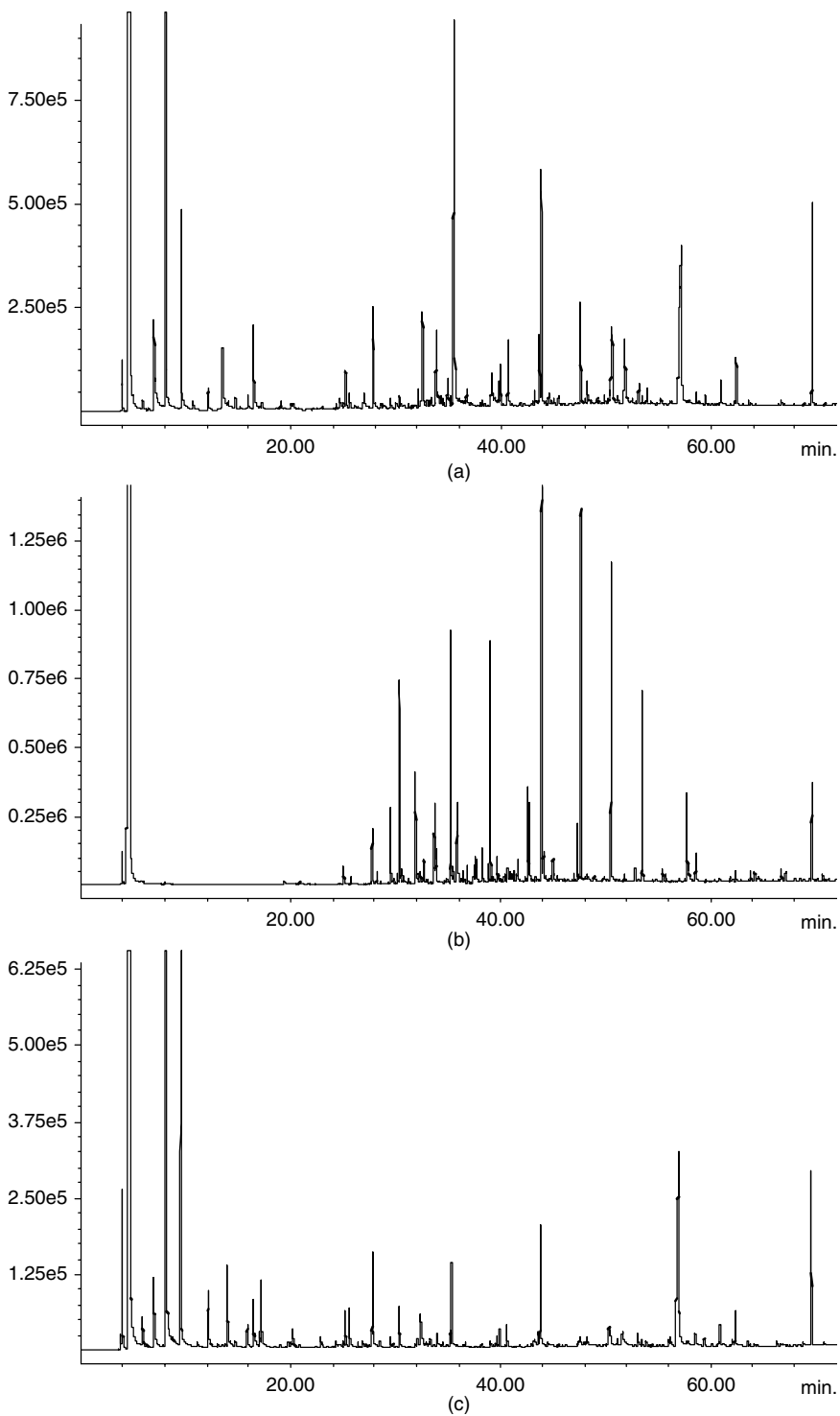


Figure 6.4 Gas chromatograms of different liquid smoke samples: (a) typical nontreated sample, (b) product enriched in phenols, and (c) product enriched in browning compounds.

Table 6.1 Chemical Constituents in Liquid Smoke Samples as Determined by the “Dilute and Shoot” Method, Relative Retention Times (RRT) Based on Fluoranthene

<i>Compound</i>	<i>RRT</i>	<i>CAS-No.</i>
Butandione, 2,3-(diacetyl)	0.084	431-03-8
Propanoic acid methylester	0.086	554-12-1
Acetaldehyde, hydroxy-	0.105	141-46-8
Acetic acid	0.115	64-19-7
Acetol; (hydroxypropanone)	0.136	116-09-6
Ethene, 1,2-dihydroxy-	0.147	
Acetoin; (butanone-2, 3-hydroxy-)	0.159	513-86-0
Propanoic acid	0.174	79-09-4
Ethleneglycol	0.193	107-21-1
Cyclopentanone	0.198	120-92-3
Butanone, 1-hydroxy-2-	0.201	5077-67-8
Propionaldehyde, 3-hydroxy	0.204	
Furanone, 2(3H)-	0.215	2082-571-2
Butanoic acid, 2-propenyl ester	0.228	
Butenoic acid	0.237	
Tetrahydrofuran, 2,5-dimethoxy-trans	0.243	
Cyclopenten-1-one, 2-	0.245	930-30-3
Furaldehyde, 2-; (2-furfural)	0.247	98-01-1
Furfuryl alcohol, 2-	0.278	98-00-0
Acetyloxypropane-2-one, 1-	0.283	592-20-1
Cyclopentene-1-one, 2-methyl-2-	0.287	1120-73-6
Furan, 2-acethyl-	0.299	1192-62-7
Cyclopenten-1-one, 2-hydroxy-2-	0.328	
Furaldehyde, 5-methyl-2-	0.349	620-02-0
Cyclopenten-1-one, 3-methyl-2-	0.359	2758-18-1
Butyrolactone, gamma-	0.361	96-48-0
Furanone, 2(5H)-	0.367	497-23-4
Cyclopenten-one, dimethyl- (not 4,4- or 2,3-)	0.369	
Furan-2-one, 5-methyl-(5H)-	0.378	591-11-17
Pyran-4-one, 3-hydroxy-5,6-dihydro-(4H)-	0.386	
Cyclopenten-1-one, 2,3-dimethyl-2-	0.398	1121-05-7
Cyclopenten-1-one, 2-hydroxy-3-methyl-2-	0.399	80-71-7
Furan-2-one, 2,5-dihydro-3,5-dimethyl-	0.400	
Furanone, 3-methyl-2(5H)-	0.400	22122-36-7
Cyclopenten-one, trimethyl-	0.405	
Cyclopentanone, dimethyl-	0.409	
Furan-2-one, 2,5-dihydro-3,5-dimethyl-	0.409	
Phenol	0.423	108-95-2
Cyclopenten-one, trimethyl-	0.432	
Guaiacol	0.435	90-05-1
Cyclopenten-one, trimethyl-	0.438	
Cyclopenten-1-one, 3-ethyl-2-	0.441	568-26-99
Cyclopenten-one, trimethyl-	0.448	
Cresol, o-	0.458	95-48-7
Cyclopenten-1-one, 3-ethyl-2-hydroxy-2-	0.460	21835-01-8
Cyclopenten-one, trimethyl-	0.464	

(Continued)

Table 6.1 (Continued)

<i>Compound</i>	<i>RRT</i>	<i>CAS-No.</i>
Phenol, 2,6-dimethyl-	0.469	576-26-1
(5H)-Furan-2-one, 4-methyl-	0.478	
Cresol, <i>p</i> -	0.482	106-44-5
Cresol, <i>m</i> -	0.483	108-39-4
Guaiacol, 3-methyl-	0.485	
Lacton derivative (base mass 85)	0.487	
(5H)-Furan-2-one, dimethyl-	0.495	
Guaiacol, 4-methyl	0.506	93-51-6
Phenol, 2-ethyl-	0.509	90-00-6
Anhydrosugar (unknown)	0.510	
Phenol, 2,4-dimethyl-	0.514	105-67-9
Phenol, 2,5-dimethyl-	0.515	95-87-4
Toluene, 3,4-dimethoxy	0.522	494-99-5
Phenol, 2,4,6-trimethyl-	0.528	527-60-6
Phenol, 2,3-dimethyl-	0.537	526-75-0
Phenol, 3,5-dimethyl-	0.539	108-68-9
Phenol, 4-ethyl-	0.541	123-07-9
Phenol, 3-ethyl-	0.542	620-17-7
Guaiacol, 3-ethyl-	0.549	
Phenol, 3,4-dimethyl-	0.557	95-65-8
Guaiacol, 4-ethyl-	0.560	2785-89-9
Phenol, (2,3,4- or 2,4,5-)trimethyl-	0.568	
Anhydrosugar (unknown)	0.570	
Anhydrosugar (unknown)	0.573	
Dianhydro- α -D-glucopyranose, 1,4:3,6-	0.583	
Phenol, 4-propyl-	0.596	645-56-7
Guaiacol, 4-vinyl-	0.597	
Guaiacol, 4-allyl-; (eugenol)	0.612	97-53-0
Guaiacol, 4-propyl-	0.613	
Furaldehyde, 5-(hydroxymethyl)-2-	0.620	67-47-0
Lactone derivative	0.627	
Syringol	0.630	91-10-1
Furanone, dihydro-4-hydroxy-2(3H)-	0.639	5469-16-9
Guaiacol, 4-propenyl-; (Isoeugenol) <i>cis</i>	0.646	97-54-1
Anhydro- β -D-xylofuranose, 1,5-	0.673	
Guaiacol, 4-propenyl-; (Isoeugenol) <i>trans</i>	0.678	5932-68-3
Syringol, 4-methyl-	0.684	
Vanillin	0.688	121-33-5
Hydroquinone	0.692	123-31-9
Syringol, 3-ethyl-	0.702	
Benzene, dihydroxy-methyl-	0.716	
Syringol, 4-ethyl-	0.725	
Deoxysugar (unknown, unspecific spectrum)	0.726	
Acetoguaiacone; (Phenylethanone, 4-hydroxy-3-methoxy-)	0.735	498-02-2
Anhydrosugar or deoxysugar (unknown)	0.741	
Deoxysugar (unknown)	0.743	
Syringol, 4-vinyl-	0.758	

Table 6.1 (Continued)

<i>Compound</i>	<i>RRT</i>	<i>CAS-No.</i>
Guaiacyl acetone	0.762	2503-46-0
Syringol, 4-allyl-	0.767	6627-88-9
Syringol, 4-propyl-	0.768	
Syringol, 4-(1-propenyl)-cis	0.796	627-88-9
Levoclcucosan; (Anhydro- β -D-glucopyranose)	0.822	498-07-7
Syringol, 4-(1-propenyl)- trans	0.828	
Dihydroconiferyl alcohol	0.830	
Syringaldehyde	0.841	134-96-3
Homosyringaldehyde	0.863	
Acetosyringone	0.875	2478-38-8
Anhydro- β -D-glucofuranose, 1,6- or galactofuranose, 1,6-	0.892	
Syringyl acetone	0.895	
Propiosyringone	0.912	
Isomer of sinapyl alcohol	0.921	
Dihydrosinapyl alcohol	0.958	
Fluoranthene = IS	1000	206-44-0

As not every constituent is commercially available, the response of chemically similar components can be estimated from known constituents. Once all response factors are collected, quantitation of the sample can start, using the following formula:

$$\text{Weight (mg)} = \frac{\text{area}_{\text{sample}} \times \text{RRF}}{\text{area}_{\text{IS}}} \times \text{amount}_{\text{IS}} \text{ (mg)} \quad (6.4)$$

In general, 85–95% of the chromatogram area—representing the volatile fraction—can be determined using this method. It must be emphasized that the detectable part of the LSF depends on the amount of oligomeric components left in the sample. These components are derived from lignin and represent the “pyrolytic lignin,” which is an integral part of all crude pyrolytic liquids from wood. Depending on the production process of the LSF, the pyrolytic lignin portion can be in the range of 15 wt% for crude primary smoke products.

6.3.3.2.2 Liquid–Liquid Extraction

Because of the wide polarity of the LSF components, liquid–liquid extraction methods have been used to separate chemical groups such as acids, phenolics, carbonyls, basic, and neutral fractions [42]. Extraction techniques are useful to facilitate chromatographic separation and to get a closer look into components with low concentrations. This technique has also been used for the separation of other pyrolysis liquids from wood [56]. Other approaches use only a water-immiscible solvent such as methylene chloride to analyze the organic [57] and the aqueous fraction [58–60]. As a consequence, Guillén and coworkers could separate nitrogenated as well as dimeric and trimeric lignin-derived products. Separation and detection of the compounds is by GC-MS. Details can be found in the corresponding literature.

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Chapter 7

Colorants

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7.1 Introduction

Color and appearance are the most important quality attributes in foods. They are the first characteristics evaluated by consumers looking to consume or buy foods. Color is also associated with flavor. Nonetheless, most naturally occurring pigments are unstable during processing or storage depending on light, oxygen, temperature, water activity, and pH conditions.¹

The colors of foods are the result of natural pigments or of added colorants. The natural pigments are a group of substances present in animal and vegetable products. The added colorants are regulated as food additives; some of them are synthetic colors. The naturally occurring pigments encompass those already present in foods as well as those that are formed on heating, storage, or processing.²

7.2 Colorants in Poultry and Meat Processing

7.2.1 Natural Processed Meat Color: Curing

The normal processing of most meat and poultry products involves curing and heat treatment. Curing refers to the addition of curing salts (sodium nitrate or nitrite) to develop the characteristic pink coloration, which is achieved by subsequent heat treatment. Besides the formation of the characteristic pink color, the curing process also contributes to flavor and to the inhibition of the spore-forming bacterial pathogen *Clostridium botulinum*.

7.2.1.1 Curing Process Reactions

In poultry and meat, pigments such as myoglobin and derivatives (mainly oxymyoglobin and metmyoglobin) can be considered as natural occurring colorants that determine fresh meat color. The reactions (iron oxidation and ligand compound) of these pigments determine the particular coloration of different processed products. An important aspect of the curing process is the immobilization of iron in the heme complex, which retards its catalytic activity.

The curing process involves many complex reactions, of which three reactions can be considered as central steps. The first involves curing salts (sodium nitrate) reacting with a reducing substance (sodium ascorbate or erythorbate). Since sodium nitrite is a strong oxidant, the reaction with sodium ascorbate or erythorbate accelerates the conversion of nitrite to nitric oxide. The reverse reaction is suppressed, resulting in a more complete conversion of the muscle pigment to the cured pigment form. In the second stage, nitric oxide is transferred to myoglobin to yield nitrosylmyoglobin. And finally, nitrosohemochrome is formed during cooking, giving the particular pink coloration.^{3,4}

Nitrates can also be reduced by bacteria in some ripened meat products, as Parma or Serrano hams. The microbial conversion of myoglobin into red derivatives may be involved, since several lactic acid bacteria (like *Kurthia* sp., *Lactobacillus fermentum*, and *Staphylococcus xylosus*) have proved capable of reducing myoglobin to ferrohemeochrome, changing the color from brown to bright red.^{5,6}

7.2.1.2 Problems Associated with Curing

Nitrites can react with secondary and tertiary amines during cooking or ingestion, forming nitrosamines, which are carcinogenic N-nitrosous compounds. Control of curing by addition of salt to the formulation is an important aspect in meat product quality. Traditionally, residual nitrates

were analyzed by the Griess reaction, but other instrumental techniques based on high-performance liquid chromatography (HPLC) have been developed,^{7,8} and more recently spectrophotometrically by sequential injection analysis.⁹ Control of ongoing nitrite levels, the use of reducing agents (such as ascorbate or erythirbate), and adherence to good manufacturing practices will substantially reduce the problem.¹⁰

The second problem, color stability in meat products, is influenced by a large number of factors, including packaging and storage conditions. The effects of external factors during packaging and storage can be diminished by the choice of packaging and storage conditions (considering the permeability of the packaging film and consequent gas absorption) to improve the color and shelf life of meat products.¹¹ Color retention in processed meat products is different from that of fresh meat. Cured meat products are more sensitive to chemical and biochemical reactions than fresh meat as a consequence of storage conditions. Color changes in processed meat products include decoloration by dehydration as a result of low moisture and high temperature during storage.¹² The degree of photo-oxidation of the nitrosyl meat pigments in cured product is highly affected by the oxygen pressure above the cured meat products; controlling the oxygen can minimize these color changes.¹³

In view of these considerations, the meat industry has looked for nitrite reduction or substitution, including the use of nitric oxide gas or nitrate-containing spices, to improve control of nitrate addition in product formulation. Nonetheless, none of these methods are commercially widespread.¹⁴ Consequently, the use of colorants seems to be a reasonable alternative to improve or maintain the color of processed meat products.

7.3 Colorants

“Colorant” is the collective term for all soluble or solubilized color agents (dyes or pigments), as well as insoluble pigments, employed to impart color to a food. Two approaches are commonly taken into consideration regarding food colorants classification. The first, based on the origin of the colorant, relates to whether a food colorant is natural, nature-identical, or synthetic. A natural colorant is the one that is synthesized, accumulated, or excreted from a living cell. Nature-identical colorants are those produced by chemical synthesis to match the chemical structure of colorants found in nature. The second approach is based on chemical structure of the colorants.¹⁵

Colorants are regulated and are categorized either as “certifiable” (those derived primarily from petroleum, known as coal-tar dyes) or “exempt from certification” (those obtained largely from mineral, plant, or animal sources). There are no generally recognized as safe (GRAS) exemptions for color additives, and all color additives are subject to premarket approval requirements.¹⁶ In Europe, directive 94/36/EC defines color additives as substances which add or restore color in a food; they include natural constituents of foodstuffs and natural sources which are normally not consumed as foodstuffs as such and are not normally used as characteristic ingredients in foods, that is, preparations obtained from foodstuffs and other natural source materials obtained by physical and/or chemical extraction resulting in a selective extraction of the pigments relative to the nutritive or aromatic constituents.

Colorants as food additives can be divided into intentional additives and incidental additives. Intentional additives are chemical substances that are added to food for specific purposes; these are regulated by strict government controls. According to the Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act of 1958, a food additive is defined as any substance the intended use of which results, or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food. This law recognizes

the following three classes of intentional additives: (1) additives GRAS, (2) additives with prior approval, and (3) food additives.¹⁷

7.3.1 Colorant Regulation

As chemical substances, colorants can cause adverse reactions in human metabolism, causing allergenic or toxicological reactions. In addition, colorants could be added to mask food defects or alterations.

The regulatory process is intended to ensure that colorants have a good safety record; regulatory agencies must determine whether there is a reasonable certainty of no harm from the color additive under its proposed conditions of use. For example, if the color additive is approved, the United States Food and Drug Administration (U.S. FDA) issues regulations that may include the types of foods in which it can be used, the maximum amounts to be used, and how it should be identified on food labels. Color additives proposed for use in meat and poultry products must also receive specific authorization by the U.S. Department of Agriculture. The European Community adopted Framework Directive 89/107/EEC, which set out the criteria by which additives would be assessed, including the adoption of the specific technical Directive 94/36/EC on colors. This directive establishes a list of additives that can be used and the foods in which they can be used, as well as maximum levels. To obtain an E-number (approval of an additive by the European Union), the additive must have been fully evaluated for safety by the European Food Safety Authority (EFSA). The E-number system serves as a simple and convenient way to label permitted additives across the range of languages in the European Union. Some of these colorants are listed in Table 7.1.

Table 7.1 List of Some Approved Food Colorants by U.S. FDA and Economic European Community (EEC)

<i>Color Additives Exempt from Certification</i>			
<i>FDA</i>	<i>EEC</i>	<i>Straight Color</i>	<i>Use and Restrictions</i>
73.40	E162	Dehydrated beets (beet powder)	Foods generally
73.100	E120	Conchineal extract; carmine	Foods generally
73.200	E172	Synthetic iron oxide	Sausage casings, NTE 0.1% (by weight); dog and cat food, NTE 0.25% (by weight)
73.250		Fruit juice	Foods generally
73.260		Vegetable juice	Foods generally
73.340	E160c	Paprika	Foods generally
73.345	E160c	Paprika oleoresin	Foods generally
73.585	E160	Tomato lycopene extract; tomato lycopene concentrate	Foods generally
73.600	E100	Turmeric or curcumin	Foods generally
Color Additives Subject to Certification			
74.250		Orange B	Casings or surfaces of frankfurters and sausages, NTE 150 ppm
74.303	E127	FD&C Red No. 3	Foods generally
74.340	E129	FD&C Red No. 40	Foods generally
74.705	E102	FD&C Yellow No. 5	Foods generally
74.706	E110	FD&C Yellow No. 6	Foods generally

Note: NTE, Not to exceed.

7.3.2 Colorant Analysis

Colorant analysis is important because colorants can be added to a food to mask certain defects in raw materials or that occur during processing. In the same way, as stated earlier, regulation of colorants must take account of any toxicity resulting from the intake of certain restricted additives. Certifiable colorants are subjected to constant monitoring and analysis.

Recently, the EFSA announced the appearance of potential health concerns regarding the colorant Red 2G, permitted in certain breakfast sausages and burger meat. It has been shown that Red 2G is converted to aniline in the body, a substance considered carcinogenic based on animal studies.⁷ While some colorants are obtained from natural sources and can be considered exempt from certification, any toxicity of the synthesized compounds must be corroborated. For example, red pigments from geniposidic acid (obtained from the fruit of *Gardenia jasminoides* Ellis) can be combined with arginine or glutamic acid under acidic conditions to yield a low-toxicity colorant.¹⁸

With the wide availability and development of sophisticated chromatographic and spectrophotometric analytical techniques, the analysis of colorant composition has become routine. In the past 40 years, many advances have been made to establish destructive and nondestructive methods for the estimation not only of colorant composition but also of color quality indices. Both the developing industry and potential buyers need analytical methods that are simple, quick, and readily available, enabling a rapid evaluation of product quality. The most important methods are for extractable color and that of chromatic attributes. Such methods furnish measurements of color. Other more reliable methods include chromatographic separation prior to quantification.¹⁹ Table 7.2 describes applications of some techniques employed for food colorant analysis.

7.3.2.1 Light Reflectance and Color Systems

Noninvasive techniques include the determination of reflected light on food surface. The reflection of light from opaque and translucent materials depends on the ratio of absorption to scatter as affected by pigmentation, refractive index, and the light-scattering properties of the material, as described by the Kubelka–Munk theory.⁵ The Kubelka–Munk theory was originally developed for paint films but works quite well in many circumstances. A limiting assumption is that the particles making up the layer must be much smaller than the total thickness. Both absorbing and scattering media must be uniformly distributed through the sheet. Ideally, illumination should be with diffuse monochromatic light, and observation should be of the diffuse reflectance of the surface. The theory works best for optically thick materials where >50% of light is reflected and <20% is transmitted.³⁴ Another widely employed technique uses the coordinates of L* (luminosity), a* (redness–greenness), and b* (yellowness–blueness) in the Commission Internationale de L’Eclairage (CIE) Lab to define the location of any color in the uniform color space.³⁵

Invasive techniques involve the extraction of coloring substances, most often employing organic solvents, for determination. The most common techniques are described next.

7.3.2.1.1 Spectroscopy

Based on the property of all chemical species—that they interact with electromagnetic radiation to diminish the intensity of the power of a radiant beam—absorption spectroscopy measures the attenuation or power decrease of radiation caused by the analyte. Absorptiometric methods are characterized according to the type of electromagnetic radiation employed, including x-ray,

Table 7.2 Methods of Analysis Employed to Determine Colorants as Food Additives

<i>Methodology and Colorants Determined</i>	<i>Details and References</i>
Noninvasive techniques	
<i>Quantitative colorimetric (CIE Lab)</i>	
Monascus	Quantitative colorimetric ²⁰
Invasive techniques	
<i>Absorbance</i>	
Tartrazine, Sunset yellow, Ponceau 4R, Amaranth, Brilliant blue	Derivative absorbance and iterative target transformation factor analysis mixture of pigments ²¹
<i>HPLC</i>	
Brilliant blue, Sunset yellow, Tartrazine	Green—no solvents—chromatography ³⁸
Tartrazine, Quinoline yellow, Sunset yellow, Carmosine, Amaranth, Ponceau, Erythrosine, Red 2G, Allura red AC, Patent blue, Indigo carmine, Brilliant blue FCF, Green S	Reverse phase ²²
Tartrazine, Quinoline yellow, Sunset yellow, carmoisine, Amaranth, Ponceau, Erythrosine, Red 2G, Allura red AC, Indigo carmine, Brilliant blue FCF, Green S, Brilliant black BN	Diode-array detection ²³
Lycopene	Accelerated solvent extraction ²⁴
<i>Capillary electrophoresis</i>	
<i>Dyes</i>	Large volume stacking ²⁵
Carmoisine, Amaranth, Ponceau 4R, Red 2G	Laser induced fluorescence detection ²⁶
<i>Anthocyanins</i>	Ultraviolet (UV)-diode array ²⁷
Tartrazine, Fast green FCF, Brilliant blue FCF, Allura Red AC, Indigo carmine, Sunset yellow FCF, New cocchine	High-performance with diode-array detection ²⁸
Cochineal, Safflower, Gardenia, Monascus, Elderberry natural	Solid phase extraction ²⁹
Tartrazine, Fast green FCF, Brilliant blue FCF, Allura red AC, Indigo carmine, Sunset yellow FCF, New cocchine, Carminic acid	Microemulsion electrokinetic chromatography ³⁰
<i>Mass spectroscopy</i>	
<i>Anthocyanins</i>	UV-diode array ²⁷
Free and acylated betacyanins	Matrix-assisted laser desorption/ionization quadruple ion trap time-of-flight mass spectrometry (MALDI-QIT-TOF-MS) ³¹
<i>Raman spectroscopy</i>	
Tartrazine, Sunset yellow, Carmoisine A, Cochineal red A	Raman ³²
<i>Voltammetric determination</i>	
Tartrazine, Sunset yellow, Allura red	Polyallylamine modified tubular electrode ³³

UV, visible, infrared, microwave, and radio-frequency radiation. Photometers provide a simple and relatively inexpensive tool for the performance of absorption analysis over a narrow band of radiation. This has some advantages, including, first, enhanced adherence of the absorbing system to Beer's law. Second, since other substances that absorb in other wavelengths can interfere, selectivity is ensured by focusing on a desired wavelength. Finally, a great change in absorbance per increment of concentration will be observed if only wavelengths that are strongly absorbed are employed, thus attaining a greater sensitivity.³⁶ Ni and Gong²¹ employed derivative spectroscopy, an efficient technique for determining compound mixtures, and chemometric approaches to determine mixtures of food colorants.

Mass spectrometers use the differences in the mass-to-charge ratio of ionized atoms or molecules to separate them from each other. Mass spectrometry is therefore useful for quantitation of atoms or molecules and also for determining chemical and structural information about molecules. Molecules have distinctive fragmentation patterns that provide structural information to identify structural components.³⁷ Mass spectroscopy has also been applied to analyze anthocyanins²⁷ and betacyanins.³¹

Raman spectroscopy is the measurement of the wavelength and intensity of inelastically scattered light from molecules. The Raman scattered light occurs at wavelengths that are shifted from the incident light by the energies of molecular vibrations. The mechanism of Raman scattering is different from that of infrared absorption, and Raman and infrared spectra provide complementary information. Typical applications are in structure determination, multicomponent qualitative analysis, and quantitative analysis.³⁷ Dyes are ideally suited to analysis by a special type of Raman phenomenon called the resonance Raman effect. If a sample is excited by a laser frequency that falls within the envelope of the visible absorption of that sample, the resultant Raman signal is enhanced over the Raman signal normally observed. Since dyes absorb in the visible region, it is relatively easy to take advantage of this resonance enhancement.³⁸

7.3.2.1.2 Chromatography

Chromatography refers to processes that are based on differences in rates at which the individual components of a mixture migrate through a stationary medium under the influence of a moving phase.³⁶ HPLC has been widely employed to analyze different types of food colorants.^{22–24,39}

7.3.2.1.3 Electrophoresis

The process of electrophoresis is defined as the differential movement or migration of ions by attraction or repulsion in an electric field provoked by positive and negative electrodes placed in a solution containing ions. When a voltage is applied across the electrodes, solute ions of different charges—that is, anions (negative) and cations (positive)—will move through the solution toward the electrode of opposite charge. Capillary electrophoresis, then, is the technique of performing electrophoresis in buffer-filled, narrow-bore capillaries, normally from 25 to 100 μm in internal diameter.⁴⁰ Different modifications of this technique have been employed in colorant analysis.^{25–29}

7.3.2.1.4 Voltammetry

Voltammetry comprises a group of electroanalytical procedures that are based upon the potential-current behavior of a small, easily polarized electrode in the solution being analyzed. Voltammetry

was developed from the discovery of polarography, adapting this principle to detection of endpoints in volumetric analyses, known as amperometric titrations. Virtually every element is amenable to polarographic analysis; in addition, this method can be extended to determine several functional groups. Polarographic data are obtained by measuring the current as a function of the potential applied to a special electrode cell, resulting in a current–voltage curve called a polarogram, which provides both quantitative and qualitative information about the composition of the solution being analyzed with the electrodes.³⁶ Silva et al.³³ employed square wave voltammetry to determine food azo colorants.

7.3.3 Use of Natural Coloring Agents

The increased demand for organic foods, with no chemical additives used in their fabrication, has raised the demand for natural sources of food ingredients. Colorants are exempt from certification if they are obtained from vegetable, animal, or mineral sources.¹⁷ Some meat products have employed natural colorants in an effort to reduce the use of curing salt without detrimental effects on sensory characteristics.

7.3.3.1 Betalains

The betalains or betanins are limited to 10 families of the order Caryophyllales. The only foods containing betalains are red beet (*Beta vulgaris*), chard (*B. vulgaris*), cactus fruit (*Opuntia ficus-indica*), and pokeberries (*Phytolacca americana*). The betanins have two major groups: the red pigment betacyanins and the yellow betaxanthins.⁴¹ The use of betanin as a colorant in meat products has shown good results. Cured meat colors can be simulated to a high degree with some levels of betalain pigments. Sausages containing beet pigment or pure betanin in their formulation exhibited a hue closely matching that of sausage containing nitrate–nitrite, and the color of betalain-containing sausages proved to be more stable to light exposure during storage than the color of those containing nitrate–nitrite salts. Sensory evaluation indicated no significant overall preference for samples prepared with nitrite–nitrate salts over samples prepared with optimum levels of betalain pigments.⁴² Cooked products with betanin were the most acceptable to consumers according to their color.⁴³

7.3.3.2 Carminic Acid

Cochineal extract is obtained from the bodies of female cochineal insects, particularly *Dactylopius coccus* Costa, by treating the dried bodies with ethanol. After removal of the solvent, the dried residue contains about 2–4% carminic acid, the main color component.⁴¹ Use of carminic acid in processed meat products increased redness of raw batter and the red color in frankfurters as compared with samples formulated with sodium nitrates.⁴³

7.3.3.3 Curcumin

Turmeric is a colorant produced from the rhizomes of several varieties of *Curcuma longa*, a perennial shrub grown in many tropical areas around the world. It contains three main pigments: curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Curcumin is insoluble in water, but a water-soluble form can be made by complexing the compound with tin or zinc to form

an intensely orange colorant. This is not allowed in most countries,⁴¹ but its use in frankfurters increased the yellowness of samples.⁴³

7.3.3.4 *Lycopene*

Lycopene is the major pigment in tomatoes and tomato products and is one of the major carotenoids in the human diet.⁴¹ Tomato paste inclusion (12%) enhanced the preferences of consumers for frankfurters based on their color, enabling the reduction of nitrites from 150 to 100 ppm without any negative effect on the quality of the product.² In another study, the addition of lycopene from natural tomato sources to meat-stuffed product resulted in a red to brown hue but a lower tendency to rancidity. Owing to the acidic tomato products, the pH in meat stuffed products was lower, thus inhibiting the growth of microorganisms.⁴⁴

7.3.3.5 *Monascus*

Monascus colorants are produced by several fungal species of the genus *Monascus*.⁴¹ Red pigments secreted by *Monascus ruber* were incorporated in sausages and pâté, and the colorants added to these meat products remained stable when stored for 3 months at 4°C. Sensory tests revealed that Monascus colorant could replace nitrate salts or other colorants such as cochineal.⁴⁵

7.3.3.6 *Paprika Extract*

Paprika is a very old colorant and spice prepared from the dried pods of the sweet pepper *Capsicum annum*.⁴¹ Frankfurters with paprika extract had a high redness value and good acceptance by consumers according to their color.⁴³

7.3.3.7 *Carbon Monoxide*

In model cooked meat systems and hotdogs, a direct flushing with a 1% carbon monoxide gas mixture during the last stage of batter chopping produced an initial red color equal to nitrite and more intense than nitrite, since carbon monoxide binds strongly to myoglobin, forming the stable bright red carboxymyoglobin. Red color stability of carbon monoxide products was maintained to a high degree by anaerobic packaging and storage as compared with products with nitrate, although in air and under light display, color stability was inadequate.¹⁴

7.3.3.8 *Vegetable Juices*

The use of vegetable juice powder in uncured emulsified frankfurter-style cooked sausage with a starter culture of *Staphylococcus carnosus* was comparable to sodium nitrite-added control for color, lipid oxidation, cured pigment, and trained sensory measurements.⁴⁶

7.4 Concluding Remarks

Colorants are an important additive in the food industry. Meat and poultry products have been associated with the characteristic “pink” cured color produced by the reaction of nitrites

with myoglobin. The search for alternatives to mimic this typical coloration opens the way for colorants, with the concomitant development of techniques of analysis, to follow the maximum limits allowed by national and international legislation, which is important in ensuring consumer safety.

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Chapter 8

Determination of Oxidation

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8.1 Introduction: Oxidation in Muscle Foods

The oxidation of muscle and meat products is a primary concern among food technologists, as it is considered a major cause of deterioration in the quality of muscle foods.¹

The oxidative degradation of fatty acids involves several molecular mechanisms that lead to the generation of oxygen-rich precursors of reactive, chain-propagating free radicals. Initially, oxygen attacks the double bond in fatty acids to form peroxide linkages and, therefore, muscle phospholipids, which contain a high content of unsaturated fatty acids (mainly linoleic and arachidonic acids), are particularly susceptible to oxidation. In fact, the susceptibility of muscle components to oxidative deterioration is mainly due to high concentrations of unsaturated fatty acids and the presence of heme pigments and metal catalysts. Although the study of oxidation in muscle foods has traditionally been focused on the lipid peroxidation, recent studies have shown how proteins can be also affected by oxidative reactions.^{2–4} In this chapter, therefore, both lipid and protein oxidation will be considered for the review of the mechanisms and analytical techniques of interest.

After slaughter, *in vivo* antioxidant systems in muscles collapse, whereas the oxidative deterioration of muscle components, mainly lipids and proteins, is enhanced by the presence of prooxidant factors. The occurrence and intensity of oxidative reactions is enhanced during processing due to the disruption of tissues and the application of heat, which accelerates oxidative reactions. The development of oxidative reactions in muscle foods involves the loss of essential fatty acids, amino acids, and vitamins and affects many quality characteristics such as flavor, color, texture, and nutritive value.¹ Although the effect of protein oxidation in processed meats is currently poorly understood, recent studies have related the oxidation of muscle proteins to texture changes in refrigerated meat^{4,5} and frankfurters.⁶

The role played by oxidative reactions in the loss of the quality in muscle foods has challenged scientists to develop a suitable methodology to evaluate the oxidative status of muscle foods. It is generally known that the complexity of the chemistry involved in the oxidative degradation of lipids and proteins prevents the development of a single general analytical test for unambiguous evaluation of oxidative deterioration in muscle foods. However, the understanding of precise chemical pathways and the development of improved techniques for the isolation, identification, and quantification of lipid and protein oxidation products have been helpful in developing accurate methodologies. Apart from this improvement and the recent development of spectroscopy techniques for the quantitation of lipid oxidation products, the same routine methods have been employed for more than 20 years, with the most common techniques being focused on the detection and quantification of secondary lipid oxidation products such as thiobarbituric acid–reactive substances (TBA–RS) and hexanal. However, great efforts have been made to develop a suitable methodology for the analysis of protein oxidation in muscle foods. In most cases, the analytical methods used in biomedical sciences are being successfully extrapolated to muscle food systems. To date, the dinitrophenylhydrazine (DNPH) coupling method for the quantification of protein carbonyls can be considered the most common technique. The increasing interest in protein oxidation and the necessity of fulfilling demanding objectives in a pioneering field demands the development of advance techniques for the characterization of particular protein oxidation products (protein aldehydes).

8.2 Lipid Oxidation

8.2.1 Mechanisms and Factors

Lipid oxidation is a radical reaction described as a combination of various chain reactions, consisting of three phases⁷—initiation, propagation, and termination—which take place at the same time, apart from the initial step.

During the initial phase, in the presence of initiators or the reactive oxygen species (ROS), unsaturated lipids lose a hydrogen radical to form a lipid free radical.⁸

The direct reaction between the fatty acid and molecular oxygen is highly improbable since lipid molecules have a singlet electronic state and the oxygen molecule has a triplet ground state. This spin barrier between lipids and oxygen can be overcome by the presence of initiators that can produce radicals by different mechanisms: (i) thermal dissociation, (ii) decomposition of hydroperoxides catalyzed by redox metals, and (iii) exposure to light in the presence of a sensitizer such as ketone.⁸ Unsaturated lipids are easily oxidized by the ROS, which include oxygen radicals and nonradical derivatives of oxygen.⁹ ROS having a reduction potential of greater than 1000 mV are thermodynamically capable of oxidizing polyunsaturated fatty acids (PUFA), which has 600 mV.¹⁰ The hydroxy radical, which is the strongest oxidant, with 2300 mV of reduction potential, is mainly responsible for the initiation of lipid oxidation.⁹

During the propagation stage, the alkyl radical of an unsaturated lipid containing a labile hydrogen reacts very rapidly with molecular oxygen (O_2) to form peroxide radicals. This reaction is always much faster than the following hydrogen transfer reaction with unsaturated lipids to form hydroperoxides (ROOH), which are considered the primary products of lipid oxidation.⁸ The newly formed hydroperoxy radical can abstract hydrogen from an adjacent unsaturated fatty acid since the reaction sequences goes through 8–14 propagation cycles before termination.¹¹ Hydroperoxides are considered the most important initial reaction products from lipid oxidation. They are labile species, of very transitory nature, which undergo changes and deterioration with the radicals. Their breakage results in secondary products such as pentanal, hexanal, 4-hydroxynonal, and malonaldehyde (MDA).⁷

At the last stage of oxidation, the radical species react with each other and self-destruct to form nonradical products by different mechanisms. At atmospheric pressure, termination occurs first by the combination of peroxy radicals to an unstable tetroxide intermediate, followed rapidly by its decomposition by the Russell mechanism, which yields nonradical products. Alkoxy radicals can react with unsaturated lipids to form stable and innocuous alcohols or undergo transformation into unsaturated aldehydes such as MDA and other unstable compounds.

Morrissey et al.¹ considered three different phases of lipid oxidation in muscle—when the animal is alive, throughout the conversion of muscle in meat, and during meat processing. When the animals are alive, there are several mechanisms limiting the exposition to ROS and therefore slowing down the lipid oxidation phenomena. These mechanisms include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase; storage and transport proteins; and retinol. In addition, the so-called chain-breaking antioxidants (vitamin E, C, β -carotene, thiol, etc.) and ascorbate play an important role in the protective mechanisms against oxidation. However, during the post-slaughter period, which involves the conversion of muscle into meat, the balance between prooxidative and antioxidative factors favors the prooxidative ones. Anaerobic metabolism, the end of blood and nutrient circulation, and the lack of enzyme protective mechanisms, together with the occurrence of several post-slaughter events related to pH drop, carcass temperature, and tenderization techniques, lead to structural modification in membrane cells which promotes lipid oxidation. During meat processing, handling, storage, and cooking, iron is released from high molecular-weight compounds such as myoglobin and hemoglobin and is available to form chelates through interaction with low molecular-weight compounds such as amino acids, nucleotides, and phosphates. This chelate compounds have a high ability to catalyze lipid oxidation, as do the high molecular-weight iron sources.¹

8.2.2 Assessment of Lipid Oxidation

Food lipids are susceptible to oxidation and, as such, analytical protocols are required to measure their quality. There are many analytical methods for measuring the oxidative status of meat and

meat products, ranging from simple sensory evaluations to more complex chemical methods. Chemical methods can be classified into two groups according to what they measure—primary oxidative changes (mainly formation of primary oxidation products) and secondary oxidation events (determination of secondary oxidation products originating from primary oxidation product decomposition).

8.2.2.1 Primary Oxidation Products: Hydroperoxides

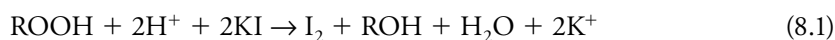
The primary oxidation products formed during the autoxidation of unsaturated lipids, the hydroperoxides, have little or no direct impact on the odor and flavor of the food product. However, hydroperoxides are easily decomposed to secondary oxidation products, of which some are volatiles with very low sensory thresholds. The analysis of lipid hydroperoxides in meat and meat products generally requires a prior lipid extraction with solvents, which must be carefully removed to avoid the decomposition of hydroperoxides or loss during solvent evaporation.⁸ Various procedures and solvent combinations have been employed to extract lipids in hydroperoxides determination. Usually, the extraction is performed with a mixture of polar and nonpolar solvents.^{12–14} Moreover, other new methods such as accelerated solvent extraction (ASE) have been described in fresh¹⁵ and cooked meat.¹⁶ Extraction with ASE could be more convenient because it is rapid, solvent saving, and, at the same time, oxidation protecting, given that the extraction occurs under flow of nitrogen.

Analytical procedures for the measurement of lipid hydroperoxides in meats and meat products can be divided into two groups: analytical methods for determining the total amount of hydroperoxides and those based on chromatographic techniques, which give information on the structure and amount of specific hydroperoxides present in a certain sample.¹⁷

8.2.2.1.1 Chemical Methods Based on Redox Reactions: Peroxide Value

The peroxide value (PV) of a fat is defined as the quantity of peroxide oxygen present in the sample. The time to reach a certain PV may be used as an index of oxidative stability for food lipids. Thus, a longer time period to reach a certain PV is generally indicative of a better oxidative status. However, the ability to measure the content of hydroperoxides as an indicator of lipid oxidation is limited due to the transitory nature of hydroperoxides. The peroxide content increases only when the rate of peroxide formation exceeds that of its destruction. Thus, a low PV may represent either early or advanced oxidation. In cases where peroxide breakdown is as fast as or faster than hydroperoxide formation, lipid hydroperoxides are not a good indicator of lipid oxidation.

8.2.2.1.1.1 Iodometric Assays The classical iodometric method is a volumetric analysis based on the titration of iodine released from potassium iodide by hydroperoxides (Equation 8.1) using a standardized thiosulfate solution as the titrant and a starch solution as the indicator. The amount of iodine present is determined by titration with a standard sodium thiosulfate solution using a starch indicator (Equation 8.2), thereby reflecting how much hydroperoxide is present in the lipid extract. The PV is generally expressed in terms of milliequivalents (mEq) of oxygen kg^{-1} lipid or meat. The official determination is described by the International Union of Pure and Applied Chemistry (IUPAC)¹⁸ and the Association of Official Analytical Chemists (AOAC).¹⁹



The sensitivity of this method is about 0.5 mEq/kg of lipid. The iodometric method is highly empirical and any change in procedure may cause variation in results.^{20,21} The two principal sources of error in this method are the liberation of iodine by air oxidation of the potassium iodide and absorption of iodine by fatty acid double bonds. Moreover, a 5-g test portion is required for this technique and it is sometimes difficult to obtain sufficient quantities of lipid from lean meat cuts and low-fat muscle foods. Despite these drawbacks, iodometric determination is one of the most common tests for monitoring lipid oxidation and has been used for following the stages of oxidation or the effects of antioxidants and food processing on meat and meat products.^{1,22,23}

8.2.2.1.1.2 Determination of Hydroperoxides by Measurement of Iron Oxidation Another approach for the determination of PV in meat products is a spectrophotometric method based on the ability of peroxides to oxidize iron(II) to iron(III).^{24–26} Ammonium thiocyanate reacts with ferric ions, resulting in a colored complex that can be measured spectrophotometrically at 500 nm. PVs as low as 0.1 mEq/kg sample can be determined with this method, providing a distinct advantage over iodometric titration, although the values obtained are higher by a factor of 1.5–2 relative to those of the iodometric methods.⁸

Alternatively, the determination of ferric ions can be carried out by the ferrous oxidation–xylenol orange (FOX) method.²⁷ The FOX method is based on the ability of lipid peroxides to oxidize ferrous ions at low pH. The resulting oxidation is quantitated by using a dye, which binds the resulting ferric ions to produce a blue–purple complex that can be measured spectrophotometrically, with a maximum of absorbance between 550 and 600 nm. The FOX method has been reported to have high sensitivity, comparable to or even better than that of the iodometric assay in meat products.^{28,29} FOX determination kits for food analysis are also available.²⁸ Hermes-Lima et al.³⁰ adapted the FOX method to muscle-based products, avoiding prior lipid extraction and thus minimizing the chance of hydroperoxide loss during solvent evaporation.

8.2.2.1.2 Analysis of Hydroperoxides by Chromatographic Techniques

In addition to the classic analytical methods for determining the total amount of hydroperoxides, several gas and liquid chromatographic techniques have been developed and applied to quantify and identify specific hydroperoxides in a variety of substances, including model compounds and food lipids. As compared to analytical methods for determining the total amount of hydroperoxides, chromatographic techniques have two main advantages. First, information on specific hydroperoxide structures can be obtained. Second, only a few milligrams of sample are required in most applications.¹⁷ Methods with high sensitivity and selectivity such as gas (GC)³¹ and liquid³² chromatography, often paired with various methods (mass spectrometry for gas chromatography and chemiluminescence or fluorescence detection for high-performance liquid chromatography [HPLC]), have been developed for the analysis of the structure and amount of specific hydroperoxides. However, these methods are not easily adapted to routine screening of large numbers of muscle-based products, and while some applications in the field of food lipids have been published (for a review, see Ref. 17), these techniques have more frequently been used in model systems and biological studies.

8.2.2.2 *Primary Oxidation Products: Conjugated Dienes*

Another simple and rapid technique to assess lipid oxidation in muscles is the measurement of conjugated diene formation. In organic chemistry, the term “conjugated diene” refers to two double bonds separated by a single bond. When conjugated diene moiety is present in fatty acids, it shows an absorption in the ultraviolet (UV) region at 233 nm and stands out as a distinct peak that can be determined spectrophotometrically. As with PV determination, conjugated diene determination will reach a maximum during the progress of oxidation and decrease when the rate of hydroperoxide decomposition is higher than the rate at which it is formed. In muscle foods, a prior lipid extraction with hexane/isopropanol (3:2),³³ or chloroform/methanol (2:1)²⁹ is required.

The measurement of conjugated dienes is a sensitive method to follow the early stages of lipid oxidation instead of, or in addition to, PV determination. This method, standardized by IUPAC (Standard Method 2.505¹⁸) is faster than PV determination, is much simpler, does not depend on chemical reactions or color development, and is a nondestructive assay, but the magnitudes of changes in absorption are not easily related to the extent of oxidation and depend on the fatty acid composition of the analyzed lipid fraction.^{20,34} This drawback is due to the fact that the absorption peak appears as a shoulder on the strong absorption band due to other lipid components, especially the polyunsaturated fatty acids themselves. In this sense, Grau et al.²⁹ found that, in meats with high levels of polyunsaturated fatty acids, the PV and TBA techniques showed a much higher sensitivity in following lipid oxidation than conjugated diene determination.

8.2.2.3 *Other Approach to Primary Oxidative Changes Determination in Meat Products*

8.2.2.3.1 *Oil Stability Index: Rancimat Test*

A common approach to determine oxidative stability involves holding samples under accelerated conditions of storage and measuring lipid oxidation products over a period of time.²⁰ Currently, automated versions of this technique, known as the oil stability instrument (OSI) and Rancimat, are available. The measurement of lipid oxidation during accelerated storage may be performed using tests such as PV or hexanal in the sample headspace. In contrast, the OSI and Rancimat methods measure lipid oxidation by monitoring the conductivity of water in which lipid volatiles are trapped. These methods have been criticized because they run at elevated temperatures at which the mechanism of lipid oxidation changes.⁸ Although the OSI and Rancimat methods were primarily developed for oil samples, it is possible to use these methods on lipids extracted from meat products. Balev et al.³⁵ used this technique, with prior lipid extraction,¹³ to determine the effect of antioxidants against lipid oxidation in dry-fermented sausage. Rižnar et al.³⁶ studied (after Soxhlet extraction) antioxidant effects in chicken frankfurters.

8.2.2.3.2 *Changes in Lipid Substrate*

Measurement of changes in fatty acid composition in long-term-cured products, such as dry-cured loin or ham, is a useful technique to identify class of lipids and fatty acids that are involved in the oxidative and lypolytic changes during meat processing.^{37,38} These techniques require total lipid extraction from meat products and subsequent conversion to derivatives suitable for gas chromatographic analysis. Separation of lipids into neutral lipids, phospholipids, and free fatty acids may also be necessary.^{39,40}

8.2.2.3.3 Recent Developments in Primary Oxidative Changes Analysis

In the past years, new methods and techniques have been developed to improve the analysis of the early stages of lipid oxidation. Electron spin resonance (ESR) spectroscopy can measure short-lived free radicals in many types of food matrices and has been validated for prediction of the lipid stability of meats^{41,42} or cured products.⁴³ Front-face fluorescence spectroscopy is another fast and nondestructive technique that can measure lipid oxidation in various types of muscle foods.^{44,45} The basis of this method is that lipid oxidation products (hydroperoxides or aldehydes) can combine with primary amine groups in, for example, amino acids, proteins, peptides, or DNA, yielding reaction products that fluoresce when they are illuminated. Primary oxidation products may also be assessed by chemiluminescence spectroscopy. Chemiluminescence generally originates from electronically excited stages, such as singlet molecular oxygen in lipid peroxidation.²¹ This method has been employed to evaluate the oxidative status in fish meat. However, according to Olsen et al.,⁴⁵ no relationship was found between chemiluminescence determination and other methods measuring either primary or secondary lipid oxidation products in freeze-stored or mechanically recovered poultry meat. Finally, mid-Fourier transform infrared spectroscopy gives information about the different functional groups present in a sample. Production of hydroperoxydes during oxidation of lipids gives rise to an absorption band at about 2.93 μm , whereas the disappearance of a band at 3.20 μm indicates the replacement of a hydrogen atom on a double bond or polymerization. It is also suggested that the appearance of an additional band at 5.72 μm , due to C=O stretching, indicates the formation of aldehydes, ketones, or acids. Furthermore, changes in the absorption bands in the 10–11 μm regions indicate trans-isomerization and probably formation of conjugated bonds. All these techniques have been successfully used to investigate the extent of lipid oxidation phenomena in different meat systems (Table 8.1).

8.2.2.4 Secondary Oxidation Products: Malonaldehyde

MDA (1,3-propanedial), a secondary product from lipid oxidation, is a three-carbon dialdehyde with carbonyl groups at the C1 and C3 positions. The amount of MDA has been commonly used as an oxidation index in muscle foods, and different analytical techniques have been reported in the scientific literature to determine and quantify MDA.

Table 8.1 Summary of Recent Methodologies Used to Monitor Products from Lipid Oxidation in Muscles Foods

<i>Technique</i>	<i>Sample</i>	<i>Reference</i>
ESR spectroscopy	Porcine muscle	46
	Turkey muscle extracts	47
	Chicken meat	48
	Chicken meat	42
	Bovine muscle	49
Fourier transform infrared (FTIR) spectroscopy	Edible fats and oils	50,51
	Pork adipose tissue	52
Chemiluminescence spectroscopy	Pork back	53
	Poultry meat	45

8.2.2.4.1 Thiobarbituric Acid Test

The extent of lipid oxidation in muscle foods is commonly determined by monitoring MDA formation following the TBA assay.⁵⁴ The TBA test is a colorimetric technique in which the absorbance of a red chromogen formed between TBA and MDA is measured. MDA is considered the major TBA-reactive substance, although other oxidation products such as α - and β -unsaturated aldehydes (for instance, 4-hydroxyalkenals) and certain unidentified nonvolatile precursors of these substances may also be involved.⁵⁵ For this reason, this test is usually referred to as the TBA-RS method.

The reaction with TBA occurs by attack of the monoenolic form of one molecule of MDA on the active methylene groups of two molecules of TBA with the eliminations of two molecules of water, leading to the formation of a red-colored complex with an absorbance maximum at 532–535 nm. The intensity of the absorbance at this wavelength is related to the concentration of MDA.⁵⁶ TBA acid has been widely used as a reagent for the colorimetric measurement of MDA amounts due to the stability and the high molar extinction coefficient of the resulting adduct at 532 nm.

Different methods can be used to perform TBA test in muscle foods; most of them have been reviewed recently by Fernández et al.⁵⁵ Briefly, the TBA test can be performed (i) by directly heating the sample with TBA followed by separation of the pink complex produced by centrifugation, (ii) by distillation of the sample followed by reaction of the distillate with the TBA, (iii) by extraction of MDA using aqueous trichloroacetic or perchloric acid and reaction with TBA, and (iv) by extraction of the lipid portion of the sample with organic solvents and reaction of the extract with the TBA (Table 8.2).

The formation of additional MDA and other TBA-RS due to heating and acidic conditions promotes further oxidation and therefore an overestimation of TBA-RS numbers. Jardine et al.⁶⁶ reported that the presence of barbituric acid impurities in the thiobarbituric acid reagent was found to produce adducts that absorbed at 513 and 490 nm. Hence, these authors suggested purifying TBA before using it. Some investigators have used antioxidants such as propyl galate, ethylene diamine tetraacetic acid (EDTA), and butylated hydroxytoluene (BHT) during the distillation step to avoid sample autoxidation during TBA assays.⁶⁷

The major disadvantage of the TBA-RS reaction is that TBA is not specific for MDA, and other lipid oxidation compounds or compounds not related to lipid oxidation can react with the TBA,⁶⁸ thereby leading to an overestimation of the extent of lipid oxidation. Considering the aforementioned limitations, Ross and Smith⁶⁹ pointed out that the TBA-RS procedure may be used to assess the extent of lipid oxidation in general, rather than to quantify MDA.

8.2.2.4.2 Determination of Malonaldehyde by Gas Chromatography and HPLC

GC methods for the determination of MDA require the formation of a stable derivative of MDA since free MDA is not suitable for direct GC analysis. Most reported GC methods give a total measure of free MDA and its bound forms because their assay conditions are sufficient to hydrolyze or decompose bound MDA during sample preparation. Hydrazine-based reagents, such as 2,4-dinitrophenylhydrazine or *N*-methylhydrazine,⁷⁰ have been often preferred because of their capacity to form stable pyrazole derivatives.

Among the chromatography methods, the most commonly used is GC-mass spectrometry (GC-MS), though the use of GC with electron capture detection has been also reported.⁷¹

Table 8.2 TBA Methods to Evaluate Lipid Oxidation in Muscle Foods: Technique, Advantages, Disadvantages, and Reference Studies

<i>TBA Test Method</i>	<i>Analyze Conditions</i>	<i>Advantage(s)</i>	<i>Disadvantage(s)</i>	<i>Reference</i>
Direct extraction	Heating Acidic conditions		Time consuming Many solvents extraction are needed Turbidity of the samples	57
Distillation method	Extraction reagent (butanol) Heating distillation	The most widely used for meat samples More sensitive and more suitable for fat samples than direct extraction Rapid and reproducible	TBARs overestimation Requires the collection of a specific volume of distillate	58–60
Extraction method	Prior aqueous-acid extraction of the sample (trichloroacetic acid/perchloric acid)	Nonexposition to heat Faster and easier than distillation method Recommended when a large number of samples need to be analyzed High correlations with sensory evaluation scores	Interferences due to the presence of impurities (water soluble proteins, peptides, and other aldehydes) May not be able to extract all products from lipid oxidation and thus the level of TBA values could be underestimated	47,61–64
Lipid extraction procedure	Prior lipid extraction by evaporation at high temperature and under flow evaporation	Noninterfering substances are present Recommended when the susceptibility to oxidation of different types of lipids or individual lipids (phospholipids) is studied	Overestimation of TBARs	65

A developed capillary GC method that allows determination of free MDA has been reviewed by Denis and Shibamoto.⁷² The method represents an advantage over existing techniques for MDA determination because capillary GC offers the highest efficiency of separation among chromatographic methods, thus allowing a more specific and accurate measure of MDA.

Regarding HPLC techniques, Kakuda et al.⁷³ initially used this method to quantify MDA in aqueous distillate and found a linear correlation between TBA values and HPLC results.

This method was followed by Williams et al.⁷⁴ to determine meat oxidation. Bergamo et al.⁷⁵ measured MDA levels in different foods, including beef, pork, and poultry, by HPLC assay with fluorometric detection. As described earlier for GC, some authors have reported a prior derivatization of MDA before HPLC analysis. Marcincak et al.⁷⁶ described a method to evaluate the lipid oxidation in broiler meat by detection of MDA with HPLC as 2,4-dinitrophenylhydrazine derivative.

Although results obtained with HPLC are more reliable compared to spectrophotometric methods of TBA detection, the HPLC technique has not become very popular due to the extremely complex nature of sample preparation and slowness of the technique.⁷⁶

8.2.2.5 Secondary Oxidation Products: Induced Lipid Oxidation

To evaluate the liability of muscles and raw meat to lipid oxidation, one of the most widely used methods involves the incubation of homogenates of muscle tissues through different times under prooxidative conditions. Once incubation times finish, aliquots of homogenates are taken and subjected to MDA assays using the TBA test.

The method described by Kornbrust and Mavis⁷⁷ for assessing the susceptibility of microsomes from lung, heart, liver, kidney, and brain of different animal species has subsequently been modified for application in muscle model systems and fresh meat (Table 8.3). In muscle tissues, before the induced-oxidation step, it is recommended to keep the samples in ice to avoid heating of the samples during the homogenization step. Following this method, lipid peroxidation in homogenate samples is induced by the presence of prooxidant factors such as ferrous sulfate (FeSO_4) (iron-induced lipid oxidation) and temperatures around 37°C. Ascorbic acid is commonly added as a reducing component. Samples are commonly incubated in a buffer (tris-maleate buffer) and under agitation. At fixed time intervals, aliquots are taken from the homogenates for measurement of TBA-RS. The oxidative reactions are stopped by freezing the samples or by the addition of BHT. Enzymatic systems, including NADPH and adenosine diphosphate (ADP), as well as combined systems such as iron-ADP-ascorbate, have been also used to induce lipid oxidation in meat extracts and muscle model systems.

Table 8.3 Summary of Techniques for Evaluating the Susceptibility of Lipid Oxidation in Different Muscles Foods

Sample	Prooxidative component	Reference
Breast, thigh, and fat (chicken)	FeSO_4	78
Pig loins (<i>longissimus lumborum</i>)	FeSO_4	79
Porcine muscle microsomal fraction	FeSO_4	46
Turkey microsomal fraction	Fe(III)	80
Dark chicken meat	FeSO_4	81
Liposomes from phospholipids muscle	Fe(III)	82
Pig muscle (homogenates)	FeSO_4	83
Chicken muscle model system	Iron-ADP-ascorbate	84
Turkey muscle extracts	NADPH	85
Membrane broiler meat	NADPH-ADP	47

8.2.2.6 Secondary Oxidation Products: Lipid-Derived Volatiles

The measurement of volatile lipid-oxidation products has become popular due to the limitations widely reported for both TBA and measurement of primary products of lipid oxidation.⁶⁹ The main lipid-derived volatiles are aldehydes, which have been successfully used to follow up lipid oxidation phenomena in muscle foods. Lipid oxidation of unsaturated fatty acids results in a wide range of these secondary aldehyde products, such as hexanal, propanal, or 4-hydroxy-2 nonenal, which has been commonly used as index of warmed-over flavor (WOF) in meat.

Hexanal, one of the major lipid oxidation products from linoleic and arachidonic fatty acids, has been reported as a useful index of oxidative deterioration in a wide variety of muscle foods such as fried chicken⁸⁶ and cooked refrigerated meats.⁸⁷ St. Angelo et al.⁸⁸ reported that both hexanal and 2,3-octanedione as well as total volatiles showed a high correlation with sensory scores and TBA numbers in cooked beef. A systematic study carried out by Kerler and Grosch⁸⁷ to determine the odorants contributing to WOF of cooked beef patties indicated that WOF was the result of a combination of a loss of desirable odorants along with an increase in lipid peroxidation products, in particular hexanal and trans-4,5-epoxy-(E)-2-decenal. Recently, Jayathilakan et al.⁸⁹ evaluated the WOF profile, expressed in terms of mg hexanal 100 g⁻¹ fat, in cooked meat from three different species (sheep, beef, and pork). In addition to hexanal, other volatile compounds such as propanal, pentanal, octanal, and nonanal have been reported as oxidation markers in meat.^{90,91} Shahidi and Wanasundara²¹ recommended that hexanal can be used as an oxidation index in fats with high levels of ω -6 fatty acids, whereas propanal would serve as a reliable indicator in fats containing high levels of ω -3 fatty acids. However, among volatiles from lipid oxidation, hexanal is still considered the most effective index of lipid oxidation in meat.⁹²

Among the analytical methods reported to analyze the volatiles generated from lipid oxidation in muscle foods, the most established are solvent extraction, simultaneous distillation extraction, dynamic headspace or purge and trap methodology, and solid-phase microextraction (SPME). The main advantages and disadvantages of these techniques have recently been reviewed by Ross and Smith.⁶⁹ Once the volatiles are extracted, separation, identification, and quantification are carried out using GC or HPLC, usually coupled to MS.

8.2.2.7 Fluorescence Measurement as an Indicator of Lipid Oxidation

Fluorescence has been demonstrated to be a good indicator of lipid oxidation in biological materials,⁹³ but it has mainly been used on extracts of lipid and proteins, probably because fluorescence spectra from complex food system are composed by several fluorophores.⁹⁴ During the oxidation process, a great variety of oxidation products are formed, some of them autofluorescent, from the reactions between oxidizing fatty acids or lipid oxidation breakdown products (aldehydes, MDA, hydroperoxydes) and compounds containing primary amino groups (proteins, amino acid, deoxyribonucleic acid [DNA], phospholipids).⁹⁵ Three different groups of fluorophores have been described and characterized by their fluorescence excitation and emission maxima.

This method has numerous advantages: It is rapid, sensitive, and can be used as nondestructive in solid samples.⁹⁴ Compared to TBA colorimetric assay on a molar basis, the amount of MDA detected with fluorescence technique is 10–100 times more sensitive.⁸ It has been successfully used directly on the surface of minced poultry,⁹⁴ turkey,⁹⁶ and pig myofibrils.⁹⁷ Since some of these fluorescent compounds are generated as a consequence of the interaction between oxidized lipids and proteins, they have been also measured as indicators of protein oxidation, as described in Section 8.3.

8.3 Protein Oxidation

Muscle proteins are highly susceptible to oxidation, and yet there is a serious lack of knowledge regarding this particular field. Several authors have recently found that muscle proteins are oxidized during processing and storage of muscle foods^{3,4,6} but the occurrence, extent, and consequences of the onset of protein oxidation in muscles and meat products is still poorly understood. Proteins are the most abundant organic component of muscle and play an extremely relevant role in meat quality from sensory, nutritional, and technological points of view. Therefore, it is reasonable to consider that the oxidative degradation of proteins might have serious consequences on protein functionality and meat quality, although most of these have not been identified yet. There is a requirement for further studies in this field of increasing interest. It seems important, for instance, to develop accurate procedures to evaluate the oxidative reactions affecting muscle proteins. To comprehend the methodological approach of the analytical procedures, it is necessary to present a brief overview of the chemical aspects surrounding the oxidation of proteins.

8.3.1 Mechanisms and Factors

Primary and secondary lipid oxidation products, mainly hydroperoxides and aldehydes, are capable of initiating the oxidation of muscle proteins.⁹⁸ In addition, particular ROS such as $\cdot\text{OH}$, $\text{O}_2\cdot$, and $\text{ROO}\cdot$, as well as metal cations (iron, copper), can catalyze the abstraction of a hydrogen from an susceptible amino acid residue, leading to the generation of a protein radical.⁹⁹ Recent studies have reported that the susceptibility of amino acids to oxidative degradation depends on the oxidizing system and the ROS. According to these studies, sulfur-containing amino acids, particularly cysteine and methionine, are the most susceptible among muscle proteins.¹⁰⁰

Several authors have claimed that the oxidation of proteins is manifested as a free radical chain reaction similar to that of lipid oxidation.¹⁰¹ The abstraction of a hydrogen atom leads to the generation of a protein carbon-centered radical ($\text{P}\cdot$) which is consecutively converted into a peroxy radical ($\text{POO}\cdot$) in the presence of oxygen, and an alkyl peroxide (POOH) by abstraction of a hydrogen atom from another molecule. Further reactions with $\text{HO}_2\cdot$, yields alcoxyl radical ($\text{PO}\cdot$) and its hydroxyl derivative (POH). As a direct consequence of the oxidative damage of muscle proteins, the amino acid residue side chains are modified. These changes include the loss of sulfhydryl groups, the generation of oxidized derivatives (e.g., sulfoxides from methionine) and the conversion of one amino acid residue to a different one.² Furthermore, the oxidation of side chains of certain amino acids (arginine, lysine, proline, and threonine) leads to the generation of carbonyl residues through deamination reactions. Protein carbonyl derivatives can also be formed as a consequence of the fragmentation of the peptide backbone, the reaction with reducing sugars (by Schiff base formation), and by binding nonprotein carbonyl compounds (e.g., MDA).⁹⁹ Another relevant consequence derived from the oxidation of muscle proteins is the formation of aggregates through covalent and noncovalent linkages. The noncovalent forces are promoted by the exposure of nonpolar residues from proteins as a result of an oxidatively induced unfolding, leading to the generation of hydrophobic interactions between protein chains. The formation of noncovalent aggregates is enhanced by the generation of hydrogen bonds and the complexes formed between proteins and oxidized lipids.⁹⁹ The ROS-mediated covalent linkages between two amino acid residues lead to the generation of intra- and interprotein cross-linked derivatives. The mechanism involved in these nondissociable protein aggregates includes (1) the direct condensation of two carbon-centered radicals, (2) the oxidation of cysteine sulfhydryl groups to form disulfide linkages, (3) the interaction of two oxidized tyrosine residues to yield bityrosines, (4) the reaction between a protein aldehyde and

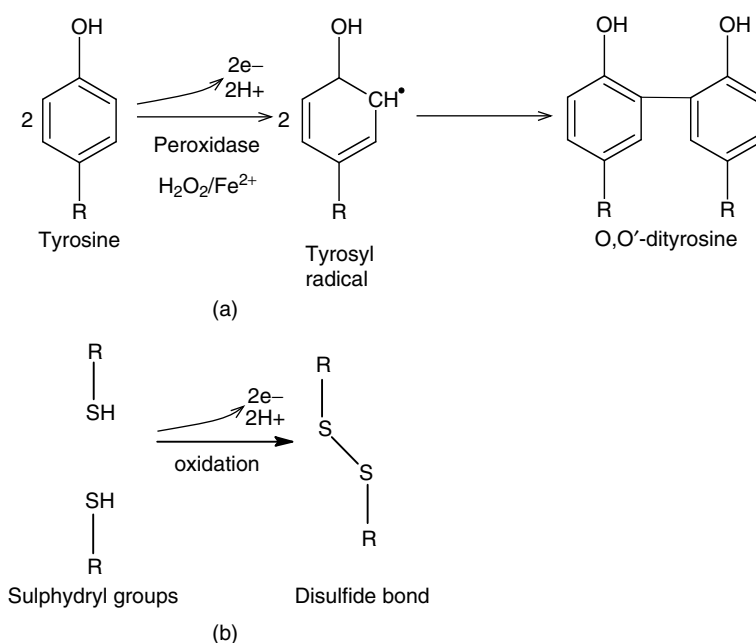


Figure 8.1 Formation of protein cross-linking—dityrosine (a) and disulfide (b) bonds.

the amino group from a lysine in the same or a different protein, and (5) the reaction of two amino groups (from two lysine residues) with a dialdehyde (e.g., malondialdehyde).^{102–105} In recent studies, the development of cross-links between myofibril proteins from chicken beef and porcine muscles subjected to prooxidant storage is mainly attributed to the generation of disulfide linkages and to a lesser extent, to the presence of bityrosines^{4,106–109} (Figure 8.1).

The oxidation of proteins and amino acids is affected by certain environmental factors such as pH, temperature, water activity, and the presence of catalysts or inhibitors. Additionally, the three-dimensional structures of proteins and their amino acid composition influence the propensity of proteins to undergo oxidative reactions.^{75,100,110}

8.3.2 Assessment of Protein Oxidation

The analytical procedures used for assessing protein oxidation have mostly been adapted from those developed for biomedical research and are focused on (i) proving the modification of oxidized proteins and amino acids and (ii) detecting protein oxidation products.

8.3.2.1 Assessment of the Oxidative Modification of Proteins

8.3.2.1.1 Loss of Tryptophan Fluorescence

Proteins and peptides display intrinsic fluorescence due to the presence of aromatic amino acids, namely tryptophan, tyrosine, and phenyl alanine. Protein fluorescence is generally excited at 280 nm or at longer wavelengths, usually at 295 nm. Most of the emissions are due to excitation

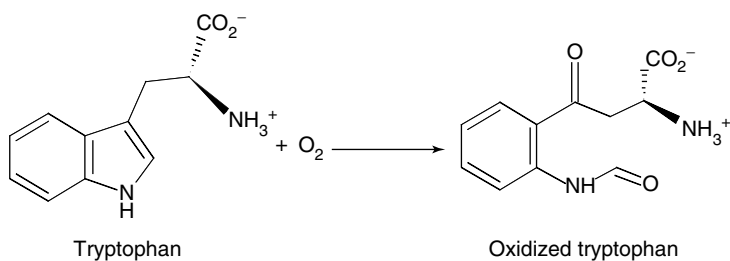


Figure 8.2 Oxidation of tryptophan amino acid.

of tryptophan residues, this amino acid being one of the most sensitive to oxidative decomposition.² Therefore, the depletion of tryptophan fluorescence is considered a reflection of the oxidative modification of proteins (Figure 8.2). In fact, the tryptophan fluorescence wavelength is a widely used tool in monitoring changes in proteins, including oxidative damage.¹¹¹ This methodology has not yet been used in muscle foods; however, recent studies have shown the viability of measuring tryptophan fluorescence with fluorescence spectroscopy to assess the oxidative deterioration of myofibril proteins in oil-in-water emulsions.¹¹² Considering that the procedure is simple and fast, it could be used as an interesting alternative for more time-consuming techniques. However, the intensity, quantum yield, and wavelength of the maximum fluorescence emission of tryptophan are dependent on several variables including the solvent, the origin of the protein, and its unfolding and oxidative status.¹¹¹

8.3.2.1.2 Determination of Protein Thiol Groups

During meat oxidation, cysteine is degraded into cysteine disulfide and sulfenic acid, whereas methionine is readily oxidized to methionine sulfoxide.¹⁰¹ Therefore, the loss of free SH groups in muscle proteins is commonly used as an indicator of protein oxidation. The original method¹¹³ uses Ellman's reagent or 5,5'-dithiobis(2-nitrobenzoate) (DTNB), which rapidly forms a disulfide bond with free thiol groups and releases a thiolate ion (TNB dianion), which is colored and has a maximal absorbance at 412 nm. Taking into consideration that the stoichiometry of protein thiol to TNB formed is 1:1, TNB formation can be used to assess the number of thiols present. This method has been used for assessing the oxidation of myofibrillar proteins in turkey,⁸⁰ chicken,¹¹⁴ beef,¹¹⁵ and porcine muscles.¹⁰⁸ The procedure can be summarized as follows: Muscle proteins are dissolved in a tris-HCl buffer, usually containing urea, sodium dodecyl sulfate (SDS), and EDTA, and an aliquot of the solution is incubated with a DTNB solution. After the reaction is complete, the absorbance measured and recorded at 412 nm can be plotted against a cysteine standard curve to determine the total amount of free thiols in proteins, although certain molar extinction coefficients have been also employed for quantitation purposes.^{108,116}

8.3.2.1.3 Sodium Dodecyl Sulfate Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) techniques are commonly used together with other analyses (detection of protein carbonyls or free thiol groups) for the detection of oxidized myofibril proteins in meat samples. The oxidative degradation of proteins is reflected as a loss of band density in the SDS-PAGE corresponding to 200 kDa (myosin heavy chain [MHC])

and 45 kDa (actin). Additionally, the electrophoresis procedure can be useful in qualitatively determining myosin polymerization, scission, and *in vitro* digestibility. The procedures recently described by a number of authors^{4,107,109} are usually applied to muscle tissue samples, whereas the analysis of oxidized muscle proteins from processed meats using electrophoresis techniques is scarcely found in the literature. Modification of the electrophoresis pattern for myofibril proteins during meat processing by the action of microorganisms and enzymes, among others, would make this technique too nonspecific to be considered a relevant tool for protein oxidation assessment in processed meat products.

8.3.2.2 Detection of Protein Oxidation Products

8.3.2.2.1 Detection of Protein Carbonyls

As described earlier, the oxidative modification of amino acids and peptides can yield carbonyl derivatives and, therefore, determination of carbonyl content in proteins can be used as a measure of oxidative protein damage. The quantitation of carbonyl compounds spectrophotometrically using 2,4-dinitrophenylhydrazine as an indicator of protein oxidation products is a widely used method for evaluating protein oxidation in muscle foods. DNPH has been used for the detection of aldehydes and ketones in oxidized lipids for decades,¹¹⁷ and it is now commonly used for labeling protein carbonyls. Carbonyl groups react with DNPH to form 2,4-dinitrophenylhydrazone, and the amount of hydrazone formed is quantitated spectrophotometrically (Figure 8.3). The original method¹¹⁸ has been successively modified⁸⁰ as follows: After the homogenization of samples and the induced precipitation of the muscle proteins with trichloroacetic acid (TCA), meat samples are incubated with a hydrochloric acid solution containing DNPH. After the remaining DNPH and muscle lipids are removed by washing the pellets with ethanol:ethyl acetate (1:1), muscle proteins are finally dissolved in a phosphate buffer containing guanidine hydrochloride. The concentration of DNP hydrazones is calculated by measuring DNPH incorporated on the basis of an absorption of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 370 nm. Concentration of protein is determined in a control sample (without added DNPH) at 280 nm using bovine serine albumin as standard. Finally, results are expressed as nmols DNP hydrazones per mg of protein.

The simplicity and convenience of this assay makes it a common measurement of protein oxidation, providing useful and meaningful information. This method has been successfully used for quantifying protein carbonyls in a large range of meat samples including beef,⁵ chicken,¹¹⁹ and porcine muscles.¹⁰⁸ It has been highlighted as a sensitive and useful method also for processed muscle foods such as dry-cured products,³ cooked meat patties,¹²⁰ and cooked sausages.⁶ However, several drawbacks of the present procedure have been also described. For example, certain oxidative

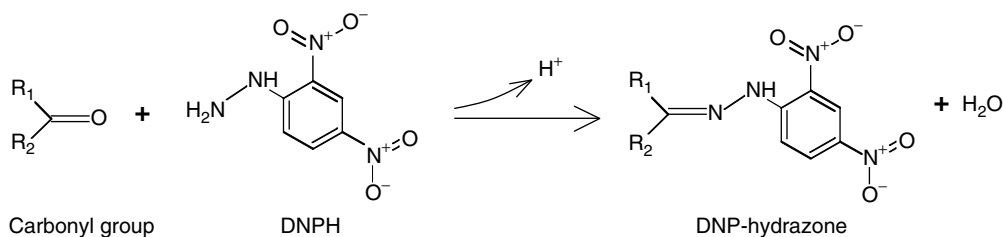


Figure 8.3 Reaction between protein carbonyl groups and DNPH.

modifications in muscle proteins (e.g., oxidation of aromatic amino acids) might not lead to the generation of carbonyl compounds.¹²¹ Additionally, carbonyl moieties can be present in proteins due to mechanisms that do not involve the oxidation of amino acid residues. For example, certain lipid peroxidation products (e.g., alkenals) may react with sulfhydryl groups of proteins to form stable covalent thioether adducts carrying carbonyl groups.¹²¹

Recently, alternative possibilities have been described to assess protein carbonyls in muscle proteins. Protein carbonyls can be detected by measuring fluorescence emitted by these compounds at approximately 450 nm when they are excited at 350 nm.⁹⁸ Recently, Chelh et al.⁹⁷ have detected fluorescent protein oxidation products in meat samples using a front-face fluorescence technique. Similarly, Estévez et al.¹¹² monitored the oxidation of porcine myofibrils in oil-in-water emulsions by using fluorescence spectroscopy. However, Rowe⁵ detected protein carbonyls in beef muscles by combining SDS–PAGE and immunoblotting.

8.3.2.2.2 Detection of Protein Cross-Links

One of the most significant oxidative modifications in proteins is the generation of covalent bonds between amino acids from different proteins. There are methods available for the determination of three different protein cross-linking structures—disulfide bonds, bityrosines, and cross-linked MHCs (Table 8.4).

The method for the determination of disulfide bonds was originally described by Thannhauser et al.¹²² and subsequently improved by Damodaran.¹²³ The disulfide bonds of peptides and proteins are firstly cleaved by excess sodium sulfite at pH 9.5 and room temperature. Guanidine thiocyanate (or other guanidine salt) is added to the protein solutions to denature them and hence make the disulfide bonds accessible. Sulfhydryl groups react with 2-nitro-5-thiosulfobenzoate (NTSB), leading to the generation of 2-nitro-5-thiobenzoate (NTB), which is easily quantified using spectrophotometry (412 nm/molar extinction coefficient of 13,600 M⁻¹ cm⁻¹). This has been described as a sensitive, quantitative, and effortless method, although several limitations have been also reported. For instance, the method could overestimate the presence of disulfide bonds as NTSB also reacts with free sulfhydryl groups. To obtain accurate results, the presence of sulfhydryl groups could be estimated (e.g., using the Ellman reagent) and subtracted from the obtained results. Following this method, Liu et al.¹⁰⁶ and Smyth et al.¹¹⁴ have investigated the presence of disulfide bonds in myofibril proteins from chicken muscles.

Dityrosine was originally discovered as a product of the peroxidase-catalyzed reaction between hydrogen peroxide (H₂O₂) and tyrosine.¹²⁴ As a marker of protein oxidation in muscle foods, dityrosines have scarcely been used, and the results obtained have been at times inconclusive.^{109,110}

Table 8.4 Summary of Techniques Used to Assess Protein Cross-Links in Muscle and Muscle Foods

<i>Cross-linking</i>	<i>Technique</i>	<i>Sample</i>	<i>Reference</i>
Disulfide bonds	Spectrophotometry	Chicken myofibrils	106
		Chicken muscle	114
Bityrosine	Spectrofluorometry	Porcine myofibrillar proteins	108
		Porcine myofibrillar proteins	109
		Porcine myofibrillar proteins	100
MHC	SDS–PAGE	Porcine myofibrils	109
		Porcine muscle	4

This moiety emits intense 420 nm fluorescence upon excitation within either 315 (alkaline solutions) or 284 nm (acidic solutions) absorption bands.¹²⁵ For measuring dityrosine release from proteins preexposed to oxygen radicals, a proteolytic or acid digestion of the oxidatively modified protein is followed by HPLC analysis with fluorescence or diode array detection.¹²⁵ Recently, new attempts for the detection of bityrosines in meat samples have been done measuring fluorescence through spectrofluorophotometry, with 320 and 420 nm being the excitation and emission wavelengths, respectively.¹⁰⁸ Decker et al.¹⁰⁴ described that formation and hence the analysis of dityrosine in muscle samples is affected by pH and ionic strength.

Finally, the formation of protein cross-links can also be evaluated by the detection of cross-linked myosin heavy chain (CLMHC) using SDS–PAGE electrophoresis. Along with the loss of MHC band (approximately 200 kDa), when performing SDS–PAGE of oxidized muscle proteins, certain authors have reported the appearance of a band corresponding to protein structures of a higher molecular weight.¹⁰⁷ Lund⁴ has identified this structure as CLMHC by mass spectrometry.

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Chapter 9

Determination of Proteolysis

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9.1 Introduction

Proteolysis constitutes an important biochemical mechanism during meat processing, especially in those processes involving fermentation and long ripening, where careful control is needed to obtain a real benefit for the final quality of the meat product. The main final products of proteolysis are small peptides and free amino acids that can contribute to an adequate taste. Other compounds, which can contribute to aroma, may be generated from amino acids through further enzymatic and nonenzymatic reactions. The enzymes involved in proteolysis are endoproteases, mainly calpains and cathepsins, which are responsible for protein breakdown,^{1,2} and exopeptidases, mainly dipeptidylpeptidases and aminopeptidases, which are able to cleave small peptides or free amino acids from proteins and peptides.³ The final result consists of the accumulation of free amino acids and small peptides in the meat product.^{4,5}

In spite of the benefits that a controlled proteolysis may exert during processing (e.g., contribution to flavor), an excess of proteolysis must be avoided, because it can affect the sensory characteristics of the meat product.³ An excessive protein breakdown may substantially affect the texture, resulting in an excessively soft meat product.⁶ In other cases, an excessive accumulation of peptides and free amino acids may result in strange tastes, for example, bitter or metallic tastes. Finally, long-ripened products, like dry-cured ham, may have some white crystals of tyrosine in the muscle⁷ or give defective textures.⁸ For all these reasons, it is important to follow proteolysis, including not only protein breakdown but also the generation of peptides and free amino acids, along the processing. This chapter gives details of the most usual methods for such control.

9.2 Extraction and Analysis of Proteins

9.2.1 Protein Extraction

Sarcoplasmic and myofibrillar proteins can be extracted by different methods. One of the most often used consists of the sequential extractions based on the different solubility of both groups of proteins.⁹ The fractionation protocol for the separation of muscle proteins is as follows. The minced meat is suspended at a dilution of 1:10 in 0.03 M phosphate buffer, pH 6.5. The suspension is homogenized for 4 min and then centrifuged at 10,000 g for 20 min at 4°C. The supernatant constitutes the sarcoplasmic proteins fraction, and the pellet is washed twice in the phosphate buffer, to recover all sarcoplasmic proteins that can be added to this fraction. Then, the remaining pellet is weighed and resuspended at a dilution of 1:10 in 0.1 M phosphate buffer, pH 6.5, containing 0.7 M KI to extract the myofibrillar proteins. The suspension is homogenized and centrifuged at 10,000 g for 20 min at 4°C. This procedure is repeated twice for full recovery of myofibrillar proteins. The extraction of muscle proteins can also be performed with a strong salt solution (0.7 M or higher) that simultaneously dissolves the major portion of the sarcoplasmic and myofibrillar proteins.

9.2.2 Protein Analysis by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The hydrolysis of sarcoplasmic and myofibrillar proteins in the extracts is usually determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.¹⁰ Sample proteins are previously dissolved with sodium dodecyl sulfate under heating. Usual percentages

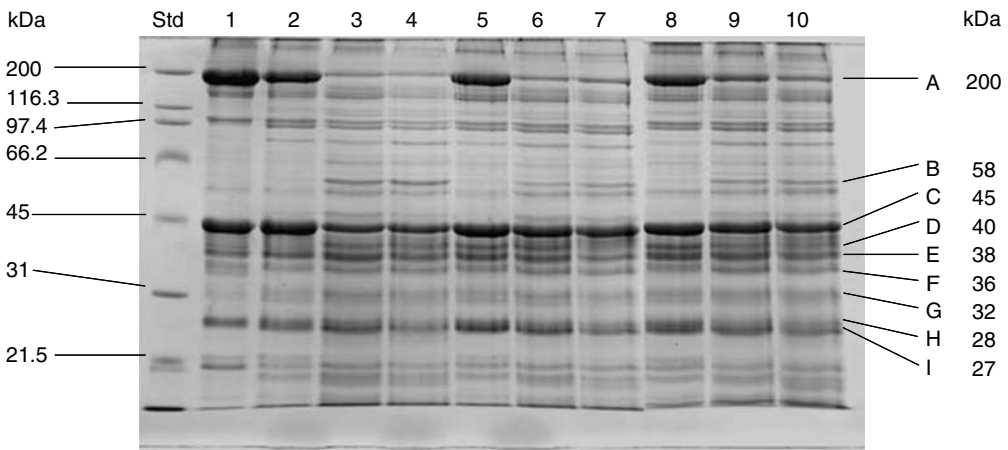


Figure 9.1 SDS-PAGE of myofibrillar proteins from control (lanes 2–4), L1 (lanes 5–7), and L2 (lanes 8–10) batches during ripening stages. Std, standards; lanes: (1) initial (0 days), (2) C-6 days, (3) C-21 days, (4) C-35 days, (5) L1-6 days, (6) L1-21 days, (7) L1-35 days, (8) L2-6 days, (9) L2-21 days, (10) L2-35 days. (Reproduced from Durá, M., Flores, A.M., Toldrá, F., *Meat Sci.*, 68: 319–328, 2004. With permission from Elsevier.)

of polyacrylamide are 3% for the stacking gel and within the range 7–12% for the resolving gel. The percentage of polyacrylamide in the resolving gel depends on the target proteins to follow; higher percentages, e.g., 12%, are chosen for a better separation of lower-molecular-weight proteins, and 7% in the case of the higher-molecular-weight proteins. Recommendations for specific proteins are given by Greaser.¹¹ Broad-range molecular-weight standards can be run simultaneously for the identification of the bands. Usual standard proteins are myosin (200 kDa), β -galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Proteins can be visualized as blue bands by staining with Coomassie Brilliant Blue R-250. Proteins can be stained with silver when more sensitivity is needed, for instance, to detect very low-density bands. The gel can be scanned for quantitative purposes so that the peak area for each respective band is calculated and compared with those from standards. An example of protein breakdown after incubation of myofibrillar and sarcoplasmic protein extracts with the yeast *Debaryomyces* is shown in Figures 9.1 and 9.2, respectively.^{12,13}

9.3 Extraction and Analysis of Peptides

9.3.1 Importance of Peptides during Processing

The isolation and analysis of muscle peptides may have a broad range of relevant applications. Peptides are generated by proteolysis during meat ageing,^{14,15} but most of them are generated during further processing such as meat fermentation and dry-curing of hams.⁴ The generated peptides can be used as markers of the product quality,^{14,16,17} whereas other peptides below 1700 Da have been reported to contribute to the taste.^{18,19} Some small peptides isolated from dry-cured ham have been sequenced recently.²⁰

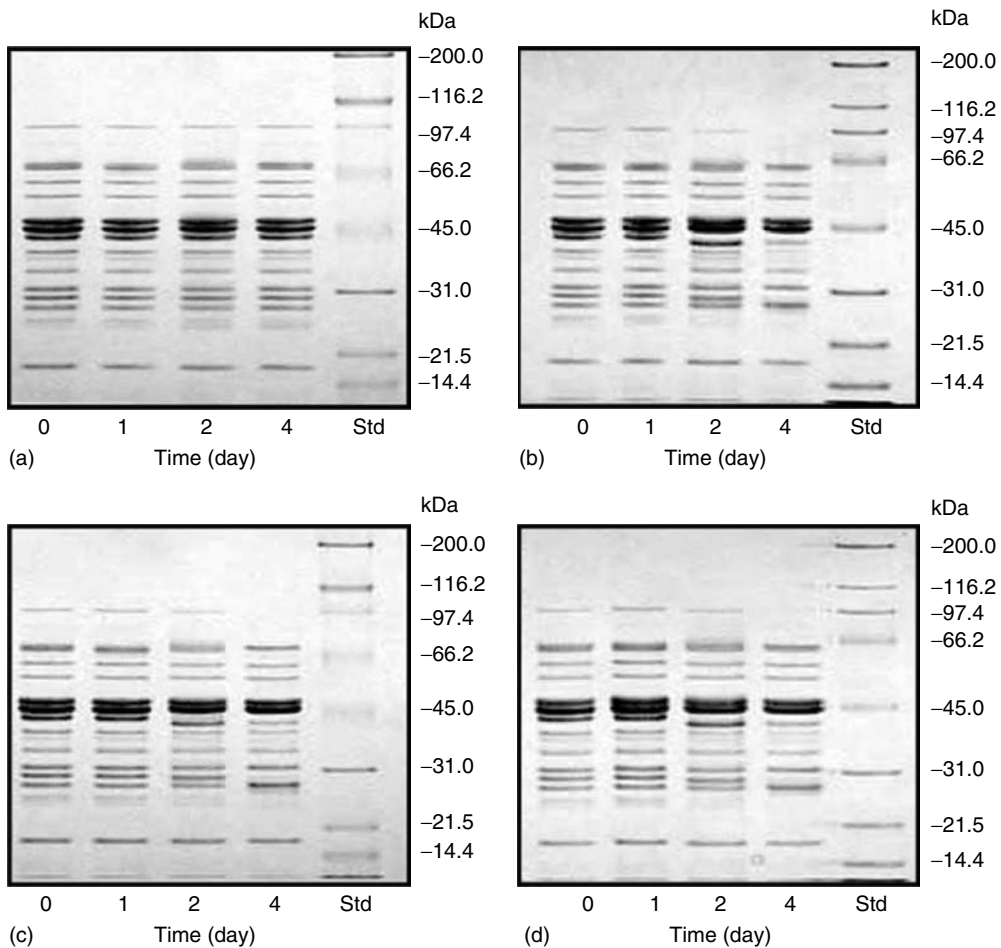


Figure 9.2 Twelve percent SDS-PAGE of sarcoplasmic proteins hydrolysis by *D. hansenii* CECT 12487 during incubation 0, 1, 2, and 4 days at 27°C. a, Control; b, whole cells; c, cell-free extract; and d, whole cells and cell-free extract; Std lane, standard proteins. (Reproduced from Santos, N.N., Santos-Mendonça, R.C., Sanz, Y., Bolumar, T., Aristoy, M.-C, Toldrá, F., *Int. J. Food Microbiol.*, 68: 199–206, 2001. With permission from Elsevier.)

9.3.2 Isolation and Extraction of Peptides

The most important techniques for the isolation and extraction of peptides in meat products are described in this section. The procedure for sample preparation is quite detailed in the literature.^{21–24} Muscle tissue must be excised from fat and other visible connective tissues. The muscle has to be finely ground, and a representative sample, at least 5–10 g, is taken for the analysis. The weighed tissue is homogenized with a sufficient amount (typically in the rate 1/2, 1/5, or 1/10 p/v) of either redistilled water, dilute saline solution, acidic solution (i.e., 0.1 N hydrochloric acid), or neutral phosphate buffer. Homogenization may be performed by vortex-mixing, or by using other instruments like Polytron, Ultra-Turrax or stomacher types. The homogenate is then centrifuged

(typically at 10,000 *g* for 20 min) at a low temperature to separate insoluble proteins, and the supernatant is filtered through glass wool or cheesecloth. The supernatant is collected and usually deproteinized by means of protein precipitation in 2.5–3-fold volume of acetonitrile, methanol, or ethanol, or by lowering the pH by the addition of perchloric acid or trichloroacetic acid.²⁵ The extraction and deproteinization can also be achieved in only one step by using a deproteinizing solvent for the extraction.²⁶

9.3.3 Fractionation of Peptides

Whereas dipeptides and tripeptides can be readily analyzed in the obtained extract (methods for the analysis of these compounds will be described in Section 9.3.4), larger peptides have needed further fractionation previous to the analysis.

Several methods of fractionation have been described based on differential properties such as size, charge, or polarity. In this section, methods based on size are described, whereas methods based on charge and polarity coincide with the analytical methods described in Sections 9.5.1 and 9.5.2.

9.3.3.1 Fractionation by Ultrafiltration

Ultrafiltration is a preparative technique based on size to isolate the peptide fraction of interest^{27–32} or to concentrate peptide extracts. There is a wide variety of cutoff sizes and materials used for membranes with good solvent resistance.

9.3.3.2 Fractionation by Gel Filtration Chromatography

In some cases, the extracted or ultrafiltered peptides need further fractionation that can be achieved on a gel filtration column. In this type of chromatography, where neither the mobile nor the stationary phases interact with the peptides, larger peptides elute first. The ranges for size fractionation depend on the type of gel. Sephadex G-25 gel (Pharmacia) is adequate for peptides within the range 500–5000 Da,^{19,32} whereas G-10 (Pharmacia) is more adequate for very small peptides (below 700 Da).³³ The elution is made with water, 0.01 N HCl, or diluted phosphate buffers at low flow rates and under refrigeration. The elution of the compounds of interest are typically monitored by ultraviolet (UV) absorption at 214 or 280 nm. Fractions can be collected for further analysis. The column is calibrated with standards of known molecular mass. Typical standards for the G-25 gel column are bovine serum albumin (68 kDa), egg albumin (45 kDa), chymotrypsinogen A (25 kDa), myoglobin (18 kDa), cytochrome C (12.5 kDa), aprotinin (6.5 kDa), ristocetin A sulfate (2.5 kDa), pepstatin (686 Da), and glycine (75 Da). Gel filtration can be also performed by high-performance liquid chromatography (HPLC) using either neutral/acid diluted phosphate or acetate buffers as eluents.^{34,35}

9.3.4 Analysis of Peptides

Small peptides like di- or tripeptides can be analyzed directly in the deproteinized extract (Section 9.3.2), whereas larger peptides may be analyzed after previous fractionation as described earlier (Section 9.3.3).

The evolution of the peptide generated during the processing of meat products can be followed by HPLC using either a reverse-phase column or a cation exchange column and detection at 214 nm. Potential presence of nucleotides or nucleosides can be detected through the spectral data from 200 to 350 nm. Capillary electrophoresis (CE), or polyacrylamide gel electrophoresis at high acrylamide percentages (above 15%) can also be used for the analysis.²⁵ Specific conditions for peptide separation have been recently reported.²⁴

9.3.4.1 *Reverse-Phase High-Performance Liquid Chromatography*

Owing to its high resolutive powder, this is the most common HPLC methodology to analyze peptidic extracts. Indeed, reverse-phase HPLC (RP-HPLC) is widely utilized to generate a peptide map from digested proteins or peptidic extracts. Peptides are separated as a function of their polarity, which is directly related to the amino acid composition. There are many types of reverse-phase columns available, with those based on silica support with octadecylsilane (C-18) covalently bonded most often used. The eluent can be monitored at different wavelengths (214, 254, and 280 nm), and spectra can be obtained using a diode array detector. Hydrophilic peptides elute first, whereas hydrophobic peptides are retained in the column and take longer to elute. An example of peptide chromatograms following the proteolysis of a microbial starter (*Lactobacillus sake* CECT 4808) incubated in myofibrillar protein extracts is shown in Figure 9.3.³⁶

9.3.4.2 *Ion-Exchange Chromatography*

This type of chromatography, which can be complementary to RP-HPLC, also offers good separation of peptides.^{16,37} The choice of the column depends on the charge of peptides. Thus, anion exchange columns can be used for the separation of acid peptides,³⁸ whereas neutral or basic peptides are separated better in cation exchange columns. The best results are obtained by using a nonvolatile salt like NaCl to achieve the elution of the retained peptides. The eluent can be monitored at different wavelengths (214, 254, and 280 nm).

9.3.4.3 *Capillary Electrophoresis*

CE has been applied for peptide mapping of protein hydrolyzates.³⁹ The main problem in real meat extracts is due to interference (mainly by amino acids and nucleotides),^{19,34} but the method works very well for kinetic studies in pure protein or peptide solutions incubated with peptidases.^{40,41}

9.4 **Rapid Spectrophotometric Methods**

There are several rapid methods for the determination of proteins and polypeptide content in a solution, as recently reported.¹¹ These methods include Biuret, Lowry,⁴² bicinchoninic acid,⁴³ and Bradford.⁴⁴ UV absorbance is a nondestructive method, but is restricted to those peptides containing amino acids absorbing at 280 nm. The main problem of the aforementioned colorimetric methods is the exact determination of peptide content, because most of them use a particular protein as standard for the calibration curve. In addition, some of these methods are subject to interference by a wide variety of chemicals.

Another relatively rapid method to analyze the degree of protein hydrolysis is based on the nitrogen analysis. This method gives the degree of hydrolysis based on the percentage of nonprotein

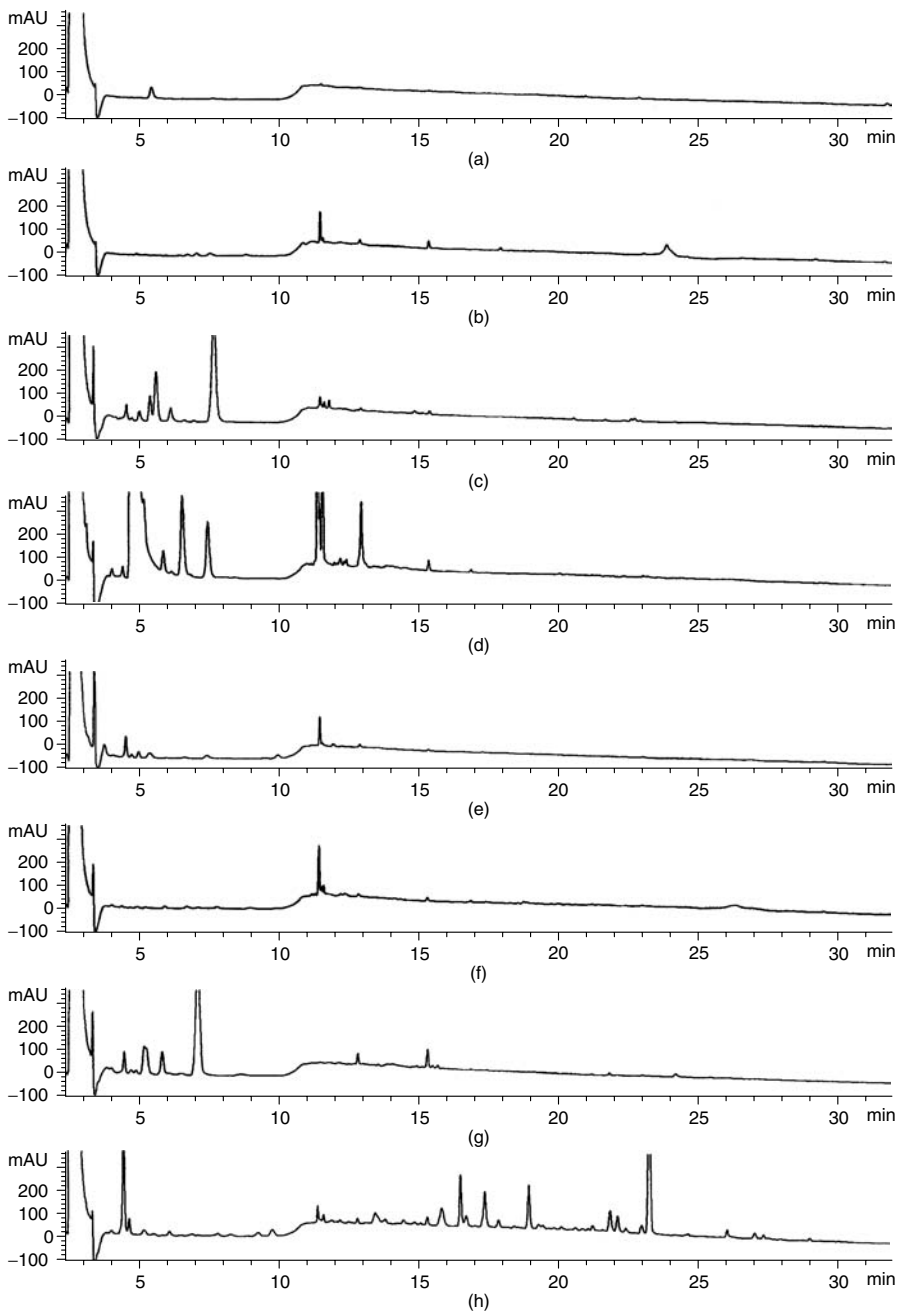


Figure 9.3 Example of proteolysis control for a microbial starter. RP-HPLC patterns of soluble peptides contained in myofibrillar protein extracts treated with *L. sake* CECT 4808 at 0 h (a, c, e, and g) and 96 h (b, d, f, and h) of incubation at 37°C. Control samples (a and b), samples containing whole cells (c and d), samples containing CFE (e and f) and samples containing whole cells plus CFE (g and h). (Reproduced from Sanz, Y., Fadda, S., Vignolo, G., Aristoy, M-C., Oliver, G., Toldrá, F., *Int. J. Food Microbiol.*, 53: 115–125, 1999. With permission from Elsevier.)

nitrogen (NPN) in relation to total nitrogen (TN). NPN gives only an approximate idea of protein hydrolysis, because it also includes ammonia nitrogen, free amino acids, small peptides, nucleotides, and nucleosides. However, it can be used to approximate the degree of proteolysis.⁸ TN can be analyzed in the crude protein or the protein extract by the method of Kjeldahl. The analysis of NPN requires a previous deproteinization of the meat extract. This can be achieved by homogenizing the extract with an acid solution (i.e., 2% trichloroacetic acid [TCA]). The homogenate is centrifuged (10,000 g for 20 min at 4°C), and the nitrogen content of the supernatant is analyzed using the Kjeldahl method.

9.5 Analysis of Amino Acids

9.5.1 Sample Preparation

No special care is required in the extraction of free amino acids and sample deproteinization. Procedures for both extraction and deproteinization are fully described in Aristoy and Toldrá.^{45,46} Further description for the amino acids analysis is given in Chapter 15 of this book.

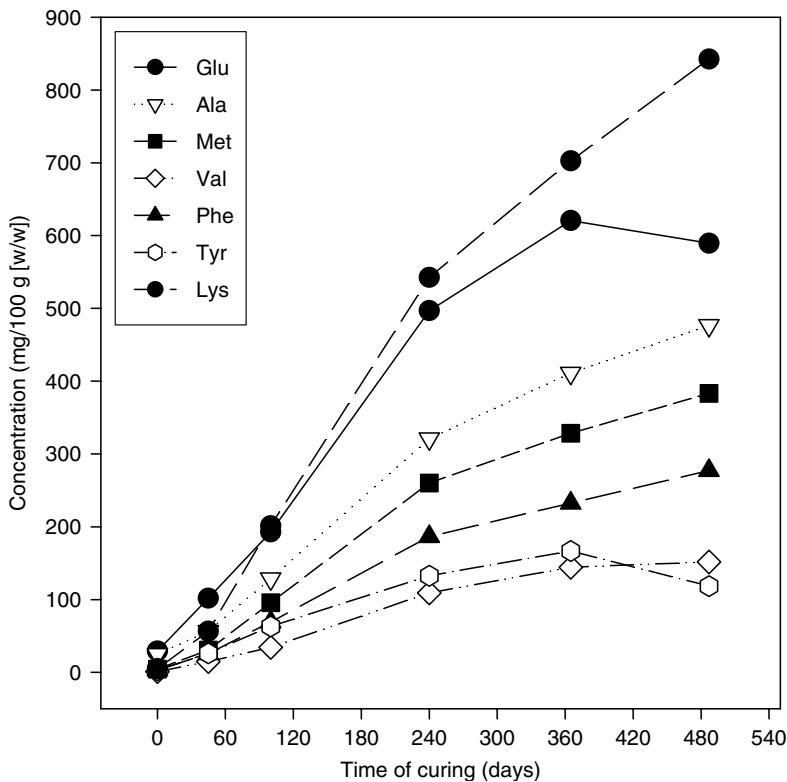


Figure 9.4 Example of typical evolution of certain free amino acids along the processing of dry-cured ham. (Adapted from Toldrá et al., *Food Research Int.*, 33: 181–185, 2000.)

9.5.2 Instrumental Analysis

The generation of free amino acids can be monitored by HPLC analysis with either a reverse-phase column or a cation-exchange column. The use of an internal standard, like α -aminobutyric acid, norvaline, or norleucine, is recommended. Samples must be deproteinized (e.g., acetonitrile or ethanol give good results) and then analyzed. Previous to further injection into the HPLC chromatograph, amino acids must be derivatized. There are several methods for derivatization, phenylthiocarbonyl derivatives^{21,47} being among those preferred for meat amino acid analysis. The derivatized amino acids are separated through the reverse-phase column, followed by UV detection at 254 nm.

In the case of using a cation-exchange column, nonderivatized amino acids are separated and then, a postcolumn derivatization is performed with ninhydrin or *o*-phthalaldehyde (OPA) before the UV or fluorescence detection.^{3,45,46} An example of the generation of certain amino acids during the processing of dry-cured ham is shown in Figure 9.4.⁴⁸

9.5.3 Rapid Spectrophotometric Methods

Most of the rapid methods for free amino acid determination generally include the precipitation of proteins, reagent addition, and colorimetric, UV absorption or fluorescent determination of the amine nitrogen in the supernatant. These methods are based on the reaction of the α -amino group with reagents such as OPA,⁴⁹ cadmium-ninhydrin,^{50,51} or trinitro-benzene-sulfonic acid (TNBS),^{52,53} which are the most often used. The reaction products are chromophores that enhance the ultraviolet response of amino acids at a higher wavelength, render them visible, or give them fluorescent characteristics. The reaction of ninhydrin with free amino groups forms a purple chromophore that is detected at 570 nm, whereas a yellow chromophore absorbing at 420 nm is formed with TNBS, and a fluorescent compound is formed with OPA.⁵⁴ The absorbance/emission intensity and the concentration of α -amino groups generally gives a linear relationship.

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Chapter 10

Determination of Lipolysis

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10.1 Introduction

Lipolysis is one of the main causes of lipid degradation in fat-containing foods. Although lipolytic activity may be regarded as a spoilage characteristic in some foods, namely milk, this phenomenon may be envisaged advantageously in traditional meat products. Many sensory traits of fermented and dry-cured meat products depend on lipid composition of muscles and adipose tissue, and on its degradation through a complex set of lipolytic and oxidative reactions during processing. For the aroma development of those products, lipolysis is the first stage of the process through the generation of free fatty acids (FFA). These have only a small impact on taste and flavor, but are important as precursors of volatile molecules, which are produced by the oxidation of FFA and play a relevant role for the aroma of fermented sausage and dry-cured ham.¹⁻⁴ However, the

relationship between lipolysis and flavor remains unclear, because the exact mechanism by which lipolysis could promote oxidation is still unknown.⁵

10.2 Agents of Lipolysis in Meat and Meat Products

Lipolysis can be defined as the enzymatic hydrolysis of lipids. The reaction is governed by a set of specific enzymes, namely lipases, esterases, and phospholipases, which are able to cleave the ester linkage between fatty acids and the glycerol core of the glycerides and phospholipids, leading to the formation of FFA. Lipases and esterases are responsible for the breakdown of glycerides (neutral lipids); esterases hydrolyze acyl ester chains from 2 to 8 carbon atoms in length, whereas lipases hydrolyze acyl ester chains of 10 or more carbon atoms. Phospholipases act on phospholipids (polar lipids); phospholipases A₁ and A₂ hydrolyze fatty acids in positions *sn*-1 and *sn*-2 of the glycerol core of phospholipids, respectively. The lipolysis of phospholipids is ended by lysophospholipases, which hydrolyze the remaining fatty acid after action by phospholipases A₁ and A₂.

The activity of lipases, esterases, and phospholipases has been widely assayed in muscles and adipose tissue according to current methodologies based on spectrofluorophotometry,⁶ and well summarized by different authors.^{1,3,5} In both adipose tissue and muscles, lipases have been described as neutral and basic lipases; moreover, muscle presents an acid lipase. These enzymes remain active during the entire process of dry-cured ham. In adipose tissue, neutral lipase remains active over 12 months whereas in muscles, neutral and basic lipases are very active during the first 3–4 months of the process; then their activities decrease slowly. In contrast, acid lipase has low activity during the entire process. All these lipases exhibit an activity equal to 10–20% of their maximal activity up to 15 months of processing. Acid and basic esterase activities have been described in ham adipose tissue and muscles. However, their role in the process seems not to be relevant, being limited by salt and low temperature, and in view of the absence of adequate substrates. Very little is known of the postmortem activity of phospholipases in muscles and of their evolution during dry-cured meat processing. Nevertheless, it has been postulated that these enzymes remain active, because the proportion of long-chain polyunsaturated FFA increases for at least 6 months in dry-cured ham, giving evidence of phospholipid lipolysis.

Numerous studies have been devoted to the lipolytic enzymes of microbial origin, and the contribution of microorganisms to lipolysis in fermented sausages has been reviewed.¹ Micrococaceae are considered the most important bacteria in the lipolysis of fermented meat, because they are able to hydrolyze triglycerides by extracellular lipases, which can act for a long period of time during ripening. The other main group of bacteria, the lactic acid bacteria, have a lipolytic activity lower than Micrococaceae, and mainly act on the mono- and diglycerides. Many molds and yeasts isolated from fermented and dry-cured meats are lipolytic.^{7–9} However, the production and the activity of lipases is strain specific, depending on substrate, pH, sodium chloride content, and temperature of incubation. Therefore, the microorganisms chosen as starter cultures should be previously checked in experimental conditions, because the enzymatic activity showed *in vitro* can be inhibited under the conditions in which dry-fermented sausages are ripened.

10.3 Lipolysis in Raw Meat

Lipolysis is one of the most important degradation phenomena during refrigeration of meat.¹⁰ In refrigerated meat, FFA generated by lipolysis account for 1–3% of the whole fatty acid content,

depending on the refrigeration period.¹¹ In refrigerated cooked meat, lipid oxidation seems to be more relevant than lipolysis. The cooking process leads to thermal denaturation of lipolytic enzymes, and this inactivation is believed to contribute to the reduction of the release of FFA in cooked meat.¹²

In raw meat, lipolysis has been studied in relation to the metabolic type of muscle fibers during refrigerated storage; in general, oxidative muscles are more prone to lipolytic processes than glycolytic muscles.^{13–15} Lipolysis of *longissimus dorsi* muscle from pig is not inhibited by freezing temperature; during a 6-month period, the highest release of FFA is observed during the first month of frozen storage.¹⁶ The effect of breed on lipolysis has not been well investigated. FFA of *longissimus lumborum* from Large White and Pietrain pigs are little influenced by breed during chilled storage,¹¹ as are those of *biceps femoris* muscle of three lines of Iberian pig and industrial genotype pigs.¹⁷ The effect of culinary practices on the lipolysis of pork loin chops fried in different fats (olive oil, refined sunflower oil, butter, and pig lard) has been studied by Ramirez et al.¹⁸ FFA profiles of loin chops were found to significantly change with frying and type of culinary fat, and reflected fatty acid composition of the fat in which frying occurred. Few studies have addressed the contribution of irradiation and high-pressure preservation technologies to lipolysis. A radiation dose-dependent increase of FFA is observed in lamb meat treated up to 5 kGy,¹⁹ and FFA content of carp fillets increases as the pressure level, from 100 to 200 MPa, and pressurization time, from 15 to 20 min, are increased.²⁰

10.4 Lipolysis in Fermented and Dry-Cured Meat

The hydrolytic processes of adipose tissue and muscle lipids have been widely investigated and reviewed in fermented and dry-cured meat products.^{1,3,5} During fermented sausage and dry-cured ham processing, lipids are progressively altered through both lipolysis and oxidation. Large differences are observed in the raw material used and in the time–temperature–relative humidity cycles, mainly during drying and ripening, according to the process used for each product in each country. These large variations in processing conditions affect the kinetics of reaction of lipolysis, and oxidation to a large extent. In general, long processes with mild drying/ripening conditions allow a relatively higher lipolytic activity and thus a higher generation of FFA.

FFA amount increases during ripening of fermented sausages. FFA content rises from 0.7–1.5% of fresh minces to 2.2–4.5% of Mediterranean and North European matured sausages.²¹ In dry-fermented sausages, lipolysis is almost exclusively brought about by adipose tissue and muscle enzymes, and the contribution of bacteria is weak because the medium conditions are far from the optimal conditions of bacteria lipases.^{22–24} Lipolysis from meat endogenous enzymes accounts for more than 60% of FFA release.²⁵ Sodium chloride reduction and partial replacement with potassium chloride show an inhibitory effect on lipolysis by decreasing FFA level.^{26,27} Inconsistent results are reported for the effect of the addition of nitrite and nitrate on lipolysis of dry-fermented sausages.^{21,26,28}

Lipolysis is directly related to flavor development and, therefore, the incorporation of exogenous lipolytic enzymes in fermented sausages has been widely considered a way to enhance flavor and reduce ripening time. The addition of pancreatic lipase and microbial lipase from *Candida cylindracea*, *Aspergillus*, and *Rhizomucor miehei* causes an acceleration of lipolytic phenomena by a significant increment of FFA fraction without inducing rancidity.^{29–32} However, the contribution to the sensory profile and the acceleration of processing are not as remarkable as expected. The most promising results of the different strategies to accelerate the ripening process and

improve the sensory quality of fermented sausages have been obtained by the incorporation of cell-free extracts from lactic acid bacteria and molds.³³

FFA amount increases during processing in both dry-cured hams and loins. Low in fresh thigh, FFA levels sharply rise from 1–2% to 10–12% of the total lipids in adipose tissue in 10-month dry-cured ham processing. In muscle, the rate of lipolysis is rapid during the first 6 months, and then slows toward the end of the process (12–24 months). At the end of the process, FFA account for 8–20% of total lipids in muscle, varying according to the technology used and the raw material. Similar results, but to a lesser extent, are reported for Spanish cured loin, in which FFA increase from 0.6% of total lipids in fresh loin up to 5.7% and 3.0% in dry-cured and pickled-cured loin, respectively.³⁴ In smoked and dried reindeer meat, FFA are also found to increase, whereas triglycerides and phospholipids decrease; the high increase of FFA shows that lipolysis has a significant role in processed reindeer meat.³⁵

In dry-cured ham, the lipolysis of most dehydrated external muscles is more pronounced than that of internal muscles.^{36,37} Nevertheless, mild dehydration and sodium chloride diffusion are associated with extensive production of FFA in Parma ham muscles; the stronger dehydration yield in 10-month-old *semimembranosus* muscle parallels a negligible rise of FFA, compared to their increase in the less dehydrated *biceps femoris* muscle.³⁸ Sodium chloride seems to have a slight promoting effect on lipolysis, at least at concentrations below 6%.³⁹ Similar levels of lipolysis are observed at the end of the process when using frozen/thawed thighs as raw material for Serrano dry-cured ham manufacture.⁴⁰

In dry-cured meat, the lipolysis of muscles concerns chiefly the phospholipids; in French dry-cured ham, two-thirds of phospholipids are degraded, whereas the triglycerides are little affected,⁴¹ as well as in Iberian dry-cured ham⁴² and dry-cured pork forelegs.⁴³ Moreover, the FFA composition is closer to the fatty acid composition of phospholipids than that of triglycerides, whatever the type of ham.

10.5 Measurement of Lipolysis

The extent of lipolysis in meat may be measured and monitored by the determination of the content and composition of the FFA generated through the enzymatic hydrolysis of both neutral and polar lipids.

The various methods used to quantify FFA may be grouped into two main classes. The first includes direct analytical methodologies, which enable the determination of the total level of FFA, most of them being simple and rapid. The second one involves multistep analytical procedures, which allow the ascertainment of FFA composition, namely identification and quantification of individual FFA, and sometimes the concomitant determination of the content and composition of neutral and polar lipid classes. Despite their high selectivity and sensitivity, these methods require particular laboratory equipments, and a pretreatment of the meat sample is necessary.

Before the total level and the composition of FFA can be determined, the lipids need to be isolated from the meat sample. Even today, the overwhelming majority of papers cite the procedure of Folch et al.,⁴⁴ or that of Bligh and Dyer⁴⁵ for extracting the lipids from the meat. These employ a mixture of chloroform/methanol (2/1, v/v) to remove the total lipid fraction, with the water derived from the sample generating a ternary solvent mixture. Briefly, the meat tissue is homogenized with the chloroform/methanol mixture at a ratio 1/20 or 1/17 (w/v), depending on the expected amount of fat of the sample. The extracted lipids are then washed by sodium or potassium chloride 0.73% aqueous solution to remove nonlipid residues. Less hazardous solvents

or techniques, such as dry column chromatography^{46,47} and supercritical fluid extraction,⁴⁸ have been proposed as alternative to the traditional chloroform/methanol extraction methods, but have not received widespread acceptance in the field of meat analysis.

10.5.1 Total Level of Free Fatty Acids

The titration with alkali is one of the oldest methods enabling the determination of the total content of FFA. There are a number of variations in the titration procedure; however, the method proposed by Pearson⁴⁹ and three official methods^{50–52} have found the largest application in meat sector. According to these procedures, 1–10 g of lipids, previously isolated from the meat sample, are dissolved in 50 mL of a neutralized mixture of diethyl ether/ethanol (1/1, v/v)⁴⁹ or ethanol,^{50–52} and 1 mL of phenolphthalein 1% aqueous solution is added. The mixture is titrated with 0.1 M aqueous sodium hydroxide or potassium hydroxide, and shaken constantly until a permanent faint pink color appears and persists for 15 s. The titration should preferably not exceed about 10 mL, or otherwise two phases are liable to separate. This does not occur if hot neutralized ethanol is used as solvent.^{50,51} The titration with alkali proposed by Kempton and Bobier⁵³ is carried out directly on the meat sample. Briefly, 10 g of meat are blended with 100 mL distilled water for 3 min, after which the emulsion is filtered through absorbent cotton. A 25-mL aliquot of the filtrate is added to 50 mL of distilled water and titrated to the phenolphthalein end point with 0.2 M aqueous sodium hydroxide.

The result is referred in terms of acidity value, defined as the number of milligrams of alkali required to neutralize the free acidity of 100 g of fat, and is expressed as grams of oleic acid/100 g lipid or milligrams of alkali/100 g lipid. Typical acidity values for some processed meats are shown in Tables 10.1 and 10.2.

A global estimation of the FFA level may be obtained by the copper soaps method, a classical colorimetric/spectrophotometric assay based on the formation of colored copper salts of FFA. Although several variants are currently known, the analytical procedures proposed by Lowry and Tinsley⁷² and Leuschner et al.⁷³ have found widest application for meat samples. In the first case, 20–30 mg of lipids extracted from meat sample are dissolved in 5 mL of isooctane, combined with 1 mL of cupric acetate–pyridine reagent, and mixed vigorously for 90 s using a vortex mixer. The upper phase is collected, and absorbance is read at 715 nm by spectrophotometer. In the second case, 10 g of ground meat is homogenized with 90 mL of distilled water for 3 min at 24,000 × g; FFA are extracted by a solvent mixture composed of chloroform/*n*-heptane/methanol (49/49/2, v/v/v). Copper salts are formed by the addition of a copper reagent, consisting of 10 mL of 1 M copper nitrate, 5 mL of triethanolamine, and 85 mL of sodium chloride saturated aqueous solution. Color development is achieved by the formation of a complex between copper salts with sodium diethyl dithiocarbamate 0.1% in *n*-butanol. The optical density is measured spectrophotometrically at 440 nm. In both cases, FFA level is calculated from a standard curve prepared using palmitic acid and expressed as milligrams of FFA per gram of lipid. The copper soaps method was used as an analytical tool to evaluate the role of bacterial and meat endogenous enzymes in lipolysis of Belgian sausages; these show between 25.9 and 39.0 mg FFA per gram of lipid after 21 days of ripening, varying from batch to batch and depending on the bacterial starter culture added.²² The same method was employed to follow the lipolytic process of sausages during the fermentation and ripening steps; Visessanguan et al.⁷⁴ observe an increase from 6.8–8.5 to 51.9–57.9 mg FFA/g lipid during 84 h of fermentation of Nham, a popular fermented pork sausage from Thailand. Kenneally et al.²³ report that the total level of FFA of fermented sausage changes from 7.7–14.7 mg

Table 10.1 Acidity Value (oleic acid/100 g lipid) of Some Processed Meats

<i>Meat Product (Origin)</i>	<i>Ripening Time (Days)</i>	<i>Acidity Value</i>		<i>Reference</i>
Parma ham (Italy)	300	9.3 ^a	S ^b	38
		12.9	<i>B.f.</i> ^c	
Iberian ham (Spain)	Green state	1.8–3.4 ^d	S	36,42,54
	588	1.6–4.8	<i>B.f.</i>	
		10.8	S	
	700	6.7	<i>B.f.</i>	
		26.5–31.9	S	
		13.3–22.1	<i>B.f.</i>	
Farmer's lard (Italy)	40	0.9		55
Chorizo (Spain)	After stuffing	0.2–1.2		56
	63	1.7–5.4		
Galician chorizo (Spain)	After stuffing	2.4		57
	30	4.2		
Chorizo de Pamplona (Spain)	20	4.0		58
Chorizo de cebolla (Spain)	—	2.7–5.9		59
Chorizo rosario (Spain)	—	1.4–4.3		
Androlla (Spain)	—	1.2–4.4		
Botillo (Spain)	—	1.7–3.7		
Lacón (Spain)	—	1.5–5.3		
Lacón (Spain)	—	2.0–2.8		60
Chistorra (Spain)	After stuffing	0.9		61
	4	2.2		
Sausage (Mediterranean type)	After stuffing	0.8–1.0		27
	24	2.5–4.5		
Sausage (Mediterranean type)	14	3.3–3.6		30–32,62
Sausage (North Europe type)	After stuffing	0.6		63
	21	4.0		
Chicken curry ^e (India)	—	0.25–0.29		64

^a Mean value.

^b Muscle *semimembranosus*.

^c Muscle *biceps femoris*.

^d Minimum–maximum value.

^e Cooked product.

FFA/g lipid for fresh mince to 41.6–87.5 mg FFA/g lipid for dry-fermented sausage ripened for 49 days, depending on the inoculated microbial starter culture.

The isolation of FFA from other lipid classes by thin layer chromatography (TLC) and their quantification by imaging densitometry is an alternative method to estimate the global level of FFA. Briefly, an aliquot of the lipid extract is dissolved in chloroform (200 mg/mL) and applied to a 0.25-mm silica gel G-60 plates (20 × 20 cm; Merck, Darmstadt, Germany). Plates are developed with petroleum ether/diethyl ether/acetic acid (80/20/1, v/v/v); a spray of ferric chloride trihydrate 0.05% solution in a mixture of water/acetic acid/sulfuric acid (90/5/5, v/v/v), followed by heating in an oven at 120°C for 20 min, is used to visualize all lipid fractions.⁷⁵ As an alternative, a mixture of hexane/diethyl ether/acetic acid (85/15/1, v/v/v) or hexane/diethyl ether/formic acid (80/20/4, v/v/v) may be used as the mobile phase to develop the plates;^{35,76} for densitometric analysis, the plates may be sprayed with a solution of 3% cupric acetate in 8% phosphoric acid or

Table 10.2 Acidity Value (mg KOH/g lipid) of Some Processed Meats

<i>Meat Product (Origin)</i>	<i>Ripening Time (Days)</i>	<i>Acidity Value</i>	<i>Reference</i>
Chorizo de cebolla (Spain)	After stuffing	0.1–0.2 ^a	65,66
	42	0.5–6.8	
Androlla (Spain)	After stuffing	1.5 ^b	67
	42	5.4	
Salchichón (Spain)	After stuffing	4.2	68
	31	17.0	
Kavurma ^c (Turkey)	—	0.8	69
Tsire ^c (West Africa)	—	0.5–0.7	70
Beef/sheep mortadella ^c (Jordan)	—	0.8	71

^a Minimum–maximum value.

^b Mean value.

^c Cooked product.

Table 10.3 Changes in Lipid Fractions (g/100 g dry matter) during Ripening of Dry Fermented Sausages

<i>Lipid Fraction</i>	<i>Day 5</i>		<i>Day 22</i>	
	<i>Batch C^a</i>	<i>Batch P^b</i>	<i>Batch C</i>	<i>Batch P</i>
FFA	0.59 ^{c,x}	0.56 ^x	1.42 ^x	2.53 ^y
TG	61.00 ^x	60.39 ^x	59.36 ^y	57.03 ^x
DG	0.39 ^x	0.42 ^x	1.03 ^x	1.37 ^y
MG + PL	3.10 ^x	3.52 ^x	3.86 ^x	5.70 ^y

^a Control batch.

^b Batch superficially inoculated with spore suspension of *P. camemberti*.

^c Mean value.

Note: FFA, free fatty acids; TG, triglycerides; DG, diglycerides; MG + PL, monoglycerides + phospholipids; x,y, values in a row of equal ripening time with different letters are significantly different ($P < 0.05$, Student–Newman–Keul's test).

Source: Adapted from Bruna et al., *Int. J. Food Microbiol.*, 85, 111, 2003.

a mixture of anisaldehyde/ethanol/sulfuric acid/acetic acid (0.5/9/0.5/0.1, v/v/v/v).^{35,76} FFA and other lipid classes are identified by comparing the retention factor values with those from standard molecules. Oleic acid, triolein, diolein, monolein, and phosphatidylcholine are commonly used as reference standards for FFA, triglycerides, diglycerides, monoglycerides, and phospholipids, respectively. The area of each lipid spot is integrated by scanner-densitometry at 390 nm, using the calibration curves for the standard employed. Lipid classes are expressed as percentage of total lipids or as g/100 g dry matter. The TLC fractionation of lipids coupled with densitometric analysis of the different lipid classes was adopted to study the lipolysis during ripening of dry-fermented sausages superficially inoculated with *Penicillium camemberti*, in an attempt to improve their sensory properties.⁷⁷ As shown in Table 10.3, the growth of that mold on the surface of the sausages results in an intense lipolysis that causes a more significant increase in the concentration of FFA than that observed in the noninoculated sausages.⁷⁷

Table 10.4 Changes in Lipid Fractions of Adipose Tissue (g/100 g adipose tissue) of Dry-Cured Ham during Processing

Lipid Fraction	Processing Time (Months)				
	0	6	12	18	24
FFA	0.1 ± 0.40 ^a	8.1 ± 1.19	7.8 ± 1.44	10.1 ± 1.04	10.5 ± 0.23
TG	89.6 ± 0.56	78.0 ± 2.02	79.1 ± 2.00	75.8 ± 1.39	75.8 ± 0.43
DG	0.0 ± 0.34	4.5 ± 0.78	4.6 ± 0.51	4.3 ± 0.27	4.5 ± 0.37
MG	0.0 ± 0.08	1.2 ± 0.19	0.7 ± 0.16	1.0 ± 0.21	1.3 ± 0.21

^a Mean value ± standard error.

Note: FFA, free fatty acids; TG, triglycerides; DG, diglycerides, MG, monoglycerides.

Source: Adapted from Coutron-Gambotti, C. and Gandemer, G., *Food Chem.*, 64, 95, 1999.

An interesting gas chromatographic method for the determination of the total level of FFA was originally proposed by Myher and Kuskin⁷⁸ for plasma, and subsequently modified by Coutron-Gambotti and Gandemer⁷⁹ for subcutaneous adipose tissue of dry-cured ham. An aliquot of 0.5–1 mg of lipid extract is silylated using 250 µL of a mixture of bis-trimethyl-silyl-trifluoro-acetamide/trimethylchlorosilane (80/20, v/v). The reaction is achieved in 30 min at room temperature. The mixture is evaporated under nitrogen, and the derivatives are dissolved in 2 mL of hexane. The silylated components (FFA, triglycerides, diglycerides, and monoglycerides) are analyzed by a gas chromatograph equipped with a flame ionization detector (FID) kept at 350°C and a capillary column (7 m length, 0.32 mm internal diameter) coated with 5% phenylmethyl–95% polysiloxane in stationary phase (0.1 µm film thickness). The oven is heated according to a temperature gradient program from 120 to 335°C. The silylated compounds are eluted according to their molecular weight in less than 25 min. Tricaprin is used as internal standard, and the results are expressed as mg/100 g sample. This method has been used to follow the changes in lipids of adipose tissue during dry-cured ham processing by measuring the total amounts of FFA, triglycerides, diglycerides, and monoglycerides at 0, 6, 12, 18, and 24 months, as shown in Table 10.4.⁷⁹

High-performance liquid chromatography (HPLC) may not be ignored as a method for lipid class analysis,^{80,81} despite only two research groups have used it to study lipolysis. HPLC together with light-scattering detection was the technique adopted by Fernandez et al.⁸² to determine the composition of lipids in terms of total levels of FFA, neutral and polar lipids, in glycolytic and oxidative muscles of Large White pigs fed or deprived of food for 24 h. The same approach was used by Leseigneur-Meynier and Gandemer⁸³ to study the lipid composition of pork muscles in relation to the metabolic type of the fibers.

Different enzymatic spectrophotometric assays are commercially available for the determination of nonesterified fatty acids (NEFA) in blood plasma. One recent paper dealt with the use of a NEFA detection kit for the measurement of FFA in cured pork loins, although its use seems not to be specifically validated for meat samples.⁸⁴

10.5.2 Free Fatty Acid Composition

The determination of FFA composition in meat and meat products involves usually three main steps: isolation of lipids, fractionation of various lipid classes, and identification and quantification of individual FFA.

The analytical methods in use for the isolation of lipids from the meat samples have been previously discussed.

10.5.2.1 Fractionation of Lipid Classes

A variety of chromatographic methods have been reported for the fractionation of lipids into the various classes. The most widespread involve normal-phase systems wherein the solutes are retained on the basis of their relative polarity. This usually takes the form of silica gel supported in a TLC plate,^{56,63} or of an amino-propylsilica solid-phase extraction (SPE) cartridge.^{85,86} In the first case, the lipid extract is dissolved in chloroform and applied in the drop form on a TLC plate covered with silica gel; the plate is developed in a mixture of petroleum ether/diethyl ether/acetic acid (80/20/10, v/v/v) or acetone in chloroform (4%, v/v), and the spots corresponding to the different lipid classes are visualized by ultraviolet light or iodine vapor. FFA are extracted by chloroform from the silica gel scraped from the plate. In the second case, 10 mg of fat, dissolved in chloroform, are applied to an amino-propylsilica SPE cartridge (100 mg), previously washed with 2 mL portion of heptane. A first fractionation with 2 mL of chloroform/2-propanol (2/1, v/v) is applied to obtain neutral lipids, and a second one with 3 mL of acetic acid in 2% diethyl ether to obtain FFA fraction.

An alternative procedure for the fractionation of lipid classes, based on ion exchange resin absorption, was originally proposed by Needs et al.⁸⁷ for the determination of individual FFA in milk; this method, modified by Gandemer et al.,⁸⁸ is widely adopted for meat samples application. Briefly, an aliquot of 50–100 mg of lipids is dissolved in 15 mL of a mixture of acetone/methanol (2/1, v/v). After addition of 100–200 mg of an anionic exchange resin (Amberlyst™ A26) and heptadecanoic acid as internal standard, the mixture is shaken for 30 min. FFA are retained by the resin, whereas non-resin-bound lipids are removed by washing the resin with acetone/methanol (2/1, v/v). Resin is then transferred into a dry tube for methylation.

The conversion of FFA to their corresponding sodium salts has been used by a few authors to isolate them from other lipid classes. According to Garcia et al.⁸⁹ and Hierro et al.,²⁵ 1 g of the lipid extract is dissolved in 2.5 mL of a ethanol/diethyl ether (1/1, v/v) mixture, and the sodium salts are formed with aqueous sodium hydroxide 5 N. These salts are extracted from the mixture by washing twice with chloroform/water (1/1, v/v) mixture and centrifuging at $1,500 \times g$ for 10 min. The aqueous phase is saturated with sodium chloride and acidified with hydrochloric acid to pH 2. The FFA are then extracted by washing with diethyl ether and dried in a rotary evaporator.

10.5.2.2 Identification and Quantification of Individual Free Fatty Acids

After their isolation from other lipid classes, individual FFA may be identified and quantified by gas chromatography (GC) or HPLC. Capillary GC coupled with FID is currently the most frequently used approach to obtain the FFA profile; HPLC is applied less frequently than GC, despite a very large number of derivatives for ultraviolet and fluorescence detection having been proposed.⁹⁰

The gas chromatographic analysis may be performed directly on FFA without derivatization or, most frequently, after their derivatization to fatty acid methyl esters (FAME). A variety of methods for methylation of FFA to FAME are in use, including the time-tested acid- and base-catalyzed reactions. The most extensively used acid catalysts are boron fluoride, hydrochloric acid,

and sulfuric acid, usually as 14, 5, and 2% in methanol solutions, respectively. Boron fluoride allows the conversion of FFA to the corresponding FAME in 2 min at 100°C; about twice as long is required with hydrochloric acid and sulfuric acid. The analytical procedures of Morrison and Smith⁹¹ and Association of Official Analytical Chemists⁹² are the most commonly used to prepare methyl esters with boron fluoride. Hydrochloric acid solution is generally adopted to methylate the FFA fractionated by ion exchange resin absorption.⁸⁷ Strong organic bases, such as quaternary ammonium hydroxides, are used to convert FFA to their salts, similar to their inorganic analogs. Unlike the latter, however, the fatty acid salts of the organic bases decompose at the high temperature of the gas chromatographic injector to form FAME.⁹⁰ The specific quaternary bases that have been used for such pyrolytic transesterification include tetramethylammonium hydroxide 20% in methanol.²³ Diazomethane may be used for the rapid methylation of FFA,⁹³ and has been employed by some authors.^{25,94} Although special reagents, procedures, and apparatus permit relatively safe operations, the use of this method is not advisable, due to the toxic and explosive nature of this compound.

The GC analysis of FAME is carried out by wall-coated open tubular (WCOT) fused-silica capillary columns, which achieve high resolution and adequately separate the majority of mixtures encountered in food samples. The WCOT capillary columns typically used for the analysis of FFA are 25–30 m long, with an internal diameter of 0.25, 0.32, or 0.53 mm. Capillary columns of 100 m may be essential for the most challenging separations, when the highest resolution is required, such as the separation of the positional and geometrical isomers of unsaturated fatty acids. The stationary phases used for the analysis of FAME range from nonpolar methylpolysiloxanes, in which the methyl groups may be partly replaced by the somewhat more polar phenyl group, to the polar polyethylene glycols or to the very polar cyanopropylpolysiloxanes. On stationary phases composed entirely or preponderantly of methylpolysiloxanes, the unsaturated FAME are generally eluted before their saturated analogs and in relation to the number of double bonds. Polar phases afford resolution by carbon number, and the unsaturated fatty acids are eluted after the saturated. Very polar stationary phases, on the other hand, retain the double bonds more strongly, and these components are eluted progressively later than their saturated counterparts as the degree of unsaturation increases. They are also most suitable for the separation of *cis* and *trans* isomers.⁹⁰ Figure 10.1 shows the chromatographic profile of a mixture of FAME (a) and FFA (after methylation with 14% boron fluoride in a methanol solution) of a sample of dry-fermented sausage (b), obtained by a WCOT fused-silica capillary column coated with polyethylene glycol as stationary phase.

Identification of fatty acid may be performed by comparison with the retention times of authentic FAME reference standards commercially available. To confirm the identity of fatty acids, it is becoming more frequent to utilize gas chromatography-mass spectrometry (GC-MS),^{19,90} to compare the mass spectrum of each signal with a reference spectrum available in the mass spectrum libraries. The combination of the retention parameters, in particular retention time determined by GC, with the structural information provided by MS, constitutes one of the most definitive methods for the identification of complex organic molecules. The major application of this method in fatty acid analysis is the determination of the position and geometry of double bonds, but branched chain or other substituents may be located as well.

The quantification of fatty acids is carried out by chromatographic signal area or height measurement, the latter in the case of an asymmetric chromatographic signal; known amounts of undecanoic, tridecanoic, pentadecanoic, or heptadecanoic acid added to the samples may be used as internal standard. The concentration of the individual FFA is calculated as percentage of total area of the identified chromatographic signals, or as milligrams per gram of fat. The level of

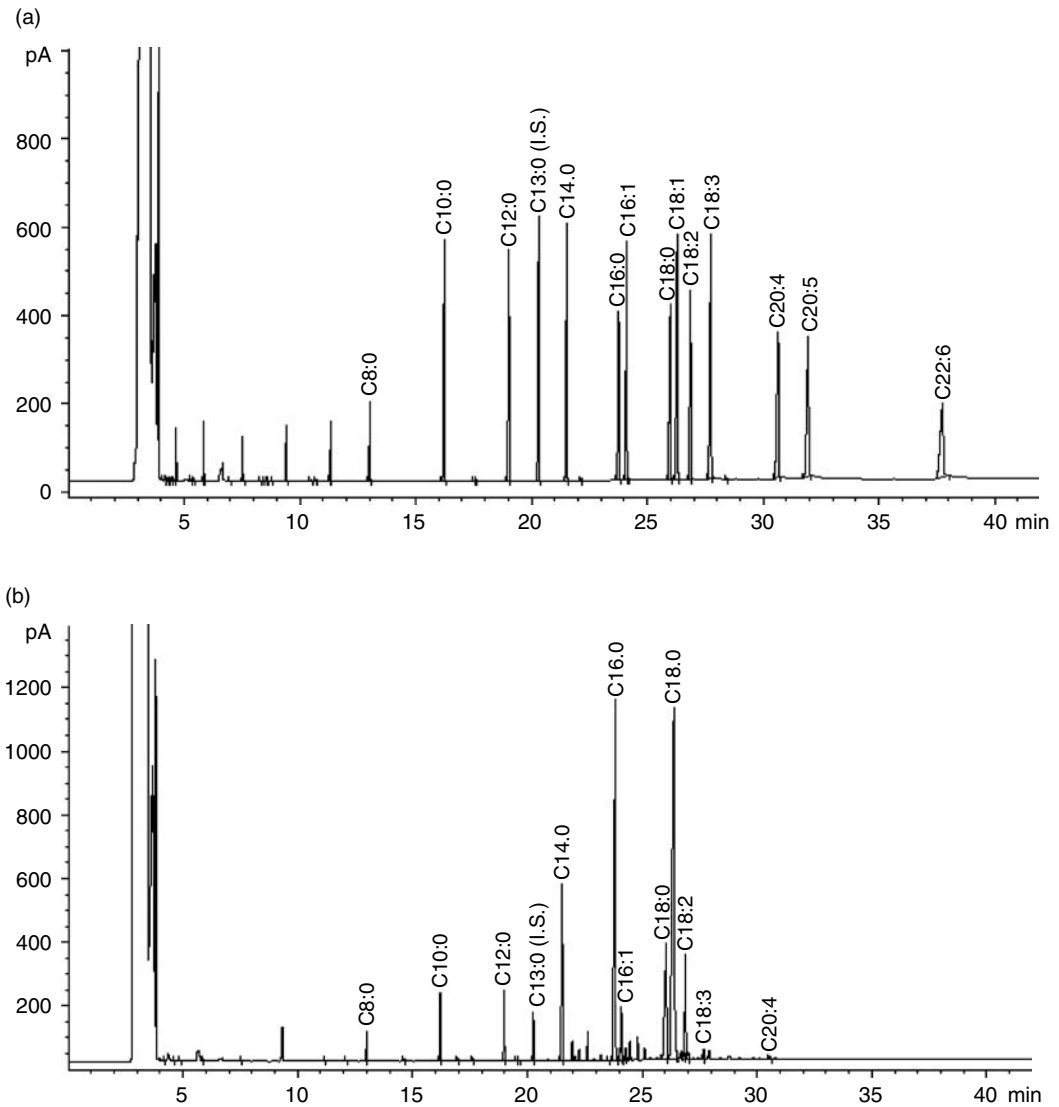


Figure 10.1 GC profile of a mixture of FAME (a) and of FFA of a sample of dry-fermented sausage (b). Analysis was performed on a gas chromatograph equipped with a WCOT fused-silica capillary column (30 m length, 0.25 mm internal diameter) coated with polyethylene glycol stationary phase (0.25 μm film thickness), a split-splitless injector, and a FID. The latter two were kept at a constant temperature of 260 and 270°C, respectively. Column oven temperature was programmed from 50 (2 min hold) to 100°C at 8°C/min, and from 100 to 260°C at 10°C/min; final temperature of 260°C was held for 25 min. The carrier (20 cm/s average velocity) was nitrogen (99.9995% purity) set at 13 psi constant pressure. The injection volume was 1 μL and the injection was performed in split mode.

Table 10.5 FFA Content (mg/g fat) of Mediterranean and North Europe Type Sausages and of Dry-Cured Iberian Ham, at the Beginning and the End of Processing

Fatty Acid	Mediterranean-Type Sausage		North Europe-Type Sausage		Iberian Ham	
	Mince	Matured	Mince	Matured	Green Ham	Dry cured
C10:0	0.16 ± 0.01 ^a	0.23 ± 0.07	0.10 ± 0.02	0.11 ± 0.01	—	—
C12:0	0.06 ± 0.01	0.19 ± 0.07	0.05 ± 0.03	0.10 ± 0.02	2.58 ± 0.10	2.56 ± 0.26
C14:0	0.24 ± 0.01	0.69 ± 0.06	0.18 ± 0.04	0.58 ± 0.09	1.00 ± 0.08	1.35 ± 0.20
C16:0	2.81 ± 0.06	6.16 ± 0.82	1.53 ± 0.47	5.53 ± 0.78	2.48 ± 0.23	7.56 ± 0.15
C16:1 (n-7)	0.22 ± 0.02	0.75 ± 0.13	0.18 ± 0.06	0.62 ± 0.12	1.12 ± 0.07	0.86 ± 0.04
C18:0	1.63 ± 0.01	3.21 ± 0.26	1.21 ± 0.21	3.19 ± 0.47	2.27 ± 0.17	2.85 ± 0.34
C18:1 (n-9)	3.24 ± 0.11	11.6 ± 2.00	2.75 ± 0.82	10.8 ± 1.77	10.19 ± 0.82	9.21 ± 0.05
C18:2 (n-6)	1.35 ± 0.04	6.47 ± 0.53	1.02 ± 0.17	5.43 ± 1.17	3.54 ± 0.28	1.80 ± 0.30
C18:3 (n-3)	0.08 ± 0.02	0.36 ± 0.12	0.13 ± 0.04	0.58 ± 0.11	0.80 ± 0.07	0.53 ± 0.04
C20:0	—	—	—	—	0.97 ± 0.06	0.95 ± 0.09
C20:1 (n-9)	0.08 ± 0.03	0.48 ± 0.02	n.d. ^b	0.32 ± 0.07	—	—
C20:2 (n-6)	0.12 ± 0.01	0.79 ± 0.16	0.26 ± 0.14	1.19 ± 0.29	—	—
C20:4 (n-6)	0.11 ± 0.01	0.80 ± 0.30	n.d.	0.27 ± 0.18	0.96 ± 0.07	0.55 ± 0.06
Σ Saturated	4.89 ± 0.05	10.5 ± 1.15	3.07 ± 0.69	9.02 ± 1.27	9.30 ± 0.61	15.27 ± 0.62
Σ Monounsaturated	3.55 ± 0.16	12.8 ± 2.11	2.93 ± 0.87	11.7 ± 1.94	11.31 ± 10.86	10.07 ± 0.72
Σ Polyunsaturated	1.66 ± 0.04	8.40 ± 0.18	1.41 ± 0.12	7.47 ± 1.54	5.30 ± 0.30	2.88 ± 0.39

^a Mean value ± standard error.

^b Not detected.

Source: Adapted from Zanardi et al., *Meat Sci.*, 66, 415, 2004 and Martín et al., *Meat Sci.*, 51, 129, 1999.

individual FFA, measured by GC analysis of the corresponding FAME, of Mediterranean and Northern European fermented sausages and of dry-cured Iberian ham is reported in Table 10.5.^{21,42}

However, raw pork,^{11,16,17} cooked pork loin chops,^{12,18} raw rabbit meat,⁹⁵ poultry meat,¹⁵ Northern European fermented sausage,^{23,26} Mediterranean fermented sausage,^{21,25,28,31,56,58,68,96,97} French dry-cured ham,^{41,79} Spanish dry-cured ham,^{39,40,98,99} dry-cured pork foreleg,⁴³ and Chinese Xuanwei ham¹⁰⁰ have been widely investigated for individual FFA levels and changes during storage and manufacturing, to elucidate the effect of different physicochemical and technological parameters on the time course of lipolysis in muscle foods.

10.6 Conclusions and Future Trends

Lipolysis is an important part of lipid degradation of processed meats and poultry. Several analytical methods are available for the measurement and monitoring of lipolysis in these products. In general, they are based on the determination of the level and composition of FFA; however, they are diverse in terms of analytical technologies applied, which range from simple and rapid routine methods to more recent chromatographic techniques that enable the concomitant determination of the content and composition of neutral and polar lipids. This diversity is important in that it provides a variety of approaches for the evaluation of enzymatic hydrolysis of meat lipids and thus of technological quality of meat products and poultry. All of the methods

are being continually perfected and evaluated, and new analytical tools are being tested and developed to enhance reliability, sensitivity, speed, and simplicity of analysis. There is potential for increased use of established technologies, such as GC-MS and liquid chromatography-mass spectrometry (LC-MS), for the measurement of the molecules produced by lipolysis, and for the enhancement of the scientific knowledge in a biochemical process strongly related to the quality and safety of lipids.

More recent research on lipids of meat addresses plasmalogens. Plasmalogens are a phospholipids subclass in which the *sn*-1 position of the glycerol core is linked with a long-chain fatty aldehyde; they account for 12–20% of total phospholipids in skeletal muscles of different animal species. Research focused on plasmalogens has increased in human medicine, due to the proposed role that they play as endogenous antioxidants and their implications in the development of several human diseases, such as Alzheimer's, heart disease, myocardial infarction, and cellular aging.^{101,102} Studies on the fatty aldehyde composition of plasmalogens and the factor affecting their occurrence and proportion in meat are scarce.^{103–106} Further research would be needed to elucidate the role of plasmalogens in lipolytic and oxidative/antioxidative processes in meat.

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NUTRITIONAL QUALITY



Chapter 11

Composition and Calories

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11.1 Introduction

The food we eat provides us with nutritive substances to build body structures and to enable them to function properly. Food also provides the body with energy from reduced organic compounds with which oxygen can react to produce chemical energy and heat. The main energy-delivering nutrients are carbohydrates, fat, and, to a lesser extent, protein. In a living being these compounds can be oxidized to H_2O and CO_2 ; protein also releases NH_3 , which is excreted as urea in mammals or as uric acid in birds.

The outstanding constituent of muscle foods, unprocessed or processed, is the protein, which varies in content among the different meat cuts and meat products. Protein content in meat and poultry, including products, ranges between about 10 and 23%.^{1,2} Another main constituent of all meat and poultry products is water, which varies from 30 to 70%. During the manufacturing of heated meat products, water (20–25%) is often added for technical and sensory reasons. The third macronutrient is fat, which may be as low as 1%, but ranges up to around 50% in some dried raw sausages.

All muscle foods contain cholesterol, again varying over a wide range, from 40–90 mg/100 g in meat and poultry. Offals such as liver, kidney, or brain contain up to several hundred mg/100 g. Meat products, depending on their composition, may contain <50 mg cholesterol/100 g, for example, in cooked ham. In liver sausages it may reach >150 mg cholesterol/100 g.

Salt (NaCl) is also added to meat products. It serves various technical purposes—binding of water and fat, and protection or retardation of microbiological spoilage (shelf life). It is also used for sensory reasons (see Chapter 4). Binding requires at least 1.5% salt, protection from spoilage >2.0% salt. The lowest concentration is necessary for sensory reasons. Sometimes a part of NaCl is exchanged (25–30%) for KCl.

Besides NaCl, minerals in meat include many trace elements, and different concentrations of many vitamins are present in heated or fermented/dried meat-poultry products (see Chapters 15 and 16).

11.2 Composition

11.2.1 Definitions

According to a worldwide accepted definition, meat is all edible parts of a carcass of a slaughtered animal (EU-2004).³ This includes the lean muscular tissue, adjacent (intra- and intermuscular) fat, and adipose tissue, usually called fatty tissue. Meat also includes blood, organs (offal), and intestines. Since, in terms of composition and ways of manufacturing products, no important differences

exist between red meat (beef, pork, lamb, sheep, goat, horse, venison, etc., i.e., meat of mammals) and poultry (mainly chicken and turkey, i.e., meat of birds), the word meat in the following means both groups unless they are directly addressed.

Meat products are processed foods that are manufactured using meat of all edible parts, as in the definition above, as the main ingredient. According to regulation EU-2004,³ meat products must show on a cut surface that the product no longer has the characteristics of fresh meat. Besides meat, other foods or additives may be added. As mentioned earlier, water and salt are often added, both for technical and other reasons. Additives such as nitrite or nitrate (for curing and protection against some pathogenic microorganisms) or smoking (for flavor and retardation of chemical spoilage and growth of mold) can be applied (Chapters 5 and 6).

Compounds supporting the action of salt as binders including emulsifiers, phosphates, salts of organic acids (e.g., citrate), nonmeat proteins, or high molecular-weight carbohydrates (e.g., starch or carragenan) are also permitted under EU-1995⁴ and EU-2006.⁵

Meat products are processed by comminuting, tumbling, fermenting, drying, smoking, heating, and other related processes. The main reasons for manufacturing meat products are (1) to prolong the shelf life of fresh meat and (2) meat products are convenience foods due to their easy and safe handling. There exist several meat product categories.

11.2.2 Category: Heat-Treated Meat Products

In the following, the basic principles of preparation and composition of the various traditional meat product types are described. Light products, meaning meat products with reduced fat content, are manufactured very similarly by reducing fat and exchanging it for meat or other additives.

11.2.2.1 Emulsion-Type Sausages

These sausages are prepared by comminution, usually in a bowl chopper, of all ingredients at temperatures <20°C (Table 11.1). The formed batter can also be mixed with pieces of meat, fat, nuts, or vegetables. The mixture is filled into casings, cans, or forms and heated to 75°C or higher. During the heating process, and with the addition of >1.5% salt or nitrite curing salt, the batter

Table 11.1 Ingredients of the Basic Emulsion-Type Sausage Batter

<i>Ingredient</i>	<i>Range (%)</i>	<i>Median (%)</i>
Lean meat without fat ^a	45–60	51
Fat	11–28	23
Water (or ice)	20–25	22
Salt or curing salt	1.5–2.2	1.8
Spices	1–1.5	1.3
Additives	0.3–0.8	0.5

^a EU defines meat in directive EU-2001. According to this definition meat may contain a considerable amount of fat. If this definition were used in the table, the meat content would be 75% and more.

Source: EU-2001, directive 2001/10/EC amended annex 1 of 28.11.2001 O.J. L 310, p. 21, 2001.

forms a heat-stable, sliceable product through a network of protein, fat, and water. Nearly all products of this group must be kept chilled at $<5^{\circ}\text{C}$ until consumption.

11.2.2.2 Cooked Sausages

For these types of sausages the meat is heated either using fresh meat and fat without additives or using precured meat (containing about 2% salt) to about 95°C . The cooked meat is comminuted and cooled to about 40°C . Uncooked liver or blood, gelatine, or cooked skin and other cooked offals are added with spices, salt (curing salt), and meat cookout, and all ingredients are mixed (Table 11.2). With liver, a further comminution is carried out. The mixture is filled into casings, cans, or forms and reheated for a second time to $>80^{\circ}\text{C}$. The products of this group are mainly liver sausage, paté, blood sausages, or aspic products. These are sliceable below 20°C . All products must be stored chilled at $<5^{\circ}\text{C}$ until consumption.

11.2.2.3 Cooked Ham and Similar Products

These products are produced from pieces of raw meat (20 g to several kilograms) to which a brine containing salt, spices, and additives is either added by tumbling in the case of smaller pieces (in a slowly revolving machine) or by brine injection of 5–15% (Table 11.3). After tumbling or injection, the meat is put into forms or plastic pouches and heated to $>70^{\circ}\text{C}$. The yield after heating in traditional products reaches 100–110% of the original weight of the meat. In nontraditional modern products the yield may reach 130% or more. The product must be kept chilled until consumption at $<5^{\circ}\text{C}$.

Table 11.2 Ingredients of Basic Type of Cooked Sausages

<i>Ingredient</i>	<i>Range (%)</i>	<i>Median (%)</i>
Lean meat without fat ^a	30–55	43
Fat	10–35	28
Offals and other ingredients (e.g., liver)	15–25	20
Cookout from meat	2–10	5
Salt or curing salt	1.5–2.0	1.7
Spices	1.0–2.0	1.3
Additions and additives	1.0–2.0	1.5

^a EU meat definition (see Table 14-1 in the footnote).

Table 11.3 Ingredients of Traditional Cooked Ham

<i>Ingredient</i>	<i>Range (%)</i>	<i>Median (%)</i>
Meat (EU definition) ^a	>85	87
Fat in the meat	1–15	4
Salt or curing salt	1.7–2.4	1.9
Brine	5–15	7

^a EU meat definition (see Table 14-1 in the footnote).

Table 11.4 Ingredients of Basic Type of Raw Sausages

<i>Ingredient</i>	<i>Range (%)</i>	<i>Median (%)</i>
Lean meat without fat ^a	50–70	59
Fat	25–50	35
Salt, curing salt or salt, and nitrate	3–4.5	3.8
Spices	1–2	1.5
Carbohydrates	<0.5	<0.5
Additives and often starter cultures	0.3–1	0.8

^a EU definition of meat (see Table 14-1 in the footnote). If meat according to the EU definition is used, then the product ready for consumption due to its loss of water will express a quantitative ingredient declaration (QUID)—“100 g of this sausage has been produced by e.g., 130 g of meat.”

Note: Percentage calculated at the time of consumption.

11.2.3 Category: Non-Heat-Treated Meat Products

11.2.3.1 Raw Sausages

For these products the mixture of raw meat (including poultry in recent years) together with salt, curing salt, or salt and nitrate, spices, 1–2% sugar or other carbohydrates, and often microbial starter cultures is ground or comminuted at -3 to $+5^{\circ}\text{C}$. The batter is filled into natural casings and fermented between 25 and 10°C . During and after this process the product loses water, which can amount to a weight loss of 30% (Table 11.4). Owing to the salt (plus nitrite) and the water loss, these products do not have to be kept under refrigeration. They may also be consumed in an unheated state.

11.2.3.2 Raw Hams

Pieces of raw meat (pork, beef, in recent years also poultry) dressed according to the required fat content is either put in dry salt or inserted into a concentrated brine (salt alone, nitrite curing salt, or salt plus nitrate) and stored at low ambient temperatures for some days or weeks. Afterwards the products are fermented or smoked. All raw hams are dried and lose between 10 and 30% of original weight. In most raw hams the fat content ranges from 1 to 20%, recognizable to the eye. Bacon and Speck have higher fat content up to 30% or higher. Salt content ranges from 3.5 to 5.5%. This high concentration is necessary for microbial stability, as these products are often stored without refrigeration. They are consumed in the unheated form.

11.3 Amount of Consumption and Uptake of Nutrients with Meat Products

The amount of meat and meat products eaten in different countries differs widely. Therefore the consumption of meat in general and meat products in particular is shown in Table 11.5,⁷ using Germany as an example.

The average German consumer (young or old, women or men) eats 82.2 g meat products, including meat preparations, per capita per day. From these figures it can be calculated that 13.5 g

Table 11.5 Consumption of Meat Products in Germany in 2006

<i>Product Type</i>	<i>Kilogram/Head × Year</i>	<i>Gram/Head × Day</i>	
<i>Fresh meat of all species</i>	29.0	79.5	
Emulsion-type sausages	14.1	38.7	} 55.9
Cooked sausages	3.7	10.1	
Cooked ham	2.6	7.1	
Non-heat-treated sausages	5.7	15.6	} 24.6
Raw ham	3.3	9.0	
Total	29.4	80.5	
Meat preparations	0.6	1.7	
Total of meat preparations and meat products	30.0	82.2	

Source: Modified from DFV, www.fleischerhandwerk.de/upload/pdf/GB2007_Verzehr_von_Fleischerzeugnissen.pdf, 2007.⁷

of protein and 19.9 g of fat are consumed per day per person. It is recommended that no more than 50–60 g of protein and 70–80 g of fat (with 1/3 being saturated fatty acids) be eaten per day by an adult female or male person. Around 20 g fat/head × day (25–28% of the daily recommended intake) is equivalent to 180 kcal, which is 9% of the daily energy need of 2000 kcal for women. If 13.5 g protein (27% of the recommended daily intake) is added (54 kcal) to the energy intake, then 234 kcal = 11.7% of 2000 kcal eaten.

Eating 55.9 g of heat-treated meat products (2% salt) per day per person results in an intake of 1.1 g of sodium chloride = 440 mg of sodium. Consuming 24 g non-heat-treated meat products per day per person with 4% salt amounts to an intake of 0.98 g of salt = 390 mg of sodium. Both add up to 830 mg of sodium/head × day. The recommended level of sodium intake per day is 2400 mg. So 830 mg is 35% of this amount.

Lean meat of all common meat species in Europe contains more than 50% unsaturated fatty acids; <40% of the fatty acids in meat fat consist of saturated fatty acids. With 20 g fat, an intake of <8 g of saturated fatty acids equals about 35% of the recommended amount. The main single fatty acid in most cases is oleic acid (up to 47%).¹ The unsaturation in fatty acids increases from lamb to cattle to pigs to poultry from about 50% in lamb to >65% in poultry.⁸

11.4 Calories: Physiological Energy

Living animals and human beings consist of chemotrophic or heterotrophic cells, meaning that they have to take in reduced organic matter as food, which is oxidized by the oxygen in air or water to CO₂ and H₂O.⁹ The latter compounds are the principal oxidized compounds of carbon (C) or hydrogen (H). Between the most reduced compound, CH₄ (methane), and the end of oxidation, CO₂ and H₂O, are the food constituents—lipids, carbohydrates, organic acids such as lactic acid, alcohol, and amino acids (protein) and nucleotides. Amino acids and nucleotides also contain nitrogen. Besides the organic matter, water and minerals must be part of the food. Minerals and water, however, do not deliver energy to the body.

All these compounds serve more than one purpose in the body. On the one hand, they provide energy to keep the physiological equilibrium of life in a steady, well organized, but energy-consuming state. A variable part of these energy-delivering compounds can be stored as fat in adipose tissue or as carbohydrates in glycogen globules. However, they are also construction

Table 11.6 Energy Units

Energy is equal to work
 1 joule (J) = 1 Newton (N) × m = 1 watt (W) × s = 10⁷ erg = 0.239 cal
 1 calorie (cal) = 4.1868 J

Source: Adapted from IUPAC Compendium of Chemical Terminology, www.goldbook.IUPAC.org/C00784.html. 1997.

Table 11.7 Energy Units of Food Compounds

<i>Compound</i>	<i>kJ/g</i>	<i>kcal/g</i>
Carbohydrate	17	4
Multiple alcohols, polyols	10	2.4
Ethyl alcohol	29	7
Organic acids	13	3
Fat	37	9
Protein	17	4

Source: After EU-1990, Council directive 90/496/EEC on nutrition labeling for foodstuffs, O.J. L 276, 06.10.1990, pp. 40–44, 1990.¹¹

material for the body—protein for muscles, fat for bilayers of cellular membranes, and minerals for bones and teeth. After being modified in the body, food constituents may also function as catalysts for the physiological equilibrium (enzymes).

Traditionally, energy is expressed in calories (cal); today another energy unit, the joule (J), is recommended (Table 11.6).¹⁰ Despite their different purposes and uses in the body, the physiological energy of fat and carbohydrates is equal to the chemical energy value under the argument that in the end all fats and carbohydrates are oxidized to CO₂ and H₂O. With protein it is different, as a considerable part is used in the construction and function of cells. This has been taken into account in setting the physiological energy value of proteins.

The physiological values have been accepted worldwide and are, for example, laid down in directive EU-1990¹¹ (see Table 11.7).

Owing to the composition of most meat products, only the energy of fat (1–50%) and protein (10–23%) has to be taken into account. The amount of carbohydrates (<1%) and organic acids (~1%) accounts at most for about 3–4 kcal (13–17 kJ) in 100 g of meat products and need not be considered. Some meat products, however, have higher carbohydrate levels. If the amount of digestible carbohydrates (mono- or disaccharides, starch, or inulin) is above 1%, it should be accounted for in considering energy value.

11.5 Analytical Methods

11.5.1 Analysis of Water

Analytically, lean meat contains 75% water. In many heated meat products, besides meat as the main ingredient (>50%), water is added for technical and sensory reasons (Tables 11.1 through 11.3). Thus, the water content of meat products may be >60%. This amount is usually determined

by drying through heating. The degree of heating for moisture determination is limited, as other compounds such as fat or protein may disintegrate at higher temperatures and release decomposition compounds in gaseous form, which would falsify the results. Thus, the determination of moisture will usually be carried out between 95 and 105°C. The drying process, however, is slow and must be repeated until a constant weight is reached, and this can take hours.

This may be too time consuming, especially within or before a manufacturing process. For these purposes rapid methods for moisture determination have been developed. Faster heating, however—for example, by microwave—has the disadvantage of higher variability, which, however, may be acceptable in a processing line. A faster method (Association of Official Analytical Chemists [AOAC International] 2005, method 950.46)¹² involves the application of 125°C in a convection oven.

Near infrared (NIR) and near infrared transmission (NIT) methods, nuclear magnetic resonance (NMR), and guided microwave spectroscopy (GMS) are new, faster methods which do not dry the food. However, they require a rather laborious calibration for each instrument and for the various meat product types, which contain varying amounts of water and which may be raw, salted, or heated products. But with these methods one can determine besides water as well as protein and fat content.

The four main components—water, fat, protein, and salt (the latter usually measured and expressed as ash)—show a very close relationship in fresh meat of all common meat species before storage, that is, without water loss.⁸ This does not apply to processed products.

11.5.1.2 *Determination of Water (Moisture) by Drying*

One of the standard reference methods is the oven drying method (AOAC 2005, method 950.46).¹² Similar methods exist in different analytical collections of methods such as the International Organization for Standardization (ISO), Euro Norm (EN), or German Institute for Standardization (DIN).

AOAC method 950.46 describes how meat samples (including meat products) will be dried after preparation according to AOAC method 983.18.¹²

11.5.1.2.1 *Sample Preparation for Drying and Further Analyses (Fat or Ash)*

Separate meat as completely as possible from any bone; pass rapidly three times through a grinder with plate openings of 3 mm, mixing thoroughly after each grinding; and begin all determinations promptly.

Take particular care with certain meat types such as meat products to assure uniform distribution of fat; take care similarly for connective tissue in samples of raw products.

11.5.1.2.2 *Drying Methods*

1. With lids removed, dry samples containing ca. 2 g dry material (equivalent to about 8–10 g of fresh meat) for 16–18 h at 100–102°C in an air oven (mechanical convection preferred) or at 125°C for 2–4 hours. Use a covered alumina dish ≥ 50 mm diameter and ≤ 40 mm deep. Cool in a desiccator and weigh until the weight is constant. Report loss in weight as moisture.
2. AOAC Official Method 985.14, moisture in meat and poultry products, rapid microwave drying method.¹²

Moisture is removed (evaporated) from the sample using microwave energy. Weight loss is determined by electronic balance readings before and after drying and is converted to moisture content by a microprocessor with digital percentage readout.

CEM (Chemie, Elektronik, Mechanik Company) designed a microwave oven specifically for moisture in foods. Samples are placed between glass fiber pads and dried for 3–5 min, after which they are reweighed. This is published as an official AOAC method (AOAC 985.14).¹²

11.5.1.2.3 Determination of Added Water to Sausages

As described earlier and shown in Tables 11.1 through 11.3, water may be added during manufacturing.¹³ The reason is that water is a solvent for salt, other ionic additives, and myofibrillar proteins (technical reasons) and for the texture of the heat-stable network. Meat itself, depending on the cut and species, contains a water:protein ratio between 3.3 to 3.8:1.^{14–17} In most countries a ratio of water:protein = 4 is taken as the limit for the natural water content of meat. All water above this value 4:1 is so-called “added” or “foreign” water. According to AOAC method 928.07,¹²

$$\text{H}_2\text{O} (\%) \text{ added} = \frac{\text{water} - 4 \times \text{protein}}{1 - 0.01 (\text{water}) + 0.04 (\text{protein})}$$

Protein is calculated as $\text{N} (\%) \times 6.25$.

Example: An emulsion-type sausage contains 12.5% protein and 59% water.

$$\frac{59 - 4 \times 12.5}{1 - 0.59 + 0.5} = \frac{9}{0.91} = 9.89\% \text{ added water}$$

For emulsion-type sausages, guidelines or codices of most countries permit 5–10% added water. This is a generous rule, as the real ratio of water:protein is ~3.7 (as mentioned earlier). This would mean in reality

$$\frac{59 - 3.7 \times 12.5}{0.87} = \frac{12.75}{0.87} = 14.66\% \text{ added water}$$

11.5.1.3 Spectroscopic Fast Methods

11.5.1.3.1 Near Infrared Methods

Near infrared reflectance (NIR) methods can be applied for moisture determination. Quantitative measurement of meat components requires measurement of known samples for calibration. Unknown samples of similar types can then be scanned and components can be determined by comparing the response to the calibration data.^{18,19} Once calibration is complete, the method provides a simultaneous measurement of fat, moisture, and protein that is extremely fast and often nondestructive.

The availability of economical microprocessors that provide for easy calibration using artificial neural networks has made NIR instruments commonly available for meat analysis. Examples

of available instruments include, for example, those from Infratec and Foss Electric (FoodScan). Once again it is important to state that reliable NIR analyses are highly dependent on proper calibration of instruments with samples similar to the unknown samples to be measured. Recalibration is necessary for any change in sample material that is outside the range of properties of the samples used for calibration. The need for careful and proper calibration is viewed by some analysts as a disadvantage of this method. Recent developments in technology have resulted in changes of most instruments from NIR reflectance measurement to near NIT. These transmission measurements utilize greater sample volume, which improves results. Correlations between NIT measurements and AOAC methods have been reported as 0.984–0.995, 0.987–0.992, and 0.949–0.957 for fat, moisture, and protein, respectively (www.aoac.org/ILM/jul_aug_07/foodscan.htm).²⁰

AOAC reports, further, that the FOSS FoodScan™ with an artificial neural network method has been granted AOAC Official MethodSM status.²¹ Study samples were chosen to represent the majority of products from the commercial meat industry (beef, pork, and poultry) and included raw meats, emulsions, and finished products. The collaborative study samples consisted of 10 meat study samples prepared as blind duplicate pairs, resulting in 20 test samples. The method is applicable to the simultaneous determination of fat, moisture, and protein in meat and meat products (fresh meat, beef, pork, poultry, emulsions, and finished products) in the constituent ranges of 1–43% fat, 27–74% moisture, and 14–25% protein.

There is an NIT system called the continuous fat analyzer (CFA) for use with mixers and grinders. The CFA utilizes an 850–1050 nm wavelength range to continuously monitor fat, moisture, and protein content during mixing, and composition can be checked and adjusted on the spot. Standard deviations of 0.3% for the measurements have been reported.²²

11.5.1.3.2 Guided Microwave Spectrometry

GMS has not been studied as extensively as NIR systems, but this approach has been developed to the point of being offered as part of meat processing equipment, similar to NIR. The GMS measurement is based on microwave energy absorption, which is used to measure differences in conductivity and the dielectric constant of water. It is used for determination of sample fat, moisture, and protein content. Calibration with known samples is necessary for GMS measurements.²³

11.5.1.3.3 Nuclear Magnetic Resonance

The most recent development in commercial instruments for fat analysis in meat has been nuclear magnetic resonance. NMR data distinguish between protons from different molecular sources and clearly differentiate between meat components such as fat and lean. Correlations between NMR measurements and known fat content in meat have been reported to be 0.967.^{24–26}

11.5.1.4 Summary for Moisture Determination

A wide variety of methods are available for measuring water content. These methods range from traditional slow wet chemistry methods that have been in use for decades to rapid, inline multi-component analyses that have been developed very recently. But repeatability, reproducibility, and bias must be determined to permit selection of a method that will meet the expectations of the analytical laboratory applying them.

11.5.2 Analysis of Fat

Fat, or more correctly lipids, of muscle foods can be divided into four main groups.⁹

1. *Triacylglycerols*. These compounds consist of the trialcohol glycerol to which three fatty acids are bound with ester bonds. These components are the main constituents of storage fat in intra- and intermuscular fat (marbling) or in adipose (fatty) tissue. They are rather lipophilic. In meat products they constitute the main part (>95%) of lipids.
2. *Phospholipids (phosphoglycerols)*. Glycerol is bound to two fatty acids. The third OH-group of glycerol is bound to phosphate, which in turn binds various alcoholic compounds; some of these contain -NH_2 groups. The best known phospholipids are the lecithins. These compounds mainly occur in cellular membranes of animals. In meat products their content is low (<1%).
3. *Sphingo- and Glycolipids*. Sphingolipids do not contain glycerol. They consist of fatty acids, amino, and sometimes phosphate groups. Some exchange amino or phosphate groups for carbohydrates. They occur in the range of phospholipids in cellular membranes. In meat products they are also low (<1%).
4. *Cholesterol (ester)*. Cholesterol is the main constituent of animal cellular membranes. About 40–55% of the membrane lipids are cholesterol. Cholesterol is a sterol compound and cholesterol itself and its esters, which occur in smaller amounts, are strongly lipophilic. Cholesterol in lean to fat meat amounts to 40–90 mg/100 g. Offals contain higher concentrations, >150 mg/100 g. Thus, meat products also contain variable concentrations, which are analyzed with extraction methods but which are negligible in the total fat value.

n-Hexane, petrol-ether, diethylether, or a mixture of methanol/methylene chloride are used for extraction. All the lipids extracted by the different solvents are commonly called “crude” fat.

11.5.2.1 Determination by Extraction

A prerequisite for the extraction with lipophilic substances is the drying of the product, as described under moisture determination.

In the classical Soxhlet extraction, the sample is extracted many times by reflux with a solvent such as diethylether, *n*-hexane, or petroleum ether; the solvent is evaporated and the extracted fat is weighed (AOAC 960.39¹² and AOAC 991.36¹²) or the specific gravity of the extract is measured (AOAC 976.21),¹² for example, with the FOSS-let fat analyzer.

Another classical method is the Folch extraction, in which the fat is extracted with a 3:1 mixture of chloroform:methanol. This more polar mixture of solvents extracts all the lipids including structural lipids, and also extracts other components. Results for total fat with this method therefore tend to be high. This extraction procedure, due to its use of hazardous organic chlorine solvents, is currently not very often used.

In the Schmid–Bondzynski–Ratzlaff (SBR) method, the sample is boiled with hydrochloric acid to hydrolyze triacylglycerols, phospholipids, and to break down lipoproteins, glycoproteins, and protein, and is then extracted with a mixture of diethylether–petroleum ether. The solvents are evaporated and the extracted fat is weighed. With this method, practically all the lipid material in the sample will be extracted, but sometimes nonlipid material is extracted as well. This method therefore tends to give high results for total fat.

The similar Weibull–Stoldt method also hydrolyzes with hydrochloric acid, but the fat is filtered off on a fat-tight filter paper, washed, and extracted. This method gives results that are

comparable to or slightly lower than those obtained using the above mentioned method, but the filtering step makes it more laborious.

The newest extraction technique is accelerated solvent extraction, which reduces extraction time considerably and uses very small amounts of solvent under pressure. Automated methods deliver the same results as the classical methods such as Soxhlet.

Fat can be extracted using supercritical fluid extraction, with carbon dioxide as the solvent. Because this solvent is nonpolar, like petroleum ether, mainly the triacylglycerols and cholesterol will be extracted.

11.5.2.2 Determination with Spectrophotometric Methods

For more details, see the description of methods in Section 11.5.1.3.

NIT and NIR are indirect methods based on absorption of light in the sample in the near infrared range from 800 to 2500 nm. Calibration by a number of similar samples, comparison with a reference method, and calculations with multivariate statistics are necessary. Equipment can be bought with built-in broad calibrations. However, it is necessary to make sure that the calibration data set covers all types of samples that are to be analyzed with NIR/NIT, as changing the matrix, for instance from pork to beef or poultry, will also change the calibration.

NMR is another indirect method based on the measurement of a spin echo of protons in a magnetic field. The samples must be heated to ensure that the entire fat phase is liquid. It is also necessary to dry the samples, as protons from water will give a signal. A linear regression with results from a reference method must be used for calibration, so the NMR technique has the same limitations as described above for NIR/NIT.

More detailed information on meat is provided in the literature.²⁷ In Section 11.5.1 (water determination), the very close relationship between water and fat in fresh meat ($r > 0.99$) is mentioned.^{8,28} This permits the measurement of moisture (water) content and calculation of the fat content without further analysis in fresh meat. As discussed earlier, this does not apply to meat products.

11.5.2.3 Determination of Cholesterol (Ester)

The lipophilic cholesterol can be determined after separation from other lipids by enzymatic analysis, gas chromatography, or high-performance liquid chromatography (HPLC) measurements (AOAC 994.10,¹² 976.28,¹² and Arneht and Hussein²⁹).

All these methods can be applied to meat and meat products.

11.5.2.4 Summary of Fat Determination

Besides the classical determination of fat by extraction and weighing, modern fast spectrophotometric methods with a minimum of sample preparation have been introduced recently. But this fast measuring technique requires a laborious calibration beforehand.

11.5.3 Analysis of Protein

In general, proteins are composed of 20 L-amino acids. The percentage of these 20 amino acids varies among proteins. The amino acid sequence is laid down in the genetic information in deoxyribonucleic acid (DNA) and it is specific for the individual being. Table 11.8³⁰ shows for beef and

Table 11.8 Amino Acid Composition of Beef and Cod Muscles

<i>Compound</i>	<i>Beef Muscle (%)</i>	<i>Cod Muscle (%)</i>
Aspartic acid/asparagin	4.0	6.8
Threonine	3.7	3.4
Serine	4.6	3.6
Glutamic acid/glutamin	9.3	8.8
Proline	4.3	3.4
Glycine	6.0	5.8
Alanine	4.9	5.9
Cystine	0.8	2.5
Valine	3.7	2.5
Methionine	2.2	2.0
Isoleucine	4.2	2.7
Leucine	5.1	5.1
Tyrosine	2.1	1.7
Phenylalanine	2.7	2.1
Tryptophan	1.2	1.1
Lysine	9.8	11.7
Histidine	4.9	3.5
Arginine	14.5	13.2

Source: Modified from Belitz, H.D., Grosch, W., and Schieberle, P., *Food Chemistry*, Springer, Berlin, p. 627, 2004.

cod muscle that almost none of the amino acids have the 5% average value ($100:20 = 5$) in beef or cod muscle (only alanine in beef and leucine in beef and cod come near this value). Many of those amino acids that carry a negative or positive charge on a side chain occur in percentages higher than 5%. The sulfur-containing amino acids show the lowest percentage in bovine and cod muscle.

The protein concentration in a food is determined by its N content. As the molecular weights (MW) of the amino acids vary widely (MW of glycine is 75 Da, tryptophan has a MW of 204 Da), the percentage of N on the total weight also varies, ranging from about 8% in tyrosine to 19% in glycine and lysine. The conversion factor from N content to amino acid content hence ranges between 5.2 and 12.5.

The total protein of meat determined through its N content is composed of myofibrillar protein + sarcoplasmic protein + other nonprotein nitrogen compounds + connective tissue protein. In analytical food chemistry, this is called “crude” protein. The proportion of the various N-containing groups in meat is shown in Table 11.9.³¹

The variability of amino acid composition of different foods leads to different conversion factors for calculation from the N determination to protein content,³² shown in Table 11.10. The factor for meat and meat product is 6.25, which is now valid for all foods, according to EU-1990.¹¹

The connective tissue of meat contains a special amino acid, 4-hydroxyproline. Its content in connective tissue is 12.4%. After measuring the hydroxyproline content in a reaction as a colored pigment, the content is multiplied by 8 to calculate the connective tissue concentration.

The connective tissue content is decisive for the tenderness of meat. The tenderizing effect of aging takes place at first within the cell. Connective tissue, however, is located extracellularly. Additionally, the most esteemed (expensive) cuts of meat in a carcass are those which are low in

Table 11.9 Proportion of N-Containing Compound Groups in Meat

<i>N-Compound Group</i>	<i>Total (%)</i>
Total protein	100
Myofibrillar protein	60–65
Sarcoplasmic protein	30–32
Nonprotein nitrogen compounds	~1.5
Connective tissue protein	2.5–12

Source: Modified from Lawrie, R.A., *Lawrie's Meat Science*, Woodhead Publishing, Cambridge, 1998, p. 59.³¹

Table 11.10 Conversion Factors from N-Determination to Protein for Different Foods

<i>Food</i>	<i>Conversion Factor</i>
Grain	5.80
Oil seeds	5.30
Milk	6.38
Mushrooms	4.17
Meat/fish/seafood	6.25
Vegetables/fruits	6.25

Source: Translated from *Ger. Chemie*, 39, 59–61, 1985.³²

connective tissue. That is why in many countries in meat and meat products, besides the total protein content, a value for connective tissue is determined and used as a part of quality characteristics of the cut.¹⁷

To measure not the crude but the “real” protein content—that is, the myofibrillar, sarcoplasmic, and connective tissue protein of meat—a homogenate of tissue must be acidified; usually perchloric or trichloroacetic acid is used. Under these conditions, the high molecular-weight proteins precipitate. Small peptides such as carnosine, anserine, and glutathion, free amino acids, and nucleotides remain in solution.

By a century-old tradition, the crude protein is determined and the small error in meat and its products is neglected. In products, however, when other N-containing ingredients are added, the error may be considerable and these nonmeat compounds must be otherwise determined. For instance, in products high molecular-weight proteins of plant origin may be added, which cannot be differentiated by precipitation. Often, other immunological or chromatographic methods must be used for determination.

11.5.3.1 Determination of Protein

11.5.3.1.1 Crude Protein Content

The method used for over a century is the Kjeldahl method, in which all nitrogen in the sample is reduced to NH_3 by heating in acid with a catalyst. After alkalization the NH_3 is distilled with water vapor and titrated with acid. The nitrogen content (not NH_3) is multiplied by 6.25 to obtain crude protein (AOAC 981.10).¹² As the heavy metal catalyst causes concern and for reasons

of automated and/or miniaturized methods, specialized equipment (for example, from Kjeltec, Labconco, Tecator) is offered on the market. Some are specified in official methods, as in AOAC 928.08, 960.52 AOAC 970.42, AOAC 977.14, and AOAC 981.10.¹²

A different approach to the protein content of food, but also by the nitrogen content, is the so-called Dumas method. At temperatures above 850°C a small sample (200–300 mg) is incinerated in an oxygen stream. The resulting H₂O and CO₂ are absorbed and the nitrogen is determined by its thermal conductivity (AOAC 992.15).¹² For this method a calibration is required. AOAC recommends ethylene diamine tetraacetic acid (EDTA) with a nitrogen content of 9.59%.

Recent developments in NIR, NIT, and NMR permit the determination of moisture, protein, and fat by spectroscopic methods. This principle is described in Section 11.5.1 for moisture determination. In all cases moisture, protein, and fat content are determined in one analysis.

11.5.3.1.2 Connective Tissue Protein

Connective tissue is an extracellular network of proteins (mainly collagen) which in meat is decisive for its tenderness. The content varies (Table 11.9) and the cross-linking between amino acids in the triple helix of collagen or between fibers enhances the toughness.³³ With cross-linking, solubility in hot water or an acidic solution is reduced, and this procedure is sometimes used for determining the soluble collagen by various nonofficial methods.³⁴ The total collagen, however, is officially determined via the determination of the specific amino acid 4-hydroxyproline, which is exclusively present in collagen.

Meat product samples (ca. 4 g weighed to the mg) are hydrolyzed in acid (3.5 M H₂SO₄ or HCl) at ~105°C. The final solution is filtered and the 4-hydroxyproline is oxidized with chloramine-T to a pyrrole. With 4-dimethylamino-benzaldehyde, a red color develops, which is measured spectrophotometrically at 560 nm. A calibration curve is required. With the usual $N \times 6.25$ protein factor it is assumed that collagen contains 12.5% 4-hydroxyproline. Collagen is therefore calculated as 8 times the concentration of 4-hydroxyproline (AOAC 990.26¹² or Lebensmittel-und Bedarfsgegenstände-und Fuffenmittelgesetzbuch [LFGB]³⁵).

11.5.3.1.3 Amino Acids

As discussed earlier, fresh meat contains a low amount of free amino acids. The determination of the free ones is therefore not very sensitive. The total amino acid composition after hydrolysis, however, is quite common, especially with regard to the nutritional value of proteins, which is expressed in its biological value for human beings related to the essential amino acid content in a protein.³⁶ The reference value is one whole chicken egg, which is equal to 100.

For this purpose the protein is hydrolyzed with acid for quite a long time and chromatographically (ion exchange or HPLC) separated; after a color reaction with ninhydrin reagent or fluorescamine it is spectrophotometrically determined.³⁷ Some amino acids like tryptophan are destroyed by the acidic hydrolysis. Some other amino acids, such as serine, threonine, valine, leucine, and isoleucine, are partially destroyed. An alkaline hydrolysis is not a perfect solution as other amino acids are high pH sensitive.²⁷ Thus, the sum of the measured free amino acids hydrolysis does not add up to what is measured by the Kjeldahl or Dumas methods.

11.5.3.1.4 Summary of Protein Determination

Protein is traditionally determined by its nitrogen content. Modern fast spectrophotometric methods have been used frequently in recent years, especially if the results must be obtained rapidly.

But, as described earlier—for example, in Section 11.5.1.4—these fast methods require a laborious calibration before measurement.

11.5.4 Analysis of Minerals (Inorganic Matter)

The whole mineral content of meat and products may be determined as ash. Ash means the oxides of all nonvolatile oxides of constituents of meat. The oxides of H to H_2O , C to CO_2 , and N to NO_2 evaporate at the temperature of incineration. Only inorganic matter, mainly metals, are present as oxides in the ash. In products the added salt or additives enhance the ash content of meat, which is $\sim 1\%$.

11.5.4.1 Determination of Ash

For ash determination the meat or meat products have to be ground to homogeneity, distributed in a thin layer on a metal (stainless steel, nickel) dish, and dried at $100\text{--}105^\circ\text{C}$, the same method used for moisture determination (see Section 11.5.1). To check the homogeneity of the material, charcoal powder may be added to reveal inhomogeneities during grinding. The charcoal is oxidized to CO_2 and evaporates and need not be taken into account. If the sample will be used for moisture determination beforehand, the added charcoal powder has to be deducted.³⁸ Some methods (e.g., AOAC 920.153)¹² add a defined amount of magnesium acetate to the ground mixture of meat. After incineration the molar equivalent of MgO has to be deducted.

The dry material is slowly heated over 5–6 h to about 550°C . After cooling down in a desiccator, the sample weight and the ash content are calculated. In meat it is around 0.8–1.3% of the fresh weight depending on fat content and species. In meat products, due to the added salt and other inorganic additives, values $>2\%$ are measured.

11.5.4.2 Analysis of Salt

$NaCl$ is usually determined by its chloride content. Na has a MW of 23 Da; $NaCl$ shows a MW of 58.45 Da. The portion of Na in $NaCl$ is $10/58.45 = 0.3975$ (ca. 40%). Na in salt is therefore calculated by $NaCl:2.5$. Meat contains only about 70 mg Na/100 g = 0.07% and nearly no chloride. To meat products $>1.5\%$ is added. Thus, the Na content of the meat part in meat products can be neglected.

11.5.4.2.1 Determination of Sodium

Other additives are added as Na salt like phosphates with ca. 0.5% (Na_2HPO_4 MW = 142 Da; Na_2 MW = 46 Da; $46/132 = 0.32$), roughly one-third. This means 0.5% phosphates add 0.15% Na to the product. Sodium citrate, with 0.3% in a product, adds Na with about 0.1%. Determining Na in meat products by its chloride content will hence often result in a lower Na content than is actually present. Sodium can be determined with atomic absorption spectroscopy or similar methods such as induced coupled plasma mass spectrometry (ICP) after incinerating the sample at 550°C (see Section 11.5.4.1) and dissolving in HCl. A calibration curve is required.³⁹

11.5.4.2.2 Determination of Chloride

Chlorides form with silver salt a precipitate of $AgCl$. After adding surplus $AgNO_3$, it is titrated with potassium chromate,⁴⁰ with potassium thiocyanate,⁴¹ or with potassium permanganate

(AAC 935.47).¹² Before titration, the meat has to be dissolved with HNO_3 or precipitated with Carrez I and Carrez II solutions (see also Chapter 4).

11.5.4.3 Analysis of Phosphates

Monophosphates exist in many forms as free acids H_3PO_4 (E338); NaH_2PO_4 to Na_3PO_4 (E339); KH_2PO_4 to K_3PO_4 (E340); CaHPO_4 , $\text{Ca}_2\text{H}_2(\text{PO}_4)_2$, $\text{Ca}_3(\text{PO}_4)_2$ (E341); MgHPO_4 , $\text{Mg}_2\text{H}_2(\text{PO}_4)_2$ (E343); diphosphate as various H, Na, K, and Ca salts $\text{X}_4\text{P}_2\text{O}_7$ (E450); triphosphates ($\text{X}_7\text{P}_3\text{O}_{10}$) as various H, Na, and K salts (E451); and polyphosphates as X_m (H, Na, K, Ca) or $(\text{PO}_3)_m$ (E452). All are permitted in EU-1995,⁴ with 5 g/kg or P_2O_5 in meat products. P_2O_5 is, for example, 64% of $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$, which is the most effective of all phosphates; 0.78% of $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ can be added. Phosphates exhibit several modes of action in meat batters, though not all to the same degree.

1. All phosphates bind many metal ions such as Fe, Zn, Mg, and Pb and prevent/retard by chelating the metal ion oxidation processes, for example, of SH groups catalyzed by free ions.
2. Alkaline phosphates enhance pH of the batter ($\text{pH} > 6.5$) and enhance water binding.
3. Diphosphates act like adenosine triphosphate (ATP) in meat; that is, they dissolve the actin/myosin complex.^{42–44}
4. Phosphates reduce raw batter viscosity by the ATP like action of diphosphates and by this the temperature of comminution in the bowl chopper remains lower.⁴⁵

11.5.4.3.1 Determination of Phosphates

Usually, total phosphate content is determined after ashing and dissolving in acid as monophosphate resp. phosphoric acid. Monophosphates form a color reaction with molybdate (yellow), which can be reduced to molybdeneum blue, which can be measured at 430 nm as phosphomolybdate or at 890 nm as molybdeneum blue (AOAC 972.72).¹² The different condensed phosphate can be detected by the thin layer chromatography (see Chapters 6 and 7).

11.6 Conclusion

Meat products of red meat or poultry contain meat as the main ingredient, which by definition includes muscular tissue, fat, organs (offals), and blood. Meat makes up ~80% of traditional meat products. In most of the heated products water and salt are added. Other ingredients are added in smaller amounts, for example, nuts and vegetables or additions such as nonmeat protein, starch, carragenan, and even smaller amounts of additives like phosphates, citrates, or nitrite/nitrate.

Most meat products are low in carbohydrates.

Calculating the energy value, meat products contain 10–23% protein, with a mean value of 15%, amounting under German conditions to 54 kcal/100 g/day per person. The fat content ranges from 1 to 50% with a mean value of 23%. In Germany, this means about 20 g fat/day per person, equal to 180 kcal/day per person. This adds up to a sum of fat and protein of 234 kcal/day with an intake of 82.2 g of meat products.

Nearly all meat products contain salt (NaCl), from 1.5 to 5.5%. About 830 mg Na is eaten per day per person in Germany by consuming meat products.

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Chapter 12

Essential Amino Acids

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12.1 Introduction

Cured meat processes include intense changes in meat structure that determine not only the development of the characteristic organoleptic properties, but also the improvement of the availability of nutritive compounds. This is the case for amino acids and especially essential amino acids. Indeed, one of the major changes reported during dry-cured ham processing is an intense proteolysis,¹ which could change the amino acid composition, and thus increase the protein digestibility of the meat.²

The quality of a given protein depends on the balance among individual amino acids, and is limited by the content of essential amino acids, which are known as “limiting amino acids.” Meat and meat products are rich in proteins of high biological value due to their essential amino acid content. These amino acids cannot be synthesized *de novo* by the organism, and therefore must be supplied in the diet (in the case of the amino acids valine, leucine, isoleucine, phenylalanine, tryptophan, threonine, methionine, lysine, and histidine). The importance of essential amino acids in nutrition and health makes their analysis highly relevant. The quality of a protein may be evaluated through different methods, such as the amino acid score, the biological value, the net protein utilization, the protein digestibility corrected amino acid score, or the protein efficiency rate (PER).³ For instance, PER values below 1.5 indicate a low-quality protein, between 1.5 and 2.0 intermediate quality, and above 2.0 good quality. Meat proteins have calculated PER values higher than 2.7, indicating high quality, and are considered of high biological value because they contain large amounts of all essential amino acids.^{4,5} There are many methods to evaluate protein digestibility. Most are based on measuring available lysine.^{6,7} This essential amino acid is usually the principal residue made unavailable particularly by early Maillard reactions but also by racemization and cross-linking reactions.⁸ However, in the case of meat, and even more so in meat products, very high free lysine content (500 mg/100 g muscle in dry-cured ham) readily available for absorption without need for further digestion has been reported.² Also, the other essential amino acids largely increase during the curing processes, as shown in Table 12.1.

The distinction between essential and nonessential amino acids is somewhat unclear, as some amino acids, such as tyrosine and cysteine, may be produced from others that are essential amino acids. Arginine is classified as a “semiessential” or “conditionally essential amino acid,” depending on the developmental stage and health status of the individual. Infants cannot effectively synthesize arginine, making it nutritionally essential for them. Adults, however, are able to synthesize arginine from ornithine and citrulline, through the urea cycle. These three amino acids, which are interconvertible, may be considered a single group. Glutamine has also been proposed as a conditionally essential amino acid, due to its importance under exceptional severe stress conditions, such as very intense exercise, infectious disease, surgery, burn injury, or any other acute traumas that might lead to glutamine depletion with consequent immune dysfunction, intestinal problems, and muscle wasting. In all these cases, supplementation with glutamine can be a matter

Table 12.1 Essential Amino Acids Content (mg/100 g w.w.) in Dry-Cured Sausage and Serrano Dry-Cured Ham versus Raw Ham

	<i>Raw Ham</i> ^a	<i>Sausage</i> ^b	<i>Dry-Cured Ham</i> ^a
Valine	4.10 ± 0.50	55.1 ± 0.3	221.4 ± 5.7
Leucine	2.43 ± 0.20	77.5 ± 0.3	246.9 ± 20.4
Isoleucine	1.74 ± 0.21	45.7 ± 0.6	155.7 ± 11.0
Phenylalanine	2.34 ± 0.24	44.9 ± 0.7	137.0 ± 20.2
Tryptophan	1.21 ± 0.21	13.7 ± 0.3	25.7 ± 5.3
Threonine	3.03 ± 0.22	24.3 ± 0.7	193.9 ± 15.3
Methionine	1.44 ± 0.38	18.7 ± 0.4	90.8 ± 8.3
Lysine	3.10 ± 0.70	19.9 ± 0.3	509.0 ± 35.1
Histidine	2.86 ± 0.37	13.5 ± 0.4	120.9 ± 3.6

^a Extracted from Toldrá, F., Aristoy, M.-C., *Int. J. Food Sci. Nut.*, 44, 215–219, 1993.

^b Extracted from Casaburi, A. et al., *Meat Sci.*, 76, 295–307, 2007.

of life or death. Tyrosine is a precursor of the neurotransmitters epinephrine, norepinephrine, and dopamine, all of which are extremely important for brain functions like transmission of nerve impulses and prevention of depression. The essential amino acid phenylalanine is the precursor for tyrosine synthesis, and thus tyrosine becomes essential in the diet of patients suffering from phenylketonuria, who must keep their intake of phenylalanine extremely low to prevent mental retardation and other metabolic complications.

Cysteine is not classified as an essential amino acid because it can usually be synthesized by the human body under normal physiological conditions if sufficient amounts of other sulfur-amino acids (i.e., methionine and homocysteine) are available. For convenience, sulfur-containing amino acids are sometimes considered a single pool of nutritionally equivalent amino acids.

Essential amino acids in meat products can be analyzed following the typical procedures described for amino acids in general.^{10–12} Nevertheless, very careful attention must be paid to some of them, especially when hydrolysis is required. Furthermore, cysteine requires specific methodologies for its analysis. All these methods are described in detail in this chapter.

12.2 Sample Preparation

12.2.1 Free Essential Amino Acids

The analysis of free amino acids goes through three stages—extraction, cleanup or deproteinization, and analysis.

12.2.1.1 Extraction

The extraction consists of the separation of the free amino acid fraction from the insoluble portion of the matrix, in this case from the muscle. It is usually achieved by homogenization of the ground sample in an appropriate solvent. The homogenization is usually achieved by using Stomacher™, Polytron™, or Ultra Turrax™ devices, or by means of a simple stirring in warm solvent. The extraction solvent can be hot water, 0.01–0.1 N hydrochloric acid solution, or diluted phosphate buffers. In some cases, concentrated strong acid solutions such as 4–5% of 5-sulfosalicylic acid,^{13–15} 2–5% of trichloroacetic acid,^{16,17} or rich alcohol-containing solution (>75%) such as ethanol^{18–21} or methanol²² have been successfully used as extraction solvents, with the additional advantage that proteins are not extracted, and therefore there is no need for further cleanup of the sample.

Once homogenized, the solution is centrifuged at least at 10,000 *g* under refrigeration to separate the supernatant from the nonextracted materials (pellet) and filtered through glass-wool to retain any fat material remaining on the surface of the supernatant.

12.2.1.2 Deproteinization

The deproteinization process can be achieved through different chemical or physical procedures. Chemical methods include the use of concentrated strong acids such as sulfosalicylic (SSA),^{14,20,23,24} perchloric (PCA),¹⁸ trichloroacetic (TCA),²⁵ picric,^{26–28} or phosphotungstic (PTA)²⁹ acids, or organic solvents such as methanol, ethanol, or acetonitrile.^{22,29–31} Under these conditions, proteins precipitate by denaturation, whereas free amino acids remain in solution. Physical methods consist

of forced filtration (mainly by centrifugation) through cutoff membrane filters (1,000, 5,000, 10,000, and 30,000 Da) that allow free amino acids through while retaining large compounds.^{20,29,32–34} These methods give a sample solution rich in free amino acids and free of proteins.

Differences among these chemical and physical methods are ascribed to several aspects such as the differences in the recovery of amino acids, compatibility with derivatization (pH, presence of salts, etc.), and separation method (interferences in the chromatogram). Some of these methods, although promising, give low recoveries of some amino acids, as is the case of PTA, which is the most efficient (cutoff is approximately 700 Da), but causes losses of acidic and basic amino acids, especially lysine. The membrane used can also affect amino acid recoveries,³⁰ and thus prewashing of filters is recommended to improve those recoveries.³⁴ It is important to consider that strong acids exert a very low pH in the medium, which can interfere with the precolumn derivatization,³⁴ where high pH is necessary to accomplish the majority of the derivatization reactions. Thus, it is essential either to completely eliminate this acid by evaporation or extraction, or to adjust the pH of the sample solution. This is not a problem when the amino acids have to be analyzed by ion-exchange chromatography and postcolumn derivatization; indeed, SSA has been commonly used before ion-exchange amino acid analysis, because it gives an appropriate pH for the chromatographic separation.¹⁵ Nevertheless, its interference in the chromatographic separation is doubtful,^{35,36} and low recoveries of some amino acids have been reported.²⁹ About 10–12% TCA is normally used to fractionate sausage extracts to study the proteolysis course during ripening, free amino acids being analyzed in the soluble fraction.^{16,37–39} This deproteinizing agent is often used before *o*-Phthaldialdehyde (OPA) derivatization.³⁷ By using 0.6 N PCA, which is easily neutralized by the addition of KOH or potassium bicarbonate, the deproteinization procedure can be very simple, and no interferences have been described.

The use of organic solvents, by mixing 2 or 3 volumes of organic solvent with 1 volume of extract, has given very good results,^{30,35,40} with amino acid recoveries approximately 100% for all them,^{29,40} with the additional advantage of easy evaporation to concentrate the sample. Some comparative studies on these deproteinization techniques have been published.^{29,41,42}

12.2.2 Total Essential Amino Acids

Sample preparation for the analysis of total amino acids includes the hydrolysis of proteins and peptides as a first step. A quantitative hydrolysis may be difficult to achieve for some essential amino acids. The main hydrolysis methods are described below, and some cautions for especially labile amino acids are pointed out.

12.2.2.1 Acid Hydrolysis

Acid hydrolysis is the most common method for hydrolyzing proteins. It consists of acid digestion by constant boiling of 6 N hydrochloric acid in an oven at approximately 110°C for 20–96 h. The hydrolysis must be carried out in sealed vials under nitrogen atmosphere and in the presence of antioxidants/scavengers to minimize the degradation suffered by some especially labile amino acids (tyrosine, threonine, serine, methionine, and tryptophan) in such an acidic and oxidative medium. Phenol (up to 1%) or sodium sulfite (0.1%) are typical protective agents, and are effective for nearly all amino acids except for tryptophan and cysteine. However, considerable tryptophan recoveries have been reported in the presence of phenol when using liquid-phase hydrolysis⁴³ or in the presence of tryptamine when using gas-phase hydrolysis⁴⁴ in the absence of oxygen.

Hydrolysis with hydrochloric acid may also be improved by optimizing the temperature and time of incubation.⁴⁴ Hydrolysis with 4 M methanesulfonic acid (115°C for 22–72 h or 160°C for 45 min, under vacuum) has been preferred for better tryptophan recovery.^{45,46} In this case, the hydrolysis is possible only in the liquid phase, due to the high boiling point of the reagent, and the use of protective reagents such as tryptamine^{47–49} or thioglycolic acid^{50,51} is also advisable to prevent oxidation. Furthermore, nitrogen flush used in this type of hydrolysis significantly increases the recovery of amino acids in general, and especially cysteine, methionine, and tyrosine.

An important fact to consider is the impossibility of evaporating methanesulfonic acid after the hydrolysis. This means that the hydrolyzate can be used for chromatographic analysis only after pH adjustment and dilution. This drawback makes fluorescence the detection of choice because of its higher sensibility. This procedure, which is generally applied to the determination of tryptophan solely, is used in conjunction with the derivatization with Dabsyl-Cl (DABS-Cl)⁴⁸ or 9-Fluorenylmethyl chloroformate (FMOC),⁴⁷ resulting in very good recoveries for all amino acids, including tryptophan. Hydrolysis with 3 M mercaptoethanesulfonic acid at high temperature for a short time (160–170°C for 15–30 min) also improves tryptophan and methionine recoveries.⁵² Cyst(e)ine is partially oxidized during acid hydrolysis, yielding several adducts—cystine, cysteine, cysteine sulfinic acid, and cysteic acid—which makes its analysis rather difficult. Several procedures have been proposed to analyze cyst(e)ine after acid hydrolysis. The previous performic acid oxidation of cysteine to cysteic acid, in which methionine is also oxidized to methionine sulfone,^{53–60} improves cysteine recoveries, making its analysis easier. Methionine can be determined as either methionine without oxidation before acid hydrolysis or as methionine sulfone. Indeed, methionine is quite stable during acid hydrolysis (24 h), with appropriate degassing allowing its analysis along with the “acid stable” amino acids, using a single-acid hydrolysis method without the need for previous methionine oxidation.⁶¹

The use of alkylating agents to stabilize cysteine before or after hydrolysis has been used as a valid alternative. Good recoveries have been reported by using 3-bromopropionic acid,⁶² 3-bromopropylamine,⁶³ iodoacetic acid,⁶⁴ and 3,3'-dithiodipropionic acid.^{34,65–67}

Another problem to take into account for a reliable essential amino acid determination in meat proteins is the remaining intact peptidic bonds left after 24 h hydrolysis time. These peptidic bonds are mainly formed by the essential hydrophobic amino acids, such as valine, leucine, isoleucine, and phenylalanine. They are more resistant to hydrolysis, requiring longer hydrolysis times of up to 96 h that cause some other amino acids to degrade. Many authors^{19,68} have overcome this problem by calculating the averages of data obtained at 24, 48, 72, and 96 h of hydrolysis for valine, leucine, isoleucine, and phenylalanine, and obtaining the data for the most labile amino acids methionine, threonine, and tyrosine from the average of values extrapolated to zero time of hydrolysis. These complex hydrolysis procedures are not practical for the meat industry.

As can be observed in this section, no single set of conditions will yield the accurate determination of all amino acids. In fact, it is a compromise of conditions that offers the best overall estimation for the largest number of amino acids. In general, 22–24-h acid hydrolysis at 110°C (vapor-phase hydrolysis, preferably), with the addition of a protective agent like phenol, yields acceptable results for the majority of essential amino acids, meeting the requirements of any food control laboratory. However, when the analysis of tryptophan or cyst(e)ine is necessary, special hydrolysis procedures, such as those described earlier, should be performed. When high sensitivity is required, pyrolysis at 500°C for 3 h⁶⁹ to 600°C overnight⁴⁸ of all glass material in contact with the sample is advisable, as well as the analysis of some blank samples to control the level of background interference present. The optimization of conditions, based on the study of hydrolysis time and temperature, acid-to-protein ratio, presence and concentration of oxidation protective agents, and importance of correct deaeration, has been extensively reported in manuscripts.^{70–75}

12.2.2.2 *Alkaline Hydrolysis*

The alkaline hydrolysis with 4.2 M of either NaOH, KOH, LiOH, or BaOH, with or without the addition of 1% (w/v) thioglycol for 18 h at 110°C, is recommended by some authors^{48,55,76–79} for a better tryptophan determination. This would be the method of choice in food samples containing high sugar concentration, but this is not the case of meat products.

12.2.2.3 *Enzymatic Hydrolysis*

Enzymatic hydrolysis with proteolytic enzymes, such as trypsin, chymotrypsin, carboxypeptidase, papain, thermolysin, and pronase, has been used to analyze specific amino acid sequences or single amino acids because of their specific and well-defined activity. By using this method, tryptophan content was analyzed in soy- and milk-based nutritional products by enzymatic (pronase) digestion of the protein to release tryptophan, which was further analyzed by isocratic reversed-phase liquid chromatography with fluorescence detection. Enzymatic digestion was completed in less than 6 h, and was accomplished under chemically mild conditions (pH 8.5, 50°C), which did not significantly degrade tryptophan.⁸⁰ Although promising, this method has not been applied to meat samples.

12.3 Analysis

After sample preparation, target essential amino acids may be analyzed either by direct spectrophotometric or by chromatographic (high-performance liquid chromatography [HPLC] or gas-liquid chromatography [GLC]) methods.

12.3.1 *Direct Spectrophotometric Methods*

Direct determinations of tryptophan without separation or even without hydrolysis of the sample are based on the acid ninhydrin method⁸¹ or on the direct measurement of the tryptophan fourth-derivative ultraviolet (UV) absorption spectrum.⁸²

During the acid hydrolysis used in amino acid analysis, some of the essential amino acids that are blocked in their native proteins revert back to the parent amino acid, leading to errors in estimates of both the amino acid content of foods and amino acid digestibility. This is a particular concern for the amino acid lysine in damaged food proteins. To overcome this fact, methods for analyzing free NH₂-lys residues that do not require the previous sample hydrolysis, have been developed. These methods use trinitrobenzenesulfonic acid^{83–85} or OPA as derivatizing reagents,⁸⁶ with significant advantages when compared to the longer and more tedious method consisting of the hydrolysis of proteins and subsequent analysis of the free lysine. This analysis, which is very often used in cereals, is not usual in meat, where lysine is not a limiting amino acid.^{83,87}

12.3.2 *Chromatographic Methods*

The separation of the individual essential amino acids in a mixture requires very efficient separation techniques, such as chromatography (liquid or gas chromatography) or capillary electrophoresis. The choice mainly depends on the available equipment or personal preferences, because each methodology has its advantages and drawbacks.

12.3.2.1 High-Performance Liquid Chromatography

HPLC is the most versatile and widespread technique to separate amino acids. Before or after this separation, amino acids are derivatized to allow their separation or to enhance their detection.

12.3.2.1.1 Derivatization

Derivatization is a common practice in the amino acid analysis. The effectiveness of a derivatizing agent is evaluated based on the following aspects: it must be able to react with both primary and secondary amino acids, give a quantitative and reproducible reaction, yield a single derivative of each amino acid, have mild and simple reaction conditions, allow the possibility of automation, provide good stability of the derivatization products, and be free of interference due to by-products or excess of reagent. The use of sufficient derivatization reagent is of special importance when dealing with biological samples since reagent-consuming amines, although unidentified, are always present.¹⁵

Some reports comparing amino acid derivatization methods for HPLC^{10,30,36,88,89} analysis of biological samples have been published. Pre- or postcolumn derivatization reagents used in the analysis of free amino acids are also useful for essential amino acids, with some exceptions. Essential amino acids such as histidine, lysine, tryptophan, and cysteine present some difficulties. The most used derivatization methods are described as follows:

Ninhydrin. This is the most used postcolumn derivatization reagent after amino acid cation exchange chromatographic analysis. The reaction takes place at high temperature (at pH 6) and renders colored derivatives detectable at 570 (primary amino acids) and 440 nm (secondary amino acids).

4-Dimethyl-aminoazobenzene-4'-sulfonyl chloride (dabsyl-Cl). This reagent forms stable (for weeks) derivatives with primary and secondary amino acids that are detectable in the visible range, presenting a maximum from 448 to 468 nm. The high wavelength of absorption makes the baseline chromatogram very stable, with a large variety of solvents and gradient systems. Detection limits are in the low picomole range.⁴⁸ The reaction time is approximately 15 min at 70°C. Reaction takes place in a basic medium with an excess of reagent. The major disadvantage is that the reaction efficiency is highly matrix dependent and variable for different amino acids, being especially affected by the presence of high levels of some chloride salts.⁴⁰ To overcome this problem and obtain an accurate calibration, standard amino acid solution should be derivatized under similar conditions. By-products originating from an excess of reagent absorb at the same wavelength and appear in the chromatogram. Nevertheless, Stocchi et al.⁴⁸ obtained a good separation of 35 DABS-amino acids and by-products in a 15-cm C18 column packed with 3- μ m particles.

Phenylisothiocyanate (PITC). The methodology involves the conversion of primary and secondary amino acids to their phenylthiocarbamyl (PTC) derivatives, which are detectable at UV (254 nm). The PTC-amino acids are moderately stable at room temperature for 1 day, and much longer in the freezer especially when dry. The methodology is well described in the literature.^{33,90,91} Sample preparation is quite laborious; it requires a basic medium (pH = 10.5) with triethylamine, and includes several drying steps, the last one being necessary to eliminate the excess of reagent, which may cause some damage to the chromatographic column. Twenty minutes of reaction time at room temperature is recommended for a complete reaction. The chromatographic separation takes approximately 20 min for hydrolyzed amino acids and 65 min for physiological amino acids.

The reproducibility of the method is very good, ranging from 2.6 to 5.5% for all amino acids except for histidine (6.3%) and cystine (10%). PTC-cystine shows a poor linearity that makes the quantitation of free cystine unfeasible with this method.⁸⁹ Detection limits are in the high picomole range. The selection of the column is critical for producing a well resolved separation, especially when the analysis of physiological amino acids is involved.

Nowadays, PTC is one of the preferred precolumn derivatizing agents for analyzing physiological amino acids from meat and meat products. The chromatograms of PTC-amino acids from dry-cured ham and sausage are shown in Figures 12.1 and 12.2, respectively.

The reliability of the method has been tested on food samples⁹² and compared with traditional ion-exchange chromatography and postcolumn derivatization.^{41,91,93}

1-Dimethylamino-naphthalene-5-sulfonyl chloride. 1-Dimethylamino-naphthalene-5-sulfonyl chloride (dansyl-Cl) reacts with both primary and secondary amines to give a highly fluorescent derivative (λ_{ex} 350 nm, λ_{em} 510 nm). The dansylated amino acids are stable for 7 days at -4°C ,⁹⁴ if protected from light. The sample derivatization appears simple, only requiring a basic pH of approximately 9.5, and a reaction time of 1 h at room temperature (in the dark), or 15 min at 60°C (132), or 2 min at 100°C . However, the reaction conditions (pH, temperature, and excess of reagent) must be carefully fixed to optimize the product yield and to minimize secondary reactions.^{94,95} Even so, this will commonly form multiple derivatives with histidine, lysine, and

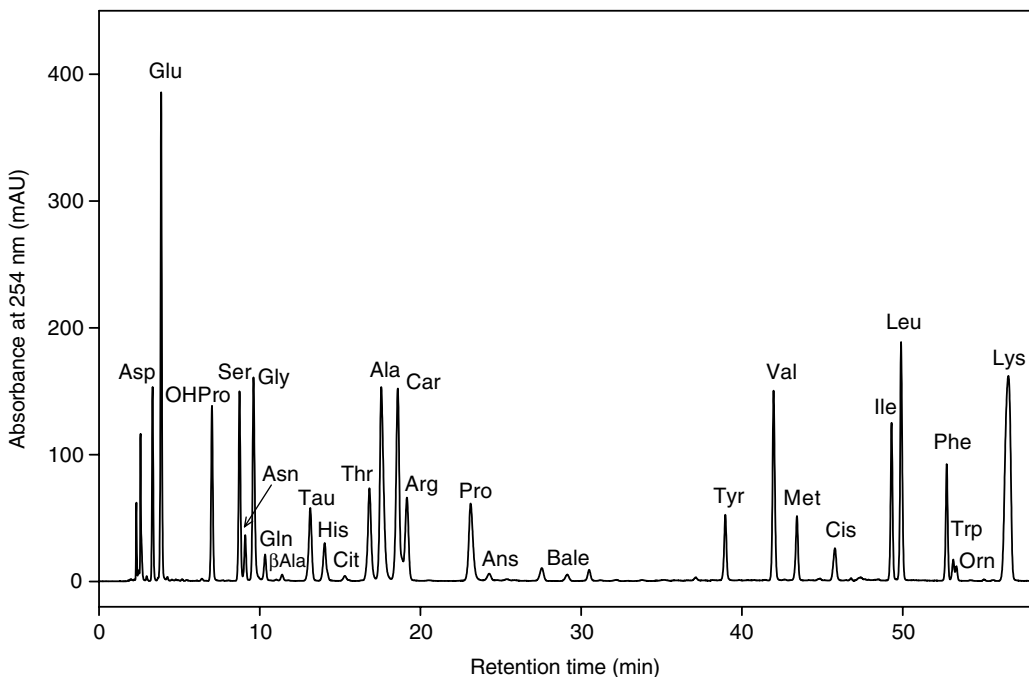


Figure 12.1 RP-HPLC chromatogram of a dry-cured ham extract after PTC derivatization of free amino acids. Asp, aspartic acid; Glu, glutamic acid; OHPPro, hydroxyproline; Ser, serine; Asn, asparagine; Gly, glycine; Gln, glutamine; β -Ala, β -alanine; Tau, taurine; His, histidine; Cit, citrulline; Thr, threonine; Ala, alanine; Car, carnosine; Arg, arginine; Pro, proline; Ans, anserine; Bale, balenine; Tyr, tyrosine; Val, valine; Met, methionine; Cis, cysteine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Trp, tryptophan; Orn, ornithine; Lys, lysine.

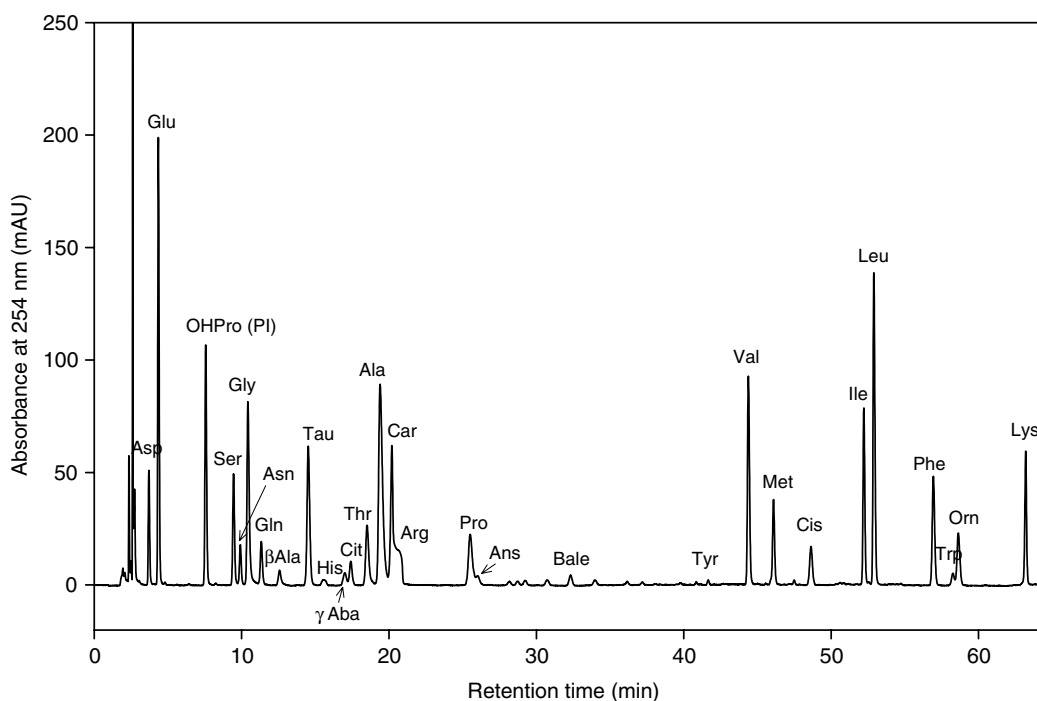


Figure 12.2 RP-HPLC chromatogram of a sausage extract after PITC derivatization of free amino acids. (Abbreviations as in Figure 12.1. γ Aba, γ -amino butyric acid.)

tyrosine. Histidine gives a very poor fluorescence response (10% of the other amino acids), reinforcing the poor reproducibility of its results.⁸⁹ Another problem is that the excess of reagent (needed to assure a quantitative reaction) is hydrolyzed to dansyl sulfonic acid, which is highly fluorescent and probably interferes in the chromatogram, manifesting as a large spike. On the contrary, this methodology reveals excellent linearity for cystine and also cystine-containing short chain peptides.^{89,96,97} This derivative has also been used to analyze taurine.⁹⁸

o-Phthaldialdehyde. This reagent reacts with primary amino acids in the presence of a mercaptan cofactor to give a highly fluorescent adduct. The fluorescence is recorded at 455 or 470 nm after excitation at 230 or 330 nm, respectively, and the reagent itself is not fluorescent. OPA derivatives can be detected by UV absorption (338 nm) as well. It may be used either for pre- or postcolumn derivatization. This last used to be coupled with cation exchange HPLC.^{50,51} The choice of mercaptan (2-mercaptoethanol, ethanethiol, or 3-mercaptpropionic acid) can affect derivative stability, chromatographic selectivity, and fluorescent intensity.^{15,50,51,99,100} The derivatization is fast (1–3 min) and is performed at room temperature in alkaline buffer, pH 9.5. OPA amino acids are not stable; this problem is overcome by standardizing the time between sample derivatization and column injection by automation. The major disadvantage when applying to essential amino acids is that the yield with lysine and cysteine is low and variable. The addition of detergents like Brij 35 to the derivatization reagent seems to increase the fluorescence response of lysine.^{101–103} Routine quantification of cystine is impossible with OPA, due to the formation of a derivative with minimal fluorescence,

and several methods have been proposed before derivatization. These methods include the conversion of cysteine and cystine to cysteic acid by oxidation with performic acid (see Section 12.2.2.1), carboxymethylation¹⁰⁴ of the sulfhydryl residues with iodoacetic acid,⁶⁴ or the formation of the mixed disulfide *S*-2-carboxyethylthiocysteine from cysteine and cystine, using 3,3'-dithiodipropionic acid¹⁰⁵ and incorporated by Godel et al.¹⁰⁶ into the automatic sample preparation protocol described by Schuster.³⁵ In these methods, cysteine and cystine are quantified together. Another proposal¹⁰⁷ consists of a slight modification in the OPA derivatization method by using 2-aminoethanol as a nucleophilic agent and altering the order of the addition of reagents in the automated derivatization procedure.³⁵

6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reacts with primary and secondary amines from amino acids, yielding very stable derivatives (for 1 week at room temperature) with fluorescent properties (λ_{ex} 250 nm; λ_{em} 395 nm). UV detection (254 nm) may also be used. Sensitivity is in the femtomole range. The main advantage of this reagent is that the yield and reproducibility of the derivatization reaction is scarcely interfered with by the presence of salts, detergents, lipids, and other compounds naturally occurring in meat products. Furthermore, the optimum pH for the reaction is in a broad range, from 8.2 to 10, which facilitates sample preparation. The excess of reagent is consumed during the reaction to form aminoquinoline, which is only weakly fluorescent at the amino acid derivatives detection conditions, and does not interfere in the chromatogram. Reaction time is short, 1 min, but 10 min at 55°C would be necessary if tyrosine monoderivative is required, because both mono- and diderivatives are the initial adducts from tyrosine. Fluorescence of tryptophan derivative is very poor, and UV detection at 254 nm may be used to analyze it. In this case, the AMQ peak appears very big at the beginning of the chromatogram, and may interfere with the first eluting peak (see Ref. 109). The chromatographic separation of these derivatives has been optimized for the amino acids from hydrolyzed proteins but, the resolution of physiological amino acids is still incomplete, and needs to be improved,¹⁰⁸ which is the main drawback of this method. Figure 12.3 shows a chromatogram of the AQC amino acids from hydrolyzed chicken meat.

Cysteic acid and methionine sulfone, which are the adducts after performic acid oxidation of cystine/cysteine and methionine, respectively, are well separated inside the chromatogram.¹⁰⁹

Some special derivatives are also proposed to determine cyst(e)ine.

7-Halogenated-4-nitrobenzo-2-oxa-1,3-diazoles can be used in the quantitative estimation of thiols and amines. For instance, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole has been used for the analysis of cysteine and cystine in foods by Akinyele et al.⁵³ This reagent reacts with cyst(e)ine in acidic medium (0.2 M sodium acetate/HCl buffer, pH 2.0), giving a greenish product that shows a maximum of absorbance at 410 nm. This method is highly specific for cysteine, and does not need a posterior chromatographic separation.

5,5'-Dithio-bis-nitrobenzoic acid is used for the precolumn derivatization of sulfhydryl and disulfide amino acids.¹¹⁰

Fluorescamine, which produces fluorescent derivatives with primary amino acids, has been used in precolumn derivatization of taurine. The column (RP-column) eluent was monitored at 480 nm (emission) after excitation at 400 nm.¹¹¹

Table 12.2 presents some disposable commercial kits and key literature citations pertaining to the application of the most common reverse-phase-HPLC (RP-HPLC) amino acid derivatization methods for free amino acids.

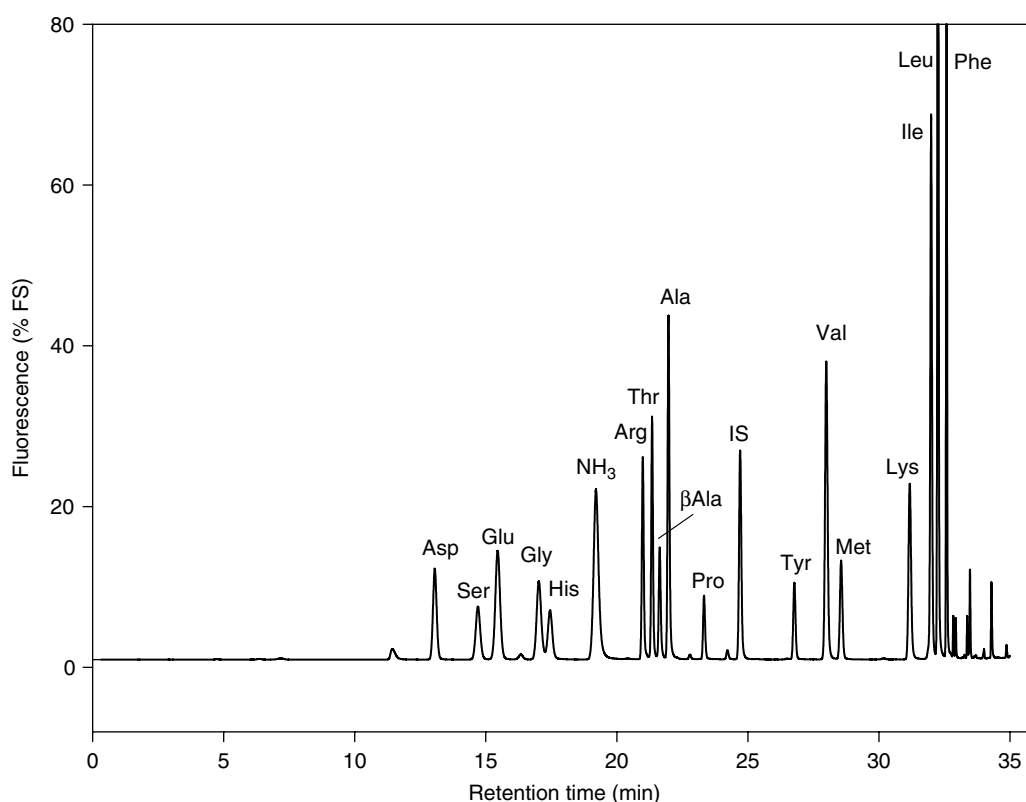


Figure 12.3 RP-HPLC chromatogram of a hydrolyzed chicken meat after AQC-derivatization. IS, internal standard (α -amino butyric acid).

Table 12.2 Selected Interesting Applications and Disposable Commercial Kits of Some Amino Acid Derivative Reagents for RP-HPLC

Reagent	Commercial Kit	Detection	Reference
PITC	Pico-Tag (Waters Associates, Milford, Massachusetts)	UV	20,29–31,33,92,112,113
OPA	AutoTag OPA (Waters Associates, Milford, Massachusetts) AminoQuant (Agilent Technologies, Palo Alto, California)	FI/UV	14,15,22,35,37,71,114
AQC	AccQ Tag (Waters Associates, Milford, Massachusetts)	FI/UV	108,109,115
Dansyl-Cl		FI/UV	97,108,109,115
Dabsyl-Cl	System Gold/Dabsylation Kit (Beckman Instruments, Fullerton, California)	Vis	34,40,69

Note: PITC, phenylisothiocyanate; OPA, *o*-phthalaldehyde; AQC, 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate; dansyl-Cl, 1-dimethylamino-naphthalene-5-sulfonyl chloride; dabsyl-Cl, 4-dimethyl-aminoazobenzene-4'-sulfonyl chloride; and FI, fluorescence.

12.3.2.1.2 Separation and Detection

The HPLC separation techniques most often used for the analysis of essential amino acids are cation exchange–HPLC (CE–HPLC) and RP–HPLC. CE–HPLC is used for the separation of nonderivatized amino acids, which are then derivatized postcolumn (ninhydrin or OPA), whereas RP–HPLC is mainly used to separate precolumn derivatized amino acids (see the earlier described reagents). The choice of the RP column is essential for sufficient separation, because many peaks appear in the chromatogram, specially in the analysis of physiologic amino acids. In the case of hydrolyzates, the sample is simpler, and the use of shorter columns is advisable to reduce the time of analysis. RP–HPLC has also been used to separate some underivatized amino acids, such as methionine, which is further detected at 214 nm,¹¹⁶ and the aromatic amino acids Tyr, Phe, and Trp, which can be detected at 214 nm but also at 260 or 280 nm. Indeed, Phe presents a maximum of absorption at 260 nm, Tyr at 274.6, and Trp at 280 nm. A chromatogram of Tyr, Phe, and Trp from a dry-cured ham extract is shown in Figure 12.4. The separation was achieved by using a gradient between 0.1% trifluoroacetic acid (TFA) in water and 0.08% TFA in acetonitrile:water (60:40). Absorption spectra from these amino acids are also shown in Figure 12.4.

For the rest of amino acids, the detector used depends on the chosen derivative, but it is worthwhile to take into account the earlier section on derivatization (Section 12.3.2.1.1), because certain derivatives from some specific amino acids elicit a poor response.

There are several different techniques for the analysis of any amino acid. An example is tryptophan, which was analyzed by CE-HPLC with postcolumn derivatization with OPA and

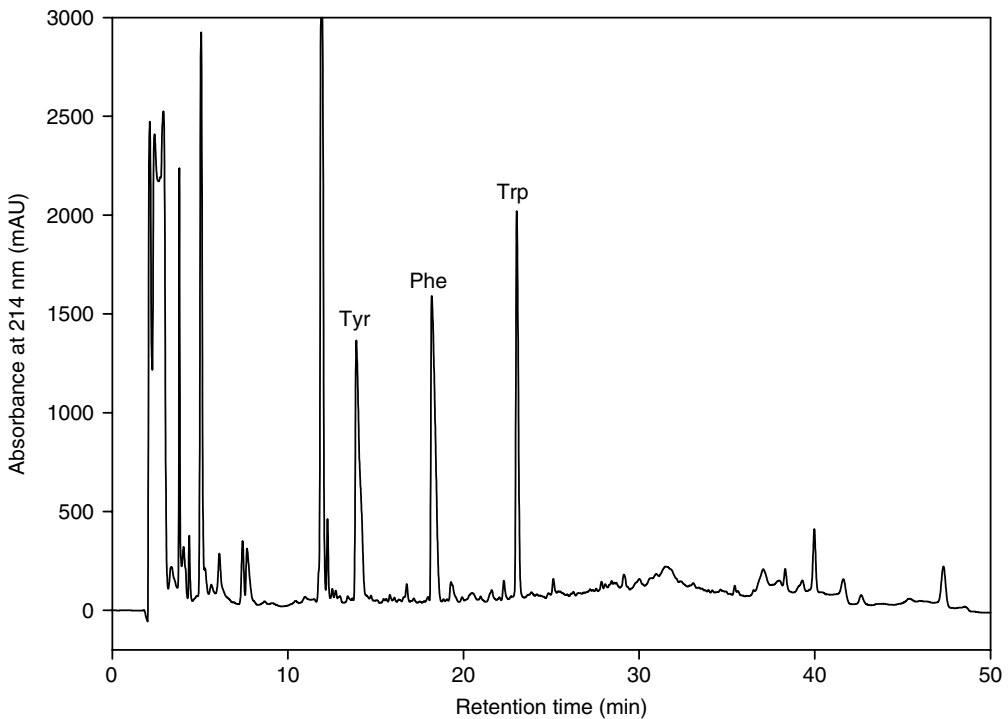


Figure 12.4 RP–HPLC chromatogram of a deproteinized dry-cured ham. (Abbreviations as in Figure 12.1.)

fluorescence detection,¹¹⁷ by RP–HPLC without derivatization and with UV or fluorescence detection,¹¹⁸ or even by RP–HPLC with precolumn derivatization (see Section 12.3.2.1.1).

12.3.2.2 Gas–Liquid Chromatography

GLC technique is in general not recommended for some essential amino acids, such as cysteine, tryptophane, or methionine. Nevertheless, a method of analysis for tryptophan in proteins based on the GLC separation of skatole produced by pyrolysis of tryptophan at 850°C was developed by Danielson and Rogers.¹¹⁹ Sample pretreatment for this method is limited to only sample lyophilization to form a dry solid, and hydrolysis is not required.

General GLC methods to analyze amino acids include their previous derivatization to enhance volatility and thermal stability and thus improve their chromatographic behavior. A main drawback is the different derivatives and derivatization conditions needed to accomplish a single derivative for each essential amino acid.¹²⁰ Proposals include the kit offered by Supelco (Sigma-Aldrich, Bellefonte, PA) that uses *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTB-STFA) as derivatizing reagent and a short (20 m) capillary column (they give the conditions to separate 24 amino acids in 8 min), and the EZ:faast™ (Phenomenex, Torrance, California) method for which a patent is pending, to analyze protein hydrolyzates and physiological amino acids from serum, urine, beer, wine, feeds, fermentation broths, and foodstuffs. This method includes a derivatization reaction (proprietary) in which both the amine and carboxyl groups of amino acids are derivatized. Derivatives are stable for up to 1 day at room temperature and for several days if refrigerated, and are further analyzed by GLC with flame ionization detection, or by GLC or LC with mass spectrometry detection. Results (50 amino acids and related compounds) are obtained in about 15 min (sample preparation included) when using the GC method or 24 min by using the LC method. Applications of both methods in meats have not yet been described.

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Chapter 13

Omega-3 and Trans Fatty Acids

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13.1 Nomenclature and Classification of Fatty Acids

The systematic nomenclature of fatty acids is based on the number of carbon atoms in the hydrocarbon chain and on the number and position of double bonds relative to carboxyl group(s). Substituted groups and their positions and geometric configuration at double bonds are designated.¹ Fatty acids are referred as derivatives of hydrocarbons of the same number of carbon atoms, in which the final letter “e” of the hydrocarbons is substituted by “anoic” for the saturated and “enoic” for unsaturated fatty acids. For example, octadecane becomes octadecanoic acid (saturated) or octadecenoic acid (unsaturated). The Greek letter Δ (delta), followed by one or more numbers, is used to designate the presence and the position of one or more double bonds, counting from the carboxyl group. The double bond positions are designated with numbers before the fatty acid name (e.g., $\Delta 9,12,15$ -octadecatrienoic acid, or simply 9,12,15-octadecatrienoic acid, or 9,12,15-18:3). Conversely, the Greek letter ω or the letter *n* are used to indicate the position of the first double bond counting from the terminal methyl group of the molecule. Thus, $\Delta 9$ -octadecenoic acid becomes 18:1 ω -9 or 18:1*n*-9; $\Delta 9,12$ -octadecadienoic acid becomes 18:2 ω -6 or 18:2*n*-6; $\Delta 9,12,15$ -octadecatrienoic acid becomes 18:3 ω -3 or 18:3*n*-3. The “ ω ” or “*n*” nomenclature has been introduced as a convenient way to express the metabolic conversions between fatty acid families. This terminology involves two assumptions.² First, all double bonds are in *cis* configuration and, second, if more than one double bond is present in the molecule, all double bonds are separated by a methylene ($-\text{CH}_2-$) group; in this case, double bonds are referred as *methylene interrupted*. An example of a methylene interrupted fatty acid is linoleic acid (18:2*n*-6; $\Delta 9,12$ -octadecadienoic acid).

The double bonds of an unsaturated fatty acid may be separated only by a carbon–carbon bond; in this case the fatty acids are referred to as *conjugated*. Among fatty acids with conjugate bonds isolated from ruminant fats, conjugated linoleic acids (CLAs) have aroused special interest in recent years since they have been linked to a multitude of metabolic effects.^{3,4} An example of a CLA is *c9,t11*-octadecadienoic acid (rumenic acid⁵). A trivial nomenclature, which consists of assigned common names usually based on the main source in nature, is also widely used. Examples include the names oleic acid (olive oil), palmitic acid (palm oil), nervonic acid (nervous cell membranes), and many others.

The naturally occurring fatty acids can be grouped on the basis of the absence or presence of double or triple bonds within the hydrocarbon chain in two wide classes called saturated fatty acids (SFAs) and unsaturated fatty acids. Most of the SFAs have an unbranched structure and an odd number of carbon atoms, generally from 12 to 24 in animal lipids, with the exception of milk lipids. The unsaturated fatty acids contain one or more double or triple bonds and can be divided into monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and acetylenic fatty acids. The terms highly unsaturated fatty acids (HUFAs) or long chain polyunsaturated fatty acids (LC-PUFAs) are sometimes used to identify fatty acids with 20 or more carbon atoms and four or more double bonds in the hydrocarbon chain. Theoretically, the double bonds can be located at any position along the hydrocarbon chain, resulting in different positional isomers. For more extensive information about classification and nomenclature of fatty acids and lipids, the reader is referred to Fahy,⁶ Lobb,⁷ and Robinson.⁸

13.1.1 Omega-3 Fatty Acids

PUFAs are grouped in classes, or families, depending on the position of the first double bond counting from the terminal methyl carbon. In the omega-3 (also known as *n*-3) family, the unsaturation starts on the third carbon atom counting from the methyl end group.

Omega-3 fatty acids, in particular α -linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), are generally known as essential fatty acids (EFA) in mammals.⁹ This designation is reserved for those fatty acids that are required for good health. In addition, they cannot be completely synthesized *de novo* in the body and consequently must be supplied by the diet. The last two decades have seen an exponential increase of interest in the health effects of omega-3 fatty acids. Several excellent reviews are available on their physiological roles and functions,^{10,11} on their tissue distribution in humans,¹² on their structural and functional role in cellular membranes,¹³ on their role in gestation and parturition,¹⁴ inflammation,¹⁵ immune response and autoimmunity,¹⁶ cortical and retinal development,^{17–19} cardiovascular disease,^{20–22} cancer,^{23–25} and cellular lifespan.²⁶

ALA is naturally found mainly in plants, where it is synthesized by a sequential desaturation of oleic acid and linoleic acid, being the first synthesized *de novo* from acetate.²⁷ In fact, many plants and algae are able to convert oleic acid (n-9) in the form of a phosphatidylcholine to linoleic acid (n-6), which is converted to ALA (n-3) as its monogalactosyldiacylglycerol derivative.²⁸ This process takes place through the action of a $\Delta 12$ and a $\Delta 15$ desaturase, respectively. By contrast, humans and animals cannot interconvert n-3 and n-6 fatty acids. In animals, except for lions and cats,²⁹ the long-chain polyene acids of the omega-3 series are biosynthesized through a combination of elongation and desaturation reactions, starting from ALA. These processes are particularly important in human or in animal systems and are required for the production of C20 and C22 PUFAs, as precursors of biologically active eicosanoids.³⁰

The elongation and desaturation pathways whereby C18 is converted to its C20 and C22 counterparts is reasonably understood and is presented in Figure 13.1. To summarize, DHA is the principal end product of elongation and desaturation of ALA, with 18:4n-3, 20:4n-3, EPA, and DPA (22:5n-3, docosapentaenoic acid) being the intermediate fatty acids in this pathway. The 20:4n-6 (arachidonic acid, ARA) is the main end product of elongation and desaturation of 18:2n-6, with 18:3n-6 and 20:3n-6 as the intermediate fatty acids in this biosynthesis. The amount of the intermediate fatty acids and end products of this biosynthesis in animal lipids depends largely on the availability of the precursor C18 fatty acids in the diet, on the elongation/desaturation ability of the organism, and on the presence of LC-PUFAs in the diet.

The reactions illustrated in Figure 13.1, with the exception of the oxidation of 24:6n-3 and 24:5n-6, which takes place in peroxisomes, occur in the endoplasmic reticulum, and the same enzyme acts on the n-3, n-6, and n-9 families. However, the binding affinity of the $\Delta 6$ -desaturase is highest for ALA, high for linoleic acid, and lowest for oleic acid. This means that desaturation and elongation of oleic acid is only observed when a combined n-3 and n-6 PUFA deficiency occurs in the diet, and that the $\Delta 6$ desaturation is the rate-limiting step in the metabolism of both n-3 and n-6 PUFAs. Therefore, there is a potential competition between 18:3n-3 and 18:2n-6 fatty acids in the synthesis of LC-PUFAs. Humans and animals are able to convert ALA into EPA and DHA, but the capacity of this conversion is limited, especially when a diet with an excess of linoleic acid is consumed.³¹

In a recent review, Sprecher³² hypothesized the participation of two different 18- and 24-carbon chain length $\Delta 6$ desaturases in DHA synthesis, but this hypothesis remains to be confirmed. It is worth mentioning that Cunnane et al.³³ have demonstrated that rats are able to synthesize longer-chain fatty acids from hexadecadienoic acid (16:2n-6) and hexadecatrienoic acid (16:3n-3), two common fatty acids in green leafy vegetables. These authors have argued that, if rats and humans metabolize hexadecadienoate and hexadecatrienoate at a similar rate, about 3–4% of the body's 18:3n-3 and less than 1% of 18:2n-6 could be synthesized from their 16 carbon counterparts in humans.

n-3 Pathway	n-6 Pathway	n-9 Pathway
9,12,15-18:3 (ω -Linolenic acid, ALA)	9,12-18:2 (Linoleic acid, LA)	9-18:1 (Oleic acid)
↓ $\Delta 16$ Desaturase	↓ $\Delta 16$ Desaturase	↓ $\Delta 16$ Desaturase
6,9,12,15-18:4 (Stearidonic acid)	6,9,12-18:3 (γ -Linolenic acid, GLA)	6,9-18:2
↓ Elongase	↓ Elongase	↓ Elongase
8,11,14,17-20:4n-3	8,11,14-20:3 (Dihomo- γ -linolenic acid, DGLA)	8,11-20:2n-9
↓ $\Delta 15$ Desaturase	↓ $\Delta 15$ Desaturase	↓ $\Delta 15$ Desaturase
5,8,11,14,17-20:5 (Eicosapentaenoic acid, EPA)	5,8,11,14-20:4 (Arachidonic acid, AA)	5,8,11-20:3n-9
↓ Elongase	↓ Elongase	
7,10,13,16,19-22:5 (Docosapentaenoic acid, DPA)	7,10,13,16-22:4 (Adrenic acid)	
↓ Elongase	↓ Elongase	
9,12,15,18,21-24:5	9,12,15,18-24:4	
↓ $\Delta 16$ Desaturase	↓ $\Delta 16$ Desaturase	
6,9,12,15,18,21-24:6	6,9,12,15,18-24:5	
↓ Peroxisomal oxidation	↓ Peroxisomal oxidation	
4,7,10,13,16,19-22:6 (Docosaesahexaenoic acid, DHA)	4,7,10,13,16-22:5	

Figure 13.1 Pathways of biosynthesis of C20 and C22 PUFA from n-3, n-6, and n-9 C18 precursors.

13.1.2 Trans Fatty Acids

A double bond in the hydrocarbon chain can have two possible geometric configurations, depending on the position of atoms or groups connected to doubly bonded atoms. Atoms or groups are in cis or trans configuration if they lie on the same or on the opposite side of a reference plane of the molecule, respectively. Using the systematic nomenclature, the prefix cis (*c*) or trans (*t*) precedes the position of double bonds.

The double bond is, in the cis configuration, asymmetric and so forces a bend into the carbon chain. As a result, the unsaturated fatty acids are unable to pack closely together, or to crystallize readily as straight-chain SFAs. This is why unsaturated oils are mostly liquid at room temperature, while more saturated fats are solid. A trans double bond does not form a sharp angle, and the carbon chain forms a straight line similar to that of an SFA, but with a small kink at the double bond site. Consequently, the trans isomer of a given fatty acid will always have a higher melting point than the cis isomer, but lower than the corresponding SFA. As a matter of fact, the physicochemical, biological, biochemical, and nutritional properties of trans fatty acids are different from those of the cis isomers.³⁴ There is some discussion concerning the definition of trans fatty acid for purposes of food labeling.

From a purely chemical point of view, a trans fatty acid is an unsaturated fatty acid that has one or more double bonds in the trans configuration. Following this definition, the European Food Safety Authority (EFSA) stated that “TFA are unsaturated fatty acids that have at least one

double bond in the trans configuration.”³⁵ However, the U.S. Food and Drug Administration³⁶ (FDA) defined trans fatty acids as “the sum of all the fatty acids with at least one nonconjugated double bond in the trans configuration.”³⁷ Thus, the main question under debate is whether or not to exclude trans CLAs from the definition of trans fatty acid.³⁸ The reader is referred to the book edited by Yurawecz et al.³⁹ for exhaustive information on conjugated linoleic acids.

13.2 Sources of Omega-3 Fatty Acids

Among n-3 fatty acids, ALA is found mainly in triglycerides of certain oily seeds, such as linseed (flax seed) and rapeseed (canola), in nuts (walnut),^{40,41} and in green leafy vegetables,⁴² mainly in glycolipids of chloroplast membranes. In plants this fatty acid is thought to be involved in important metabolic functions, as a precursor of a plant growth regulator and other active signaling compounds.⁴³ Pereira et al.⁴² analyzed 11 types of green vegetables and found a proportion of n-3 PUFAs ranging from 59 to 65% of total fatty acids.

In mammal, fish, and bird tissues ALA is found mainly in triglycerides and cholesteryl esters, while EPA and DHA are localized in triglycerides, cholesteryl esters, and phospholipids.

An important question is whether dietary intake of ALA can provide sufficient amounts of tissue EPA and DHA in animals and humans by conversion through the desaturation/elongation pathways. In other words, the main discussion is whether the true essentiality of n-3 fatty acids resides with ALA or with its long-chain derivatives, EPA and DHA.¹⁰

Long-chain n-3 PUFAs such as EPA and DHA are particularly abundant in the marine food web.⁴⁴ Most fish species living in natural conditions are well supplied with these FA, which originate from the marine algae that constitute the phytoplankton. Thus, fish oil is the main source of long-chain n-3 PUFAs, but the use of this ingredient has raised concerns over its sustainability due to limited supplies.⁴⁵ Consequently, great emphasis has been directed toward the identification of alternative, economically and ecologically sustainable production of other n-3 sources, such as algae, krill oil, or transgenic plants that synthesize n-3 PUFAs.⁴⁴ Since the early 1980s microalgae have been grown in outdoor ponds or in appropriate reactors for the production of biomass containing long-chain n-3 PUFAs. Today there are a number of patented processes designed to grow algae.

Recently, the metabolic engineering of plants to produce sources of n-3 PUFAs has been extensively investigated.^{46,47} Also the creation of transgenic pigs with high levels of n-3 fatty acids in tissues⁴⁸ has been attempted.

13.3 Sources of Trans Fatty Acids

There are three major sources of trans fatty acids in food items. The first source is the partial hydrogenation of vegetable oils (PHVO) and partial hydrogenation of marine oils (PHMO), the second is processing of edible oils at high temperatures, and the third is natural occurrence in ruminant meat and dairy products. Hydrogenation was first industrially applied to whale oil in 1903.^{49,50} The hydrogenation reaction consists of the addition of hydrogen atoms to the fatty double bonds in the presence of a catalyst.⁵¹ Currently it is generally applied by the food industry to vegetable oils, such as soybean or cottonseed oil, for two main purposes: to improve the oxidative stability of fats and to produce semisolid edible fats with better organoleptic and spreadability properties.

The major food items prepared with hydrogenated fats as ingredients are bread, crackers, pastries, dressings, and cookies. During catalytic hydrogenation, both positional and geometrical isomerization occur. Positional isomerization refers to the migration of the position

of the double bond within the hydrocarbon chain. The hydrogenation process has to be carefully controlled and depends on the appropriate selection of the processing parameters, including the type and level of catalyst, fatty acid composition of the native oil, hydrogen pressure, agitation rate, time, and temperature of reaction. When there are sufficient hydrogen atoms to cover the catalyst, the double bond opens and hydrogen atoms are added to the carbon atoms at either side, converting the unsaturated into a saturated bond. Conversely, when the catalyst has insufficient hydrogen atoms, a hydrogen atom could be removed from either side of the partially saturated bond, producing a new double bond within the chain, which may form in its original position or may be moved one carbon away, either up or down in the chain. These new double bonds could have either *cis* or *trans* configuration. Partially hydrogenated vegetable oils have a complex fatty acid profile in which *trans*-octadecenoic acid predominates. The distribution of positional and geometrical isomers of margarine, shortenings, cookies, and dietary fats from vegetable oils have been extensively studied by several authors.^{52,53}

The second possible source of *trans* fatty acid is the refining and heating treatment of edible oils,^{54,55} or from the frying process.⁵⁶ Both vegetable and fish oils must undergo refining steps before their utilization. Refining is usually divided in four steps: degumming, neutralization, bleaching, and deodorization. This last step is particularly critical; it involves high temperatures (180–270°C) and could lead to the formation of degradation products, including polymers, cyclic fatty acids, and geometrical isomers.⁵⁷ Liu et al.⁵⁸ investigated the formation of *trans* fatty acids in unhydrogenated soybean oil during heating at 160, 180, and 200°C for 4–24 h. They did not find that *trans* fatty acids formed under these heating conditions and concluded that more drastic heating conditions (>200°C and >24 h) would be required to generate fatty acid isomerization in heated soybean oil. Fournier et al.⁵⁹ studied the thermal isomerization of fatty acids during the deodorization of fish oil. They found that the oil deodorized at 220°C and 250°C for 3 h under a pressure of 1.5 mbar contained 4.2 and 7.6% *trans* isomers, respectively. They demonstrated that in EPA the central double bond ($\Delta 11$) is preferentially isomerized, while in DHA the two central double bonds ($\Delta 10$ and $\Delta 13$) are more prone to isomerization.⁶⁰ They suggested that deodorization of fish oil should be conducted at a maximum temperature of 180°C.

The third important source of *trans* fatty acids is the biohydrogenation of dietary unsaturated fatty acids in the gastrointestinal tract of ruminants, specifically in the rumen, and consequently *trans* fatty acids could be found in milk and meat products. This ruminal biohydrogenation was first demonstrated by Reiser⁶¹ and by Shorland and Weenink;⁶² it requires a free acid to proceed, and the products are then absorbed and incorporated into ruminant milk and meat fat. The major biochemical pathways for the biohydrogenation of these acids are extensively described by Hartfoot and Hazlewood⁶³ and Griinari et al.⁶⁴ The major substrates of this reaction are linoleic and α -linolenic acids. The initial step typically involves an isomerization of the 12*c*-double bond to an 11*t*-double bond, resulting in conjugated dienoic and trienoic fatty acids. Next there is a reduction of the 9*c*-double bond resulting in an 11*t*-fatty acid. The final step is a further hydrogenation of the 11*t*-double bond, producing stearic acid (18:0). To our knowledge, the extent to which the various pathways of biohydrogenation are associated with specific enzymes and species of bacteria, and the extent to which double bonds migrate during the enzymatic biohydrogenation in the rumen, has not been investigated in any detail. The key biohydrogenation intermediates are 11*t*-octadecenoic acid (vaccenic acid), which is formed from linoleic and α -linolenic acids, and 9*c*,11*t*-octadecadienoic acid (rumenic acid), a CLA formed in the biohydrogenation of ALA. These intermediates are present in appreciable quantities in ruminant fat at a ratio of about 3:1.⁶⁵ Rumenic acid is also formed by desaturation of 11*t*-octadecenoic acid in the mammary gland by a $\Delta 9$ -desaturase.⁶⁶ Thus, a portion of *trans*-octadecenoic acid found in ruminant fat may be derived from ruminal

biohydrogenation of unsaturated fatty acids, or it may originate in the mammary gland and adipose tissue from endogenous synthesis involving $\Delta 9$ -desaturase with rumen-derived vaccenic acid as the substrate. It is worth mentioning that the production of CLA has also been demonstrated in human tissues,⁶⁷ and positional and geometrical isomers of monoenoic fatty acids have been found in microsomal preparations from rat liver⁶⁸ and in rat tissues.⁶⁹ The distribution pattern of positional and geometrical isomers in ruminant milk and meat fat is consistently different from the distribution of isomers in partially hydrogenated vegetable oils. Both fats contain *trans*-octadecenoic fatty acids, with the double bond position ranging from $\Delta 6$ to $\Delta 16$. Nevertheless, in PHVO the predominant *trans*-18:1 positional isomers form a *Gaussian* distribution that centers around the $\iota 9$ -18:1, $\iota 10$ -18:1, and $\iota 11$ -18:1,⁷⁰ while in ruminant fat vaccenic acid ($\iota 11$ -18:1) is the predominant *trans* isomer, and consists of 50–70% of total *trans* fat in milk fat.⁷¹ Also, the *trans* fatty acids in ruminant-derived fats include CLA (1–2% of total fat), predominantly the $\epsilon 9, \iota 11$ isomer (>80% of total CLAs).

13.4 Analysis of Omega-3 and Trans Fatty Acids

Usually, the analysis of fatty acids in a food matrix involves three steps: lipid extraction, preparation of fatty acid derivatives, and gas chromatographic analysis. For decades, gas chromatography (GC) has been the most frequently applied method for fatty acids analysis.^{72–75} The success of GC is based overall on the ability of this technique to separate several decines of compounds, depending on the type and the length of the column, and on the economical accessibility of the gas chromatographic instrumentation, which is actually present in all analytical laboratories. The advent of the wall-coated open tubular (WCOT) capillary column, available in a wide range of different stationary phases, has led to an excellent resolution capability of this technique. Specific separation problems connected to specific food matrices or specific applications could be solved by alternative methodologies of sample preparation, which will be briefly discussed in this chapter.

13.4.1 Lipid Extraction and Preparation of Fatty Acid Esters

A discussion of appropriate procedures for sample storage and handling is beyond the scope of this chapter. Nevertheless, it is worth noting that lipids are prone to oxidation and should be analyzed immediately after sampling to minimize changes occurring in lipid components.

When immediate extraction is not feasible, the sample should be frozen as soon as possible, possibly in liquid nitrogen or dry ice, and stored in glass containers under nitrogen at -80°C . Both wet animal tissue and organic solvents must not come in contact with any plastic ware, since plasticizers are very easily leached out and could be co-chromatographed with fatty acids, causing severe interference in the chromatograms. Such compounds (usually esters of phthalic acid) are characterized by an abundant base peak at m/z 149 in their mass spectra. Also, any source of contamination by mineral oils, greases, and detergents should be avoided. It is usually advisable to add an appropriate antioxidant, such as butylated hydroxy toluene (BHT), at a level of 50–100 mg L⁻¹ to the storage solvent when fatty acid analyses are planned. Optimal conditions for sample handling and lipid storage were extensively reviewed by Christie.⁷⁶

The most often cited methods for lipid extraction in research papers are the Bligh and Dyer⁷⁷ and the Folch⁷⁸ methods. These methods are based on the use of a chloroform/methanol mixture (2:1, v/v), with the water content of the tissue as a tertiary component, or with an appropriate addition of water to obtain a tertiary system. The food matrix is usually homogenized in the presence of such mixtures using an Ultra-Turrax or a Waring blender.

Many modifications of the original procedures have been published^{79,80} to improve lipid extraction in certain food matrices or for particular applications. Iverson et al.⁸¹ compared the two methods for total lipid extraction in a broad range of marine tissue, and found that the Bligh and Dyer method produced significantly lower amounts of lipid content, as compared to the Folch method, in samples containing >2% lipid. This may be due to the limited solubility of triacylglycerols in the chloroform/methanol mixture (2:1, v/v). To overcome this problem they suggested, in the presence of fatty samples, adding a preliminary extraction step with a nonpolar solvent, such as chloroform or diethyl-ether, prior to the Bligh and Dyer procedure. Christie has reasonably argued that this extraction method is often misunderstood and therefore misused.⁷⁶

Prior to GC analysis, the lipid sample has to be hydrolyzed (saponified) and fatty acids converted into nonpolar derivatives, usually fatty acid methyl esters (FAMES). This is usually obtained by direct transesterification of lipids, which proceeds more rapidly than saponification-esterification, and the reaction takes place in one step with only one reagent. Several types of acid-catalyzed or base-catalyzed reactions are suitable for the direct transesterification of lipids.⁸² According to Christie, methanolic hydrogen chloride (5%) or methanol-sulfuric acid (1%) are the best general purpose esterifying reagents.⁸³ They transesterify *O*-acyl lipids (5–10 mg) efficiently under reflux for 2 h or overnight in a stoppered tube at 50°C. The derivatization procedure has not been reported to cause any isomerization of MUFAs. However, there is some controversy over whether derivatization causes isomerization of geometrical isomers of CLAs. Kramer et al.⁸⁴ have evaluated acid and base catalysts in the methylation of milk fat. They concluded that acid-catalyzed methods (HCl, BF₃, acetyl chloride, and H₂SO₄) caused extensive isomerization of conjugated dienes and formed allylic methoxy artifacts, and therefore they do not recommend the use of these reagents for the derivatization of fatty acids when the determination of CLAs has to be performed.

In our laboratory the lipid sample (5–10 mg) is dissolved in 0.1 mL of toluene in a test tube and 10% methanolic hydrogen chloride (2 mL) is added. The sample is then left overnight at 50°C in a stoppered tube. After the addition of a potassium carbonate solution, the fatty acid methyl esters are extracted with hexane containing BHT at the 50 mg L⁻¹ level. In these conditions FAMES can be stored at -20°C for several days.

13.4.2 *Direct Gas Chromatography*

The gas chromatographic analysis of animal fat, excluding fish and marine oils, generally requires simultaneous separation and quantitation of 20 or more fatty acids. Obviously, the number of fatty acids detected depends on many factors, principally the type of column used, separation conditions, sample loading, availability of authentic standards, and the skill of the analyst who is required to investigate a larger number of minor fatty acids.

Basically, the separation of fatty acid methyl esters can be performed on three types of WCOT capillary column coated with nonpolar, polar, and very polar stationary phases, depending on the type of lipid sample to be analyzed and on the objectives of the study.^{72,74,75} The choice of stationary phase affects the retention times and the resolution of the analytical method. The use of an apolar column, such as DB-5 (5% phenyl 95% dimethyl polysiloxane, Agilent J&W), leads to a separation profile that is rather different from that obtained with polar columns, with unsaturated fatty acids eluted ahead of SFAs of the same chain length. The main disadvantage of these columns is the partial overlapping of some unsaturated fatty acids. In fact, linoleic acid (18:2n-6) is not fully resolved from oleic acid (18:1n-9) and co-elutes with 18:3n-3; this is also true for the corresponding C20 and C22 fatty acids.⁸⁵ For these reasons these columns are less often used for

the separation of FAMES, although they could have some advantages in GC-mass spectrometry applications (GC-MS), due to their low grade of bleed and their stability at high temperatures.

Among polar columns, two main types of stationary phases of progressive polarity can be chosen for the analysis of FAMES. In the polyethylene glycol stationary phases (i.e., DB-Wax—Agilent J&W, Supelcowax-10 and Omegawax—Supelco, AT-FAME—Alltech), a broad range of fatty acids from C4 to C24 can be separated according to the number of carbon atoms and the degree of unsaturation. The use of polyethylene glycol columns is widely accepted; these columns are used for the analysis of a wide range of samples, such as vegetable oils, animal fats, and fish and marine oils,^{86–89} with excellent separation of n-3 and n-6 fatty acids. Unfortunately, the separation of geometrical cis and trans isomers cannot be obtained on these columns. Therefore, for the separation of complex mixtures of unsaturated FAMES containing many positional and geometrical isomers of monoenoic, dienoic, and trienoic fatty acids, as in the case of PHVO or ruminant fat, additional resolution is needed. A better resolution and separative performance is obtained using capillary columns coated with 50% (DB-23—Agilent J&W) to 100% of cyanopropyl polysiloxane phase, such as SP2340,^{90–92} SP-2380, SP-2560^{93,94} (Supelco), CP-Sil 88^{94–102} (Chrompack), HP-88³⁷ (Agilent J&W), BPX-70¹⁰³ (SGE), or AT-Silar-100¹⁰⁴ (Alltech). It is generally recognized that columns coated with cyanopropyl phase are mandatory for GC analysis of cis and trans isomers. Both the American Oil Chemists' Society Official Method Ce 1h-05¹⁰⁵ (developed for the determination of cis, trans, saturated, monounsaturated, and PUFAs in vegetable or nonruminant animal oils and fats⁹³) and the AOAC International Official Method of Analysis 996.06¹⁰⁶ (developed for the determination of total, saturated, and monounsaturated fats in foodstuffs¹⁰⁷) recommend the use of this type of high polarity column for the analysis of FAME isomers. In the latter method, lipids are first extracted from food samples by hydrolytic methods (acidic or alkaline, depending on the food matrix) and petroleum ether, followed by methylation to FAMES using BF₃. FAMES are further extracted in a small volume of hexane prior to GC analysis.

Due to the weaker interaction of the trans isomer with the cyano-dipole, the trans isomer elutes before the cis isomer with cyanopropyl phases. For example *t*9-18:1 elutes before *c*9-18:1 and *t*11-18:1 elutes before *c*11-18:1. Moreover, positional isomers of *trans*-18:1 or *cis*-18:1 elute in the order of double bond progression along the chain, starting from the carboxyl end (Δ 9 elutes before Δ 10). For example, the order of elution of the geometrical and positional isomers usually found in ruminant fat is *t*9-18:1, *t*10-18:1, *t*11-18:1, *c*9-18:1, *c*11-18:1.

However, overlaps of some cis and trans isomers with different positional isomers can occur, especially when analyzing oils with a high content of trans fat, such as PHVO. The extent of these overlaps depends mainly on the following factors: (i) the choice of the stationary phase, (ii) the length of the column, (iii) the temperature program or the isothermal temperature of the oven, (iv) the skill of the analyst, and (v) the age of the column.

Several methods for the determination of trans fatty acids in fats and oils of different origins have been published in recent years and excellent reviews are available.^{37,108–113} In Table 13.1 the gas chromatographic conditions for the separation of FAMES obtained from literature data are summarized. As a general rule, depending on the column length and phase, trans isomers up to Δ 12 elute before oleic acid (*c*9-18:1), while trans isomers from Δ 13 to Δ 15 may co-elute with *c*9-18:1.⁹² Operating isothermally at 180°C with a 100-m SP-2560 column, *t*13-18:1, *t*14-18:1, and *t*15-18:1 were resolved from *c*9-18:1, but overlapped *c*6-18:1, *c*7-18:1, and *c*8-18:1.⁹³ Furthermore, *t*13-18:1 and *t*14-18:1 always pair, and some problems in separation of 20:1 isomers and ALA could occur. Kramer et al.¹¹⁴ compared the separation profiles of FAMES from milk fat using a 60-m Supelcowax 10 column and a 100-m CP Sil 88 column. They found that the CP Sil 88 column provided better resolution of CLA isomers, 18:1 isomers, 18:2n-6, and 18:3n-3 isomers and

Table 13.1 Chromatographic Conditions Used for Gas Chromatographic Separation of Trans Fatty Acids

Food Item	Type of Esters	Procedure	Column (Brand Name), Length, I.D., Film Thickness		Stationary Phase	Carrier Gas, Flowrate, Inlet Pressure	Injection	Temperature Program	Reference
			CP-Sil 88, 50 m, 0.25 mm, 0.20 μ m	AT-Silar-90, 30 m, 0.25 mm, 0.20 μ m					
Butterfat	Fatty acid isopropyl esters (FAIPE)	Ag-TLC fractionation	CP-Sil 88, 50 m, 0.25 mm, 0.20 μ m	AT-Silar-90, 30 m, 0.25 mm, 0.20 μ m	Bis-cyanopropyl polysiloxane	Helium, nd, ^a 20 kPa	Split	65 (hold 6 min) – 185°C at 5°C min ⁻¹ or isothermal at 160°C	101, 102
Shortening	FAME	Direct GC			90% Bis-cyanopropyl 10% cyanopropylphenyl polysiloxane	Helium, 0.6 mL min ⁻¹	Split, 1:50	150 (hold 10 min) –210°C at 2.7°C min ⁻¹	104
Soybean oil, rapeseed oil, palm oil, hydrogenated soybean oil, hydrogenated palm oil, hydrogenated cottonseed oil	FAME	Direct GC	TC-70, 60 m, 0.25 mm, 0.25 μ m SP-2560, 100 m, 0.25 mm, 0.20 μ mz		Bis- cyanopropylsiloxane polysilphenylene Bis-cyanopropyl polysiloxane	Helium, 1 mL min ⁻¹	nd ^a	Isothermal at 190°C	115
Margarine (partially hydrogenated soybean oil)	FAME	Direct GC	CP-Sil 88, 100 m, 0.25 mm, 0.20 μ m SP-2560, 100 m, 0.25 mm, 0.20 μ m		Bis-cyanopropyl polysiloxane Bis-cyanopropyl polysiloxane	Helium, 1 mL min ⁻¹ or hydrogen, 0.6–0.8– 1.0 mL min ⁻¹	Split 1:100	Isothermal at 180°C	94
Beef meat	TMS-DM	Direct GC	CP-Sil 88, 100 m, 0.25 mm, 0.20 μ m		Bis-cyanopropyl polysiloxane	Helium, 2 mL min ⁻¹ , 355 kPa	nd ^a	100–170°C at 2°C min ⁻¹ (hold 15 min) then to 180°C at 0.5°C min ⁻¹	95

Milk fat	FAME	Ag-TLC fractionation	SUPELCO WAX-10	Polyethylene glycol	Helium or hydrogen	nd ^a	65°C (hold 1 min) to 195°C (hold 50 min) at 13°C min ⁻¹ , then to 240°C (hold 50 min) at 15°C min ⁻¹ to 45°C (hold 4 min) to 175°C (hold 50 min) at 13°C min ⁻¹ then to 215°C (hold 35 min) at 4°C min ⁻¹	114
Beef cuts, goat cheese, human milk, beef tallow	FAIPE	Ag-TLC fractionation	CP-Sil 88	Bis-cyanopropyl polysiloxane	Helium, nd, ^a 20 kPa	Split	65 (hold 6 min) to 185°C at 5°C min ⁻¹ isothermal at 160°C or 175°C Isothermal at 175, 180, 185, 190°C	116
Margarine (partially hydrogenated canola oil)	FAME	Direct GC	CP-Sil 88, 100 m, 0.25 mm, 0.20 µm SP-2560, 100 m, 0.25 mm, 0.20 µm	Bis-cyanopropyl polysiloxane Bis-cyanopropyl polysiloxane	Helium, 1 mL min ⁻¹ or hydrogen, 0.6–0.8–1.0 mL min ⁻¹	Split 1:100	Isothermal at 170, 175, 180, 185, 190°C	93
Shortenings and fast foods	FAME	Ag-TLC fractionation	SP-2340, 60 or 100 m, 0.25 mm	Bis-cyanopropyl polysiloxane	Helium, 1 mL/min	Split 1:100	150–200°C at 0.4°C min ⁻¹	90
Pork and beef meat	FAME	Ag-TLC fractionation	SP-2340, 60 m, 0.25 mm	Bis-cyanopropyl polysiloxane	Helium, 0.6 mL/min, 30 psi	Split 1:100	170–210°C at 2°C min ⁻¹	91
Food items containing PHVO	FAME	Ag-TLC fractionation	CP-Sil 88, 50 m, 0.25 mm, 0.20 µm CP-Sil 88, 100 m, 0.25 mm, 0.20 µm	Bis-cyanopropyl polysiloxane	Helium, nd, ^a 100 kPa	Split 1:50	Isothermal at 190°C (total trans)	117
Partially hydrogenated marine oils	FAME	Ag-TLC fractionation	CP-Sil 88, 50 m, 0.25 mm, 0.20 µm	Bis-cyanopropyl polysiloxane	Hydrogen 160 kPa Helium, 1.1 mL/min	On-column	Isothermal at 172°C (isolated fractions) 80–165°C (hold 12 min) at 25°C min ⁻¹ , to 220°C at 2°C min ⁻¹ (hold 6 min)	100

(Continued)

Table 13.1 (Continued)

Food Item	Type of Esters	Procedure	Column (Brand Name), Length, I.D., Film Thickness	Stationary Phase	Carrier Gas, Flowrate, Inlet Pressure	Injection	Temperature Program	Reference
Partially hydrogenated vegetable oils Beef tallow	FAME	Ag-TLC fractionation	SP-2340, 60 m, 0.25 mm, 0.20 μ m	Bis-cyanopropyl polysiloxane	Hydrogen, 20 psi	Split	Isothermal at 160°C	92
	FAIPE	Ag-TLC fractionation	CP-Sil 88, 50 m, 0.25 mm, 0.20 μ m	Bis-cyanopropyl polysiloxane	Helium, 20 kPa	Split	65 (6 min) to 185°C at 5°C min ⁻¹ isothermal at 160°C or 175°C	118
Food items containing ruminant fat, PHVO and PHMO	FAME or DMOX derivatives	Ag-TLC fractionation	CP-Sil 88, 100 m, 0.25 mm, 0.20 μ m	Bis-cyanopropyl polysiloxane	Hydrogen, 1.0 mL/min	Split 1:88	150–155°C (hold 50 min) at 2°C min ⁻¹ , to 170°C (hold 7.12 min) at 40°C min ⁻¹ , to 224 (hold 8.66 min) at 40°C min ⁻¹ (FAMES)	97
					Hydrogen 0.7 mL/min	Split 1:75	150 (hold 125 min) to 220°C (hold 10 min) at 10°C min ⁻¹ (DMOX derivatives)	
Milk fats	FAME	Ag-TLC fractionation and direct GC	CP-Sil 88, 100 m, 0.25 mm, 0.20 μ m	Bis-cyanopropyl polysiloxane	Hydrogen, 0.6 mL/min, 160 kPa	Split 1:100	Isothermal at 175°C	99
Food items of animal and vegetal origin	FAME	Direct GC	CP-Sil 88, 50 m, 0.25 mm, 0.20 μ m	Bis-cyanopropyl polysiloxane	Helium, 1.2 mL/min	Split 1:100	Isothermal at 195°C	119

^a Not described by authors.

concluded that this column is to be recommended for milk lipid analysis. In Figures 13.2 and 13.3, typical chromatograms of the 18:0 to 18:2n-6 FAME region using both column are illustrated.

Aldai et al.⁹⁵ characterized the total fatty acid profile of intramuscular fat of beef meat using direct saponification with KOH/methanol, followed by a derivatization with (trimethylsilyl)di azomethane. Total FAMES were analyzed on a 100-m CP-Sil 88 column. They separated and identified 43 peaks in 97 min of analysis, including 13 SFAs, 5 branched chain fatty acids, 11 MUFAs, 12 PUFAs, and the two major CLA isomers (*c*9,*t*11-18:2, *t*10,*c*12-18:2). They found

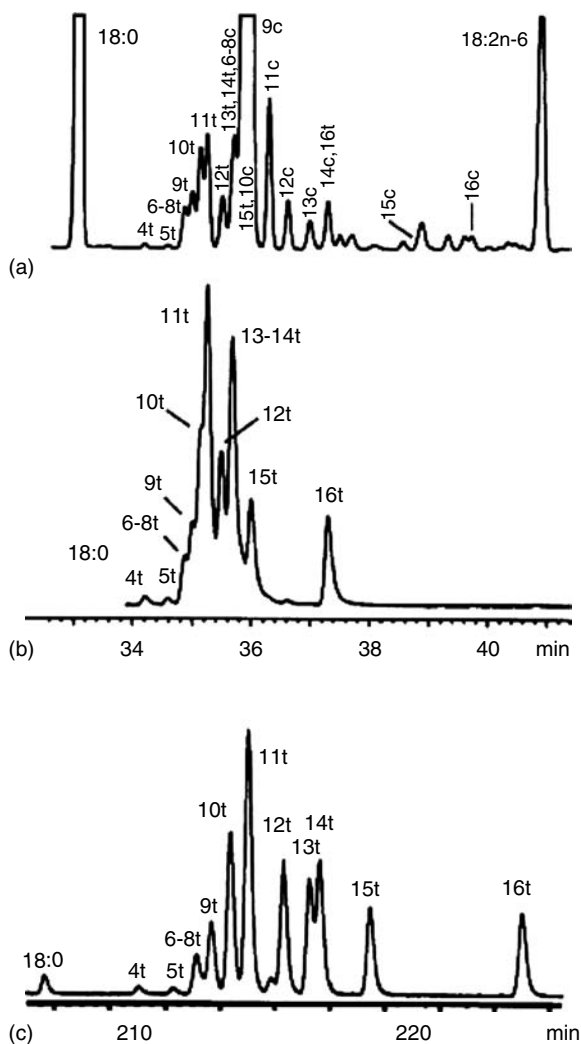


Figure 13.2 Partial gas chromatogram of the 18:0 to 18:2n-6 FAME region using a 100-m CP Sil 88 capillary column, hydrogen as carrier gas, and a typical temperature program from 45 to 215°C. (a) Total milk FAME from cows fed a control diet; (b) trans fraction isolated from the same milk fat FAME as (a) using Ag-TLC; (c) the same trans fraction as (b) but resolved using stepwise GC program starting at 120°C. (Reproduced from Kramer, J., Blackadar, C., and Zhou, J., *Lipids*, 37 [8], 823, 2002. With permission.)

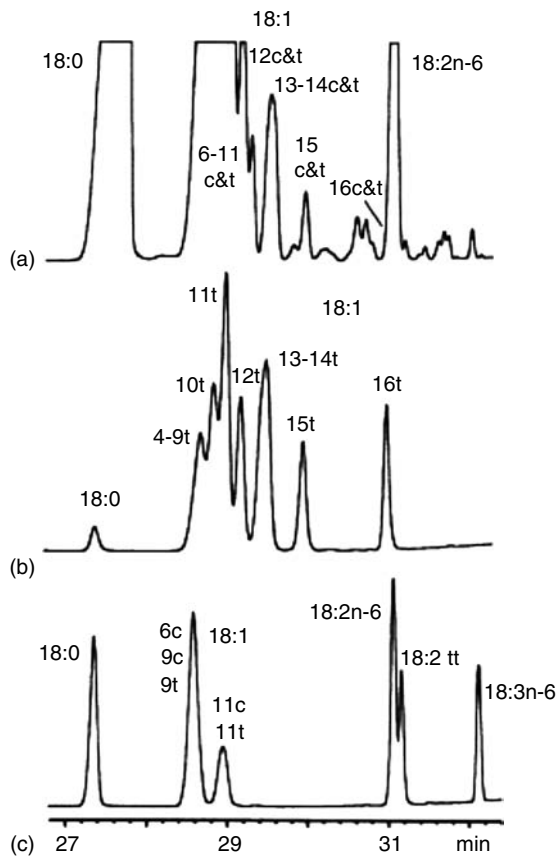


Figure 13.3 Partial gas chromatogram of the 18:0 to 18:2n-6 FAME region using a 60-m SUPELCOWAX 10 capillary column, hydrogen as carrier gas, and a typical temperature program from 65 to 240°C. (a) Total milk FAME from cows fed a control diet; (b) trans fraction isolated from the same milk fat FAME as (a) using Ag-TLC; (c) FAME standard 463 from Nu-Chek-Prep (Elysian, MN). (Reproduced from Kramer, J., Blackadar, C., and Zhou, J., *Lipids*, 37 (8), 823, 2002. With permission.)

t_9 -18:1, t_{10} -18:1, and t_{11} -18:1 in beef meat. Huang et al.¹²⁰ proposed a simple method based on the AOAC method 996.06 for routine analysis of trans fatty acids in shortenings. They used GC/MS and a 30-m AT-Silar-90 column. Δ^4 - Δ^{11} isomers were separated from oleic acid, but co-eluted almost completely.

To select the most appropriate analytical procedure, lipid extracted from food samples could be divided into categories according to their fatty acid composition—for example, (i) dairy fat, which is peculiar for its content of short chain and branched chain fatty acids; (ii) ruminant meat fat; (iii) non-ruminant meat fat and vegetable oils; (iv) marine and long-chain polyunsaturated oils; and (v) partially hydrogenated vegetable or marine oils. At this point the choice of the column depends mainly on the objective of the study. An optimal separation of a “classic” vegetable oil or animal fat can be obtained efficiently with polyethylene glycol columns, as already described. A medium-polarity cyanopropyl column, such as DB-23, is suitable for the

analysis of complex mixtures of PUFAs, including n-3 fatty acids, and partial separation of 18:1 isomers could be obtained. However, when detailed information on positional and geometrical isomers has to be acquired, the use of a 100-m column coated with 100% of a cyanopropyl phase is recommended.

Using direct GC analysis of fatty acids, the key limitation is the incomplete separation of *trans*-monoenoic from *cis*-monoenoic fatty acids. These overlaps can be partially overcome by using a 100-m cyanopropyl column operating at 180°C, as demonstrated by Ratnayake et al.⁹³ In these conditions the *l*15-18:1 usually co-elutes with *l*10-18:1 and (*l*13 + *l*14)-18:1 co-elutes with (*c*6-*c*8)-18:1. These overlaps, which represent a serious drawback in PVHO analysis, are of minor importance when considering ruminant fat (milk or meat fat), because these lipids do not contain *cis*-18:1 isomers other than *c*9-18:1 (oleic acid) and *c*11-18:1 (*cis*-vaccenic acid).¹¹³ Nevertheless, to eliminate these co-elution problems, the prefractionation of fatty acids by silver ion chromatography is often used in its two different forms, thin-layer chromatography or silver ion high-performance liquid chromatography.¹²¹

13.4.3 Silver Ion Thin-Layer Chromatography

Silver ion thin-layer chromatography (Ag-TLC) could be considered the first necessary step when analyzing a complex lipid mixture to obtain valuable information on the whole sample. It does not require expensive instrumentation and uses small volumes of organic solvents. Glass plates are used, coated with an appropriate layer (0.1–1.0 mm thickness) of supporting material (usually silica gel G for FAMES), impregnated with silver ions. Separations are performed at ambient temperature in covered tanks lined with filter paper to saturate the atmosphere with the developing mixture, which usually consists a mixture of two or three solvents. The principle on which this technique is based is the formation of charge transfer complexes between the *d* orbitals of silver and the π electrons of double bonds.³⁷ By Ag-TLC, fatty acids, which are usually subjected to separation in the form of methyl ester derivatives, could be resolved on the basis of the number, the geometrical configuration, and, to a lesser extent, the position of double bonds. The separation of a FAME mixture that contains saturated, monoenoic, dienoic, and trienoic fatty acids could be obtained with the following migration order: saturated > *trans* monoenes > *cis* monoenes + *trans,trans* dienes > *trans,cis* dienes + *cis,trans* dienes > *cis,cis* dienes > trienes. Undoubtedly, one of the most important aspects of the application of Ag-TLC to FAME analysis is the separation of *cis* and *trans* isomers. *Trans* isomers, irrespective of chain length, migrate ahead of the *cis* isomers and are completely separated from SFAs and from dienoic acids. A separation of methyl ester derivatives of unsaturated fatty acids is illustrated in Figure 13.4.¹²²

This technique has been extensively used prior to GC or GC/MS for the complete and more accurate analysis of geometrical isomers of fatty acids in a wide range of edible fats and oils.^{108,113} In particular, the utilization of Ag-TLC for the isolation of *trans*-octadecenoic fraction from lipid samples of different origin has been thoroughly evaluated by Ratnayake.¹²³ Ag-TLC has been used also for the isolation of 16:1 and 17:1 isomers from human milk,¹²⁴ and of 20:1 and 22:1 isomers from marine oils.¹⁰⁰

Molkentin and Precht¹²⁵ developed a method for quantitative analysis of *trans*-octadecenoic acids in butter, beef tallow, PHVO, and human milk by Ag-TLC and GC. They utilized pre-coated TLC plates (20 × 20 cm) with 0.25 mm of Silica Gel 60 impregnated by immersion into a 20% aqueous solution of silver nitrate (w/v) for 20 min and activated at 120°C for 30 min before use. After the application of the FAMES sample, the plates were developed in an n-heptane/diethyl ether mixture (90:10, v/v). The migration order of FAMES under these conditions was as

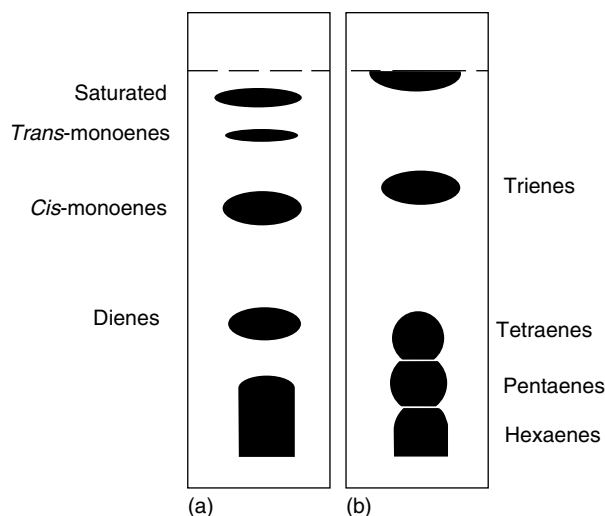


Figure 13.4 Separation of methyl esters derivatives of unsaturated fatty acids by TLC impregnated with 10% (w/w) silver nitrate. Plate A: mobile phase hexane-diethyl ether (9:1, v/v). Plate B: as plate A but solvents in the ratio of 2:3. (Reproduced from Christie, W.W., *Gas Chromatography and Lipids: A Practical Guide*, The Oily Press, Bridgwater, England 1989. With permission.)

follows: saturated, trans monoenes, and cis monoenes. Each fraction was scraped off and analyzed separately by GC on a 100-m CP Sil 88 column. The stearic acid content of the lipid sample was used as the internal standard to quantify the 18:1 isomers. The combination of Ag-TLC with GC permitted the separation and quantification of 10 trans isomers of 18:1 ($\Delta 4$, $\Delta 5$, $\Delta 6 + \Delta 7 + \Delta 8$ which co-eluted, $\Delta 9$, $\Delta 10$, $\Delta 11$, $\Delta 12$, $\Delta 13 + \Delta 14$ which co-eluted, $\Delta 15$, and $\Delta 16$; Figure 13.5).

The authors applied this method also to the determination of 16:1 and 17:1 geometrical isomers in human milk¹²⁴ and to the determination of *trans*-18:1 isomers in partially hydrogenated vegetable oils marketed in Germany⁵³ and in milk fat.⁹⁹ In more recent papers, further improvements on this method have led to separation and quantification of 16:1, 18:1, 18:2, 18:3, and 20:1 trans isomers in margarines, shortenings, and cooking fats,¹²⁶ and of 18:1, 18:2, 18:3 isomers, and *trans*-14:1, *trans*-16:1, and four isomers of ALA.

Wolff¹²⁸ fractionated fatty acid isopropyl esters prepared from butter and margarine samples on silica gel plates impregnated in a 5% (w/v) silver nitrate solution in acetonitrile for 20 min. The developing solvent was a hexane-diethyl ether-acetic acid mixture (90:10:1, v,v,v). The bands corresponding to the saturated and to *trans*-18:1 were scraped off, pooled in a test tube and analyzed by GC. The 16:0 and 18:0 present in fat were used as internal standards. The *trans*-18:1 fraction subjected to GC separation on a 50-m CP Sil 88 column consisted of 6 peaks: (*t*6-*t*9)-18:1, (*t*10-*t*11)-18:1, *t*12-18:1, (*t*13-*t*14)-18:1, *t*15-18:1, and *t*16-18:1.

Some overlaps were partially overcome by using a longer column.¹⁰² The robustness of the method was demonstrated when applying to several samples of French processed foods containing PVHO¹¹⁷ and to the study of seasonal variations in French butters.¹⁰¹ Typical chromatograms of the *trans*-18:1 acid isopropyl esters isolated from different food matrices are presented in Figure 13.6.¹⁰²

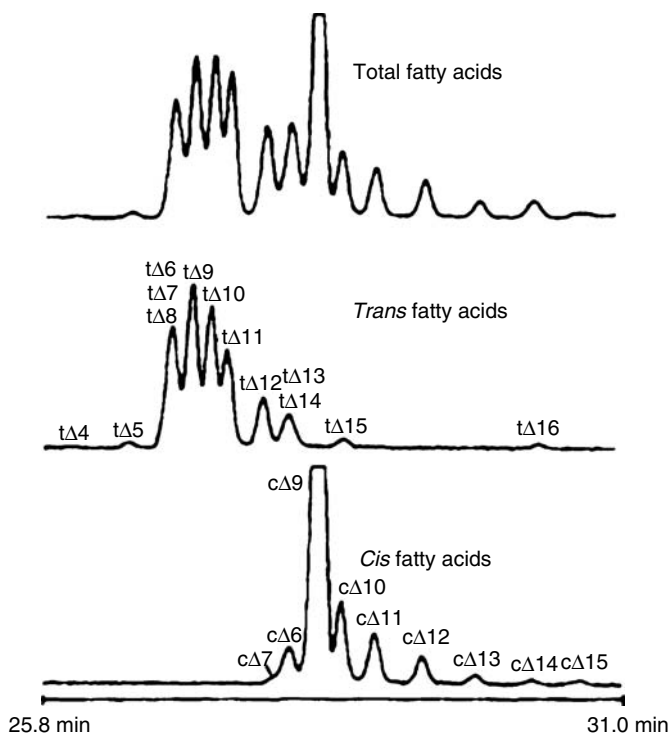


Figure 13.5 Chromatograms resulting from the separation of cis and trans isomers of FAME from a cooking fat by direct GC (total fatty acids) and by prefractionation using Ag-TLC. (Reproduced from Molkentin, J. and Precht, D., *Chromatographia*, 41 (5–6), 267, 1995. With permission.)

13.4.4 Silver Ion Solid-Phase Extraction

An alternative approach to the fractionation of FAMEs before GC analysis is the use of silver ion solid-phase extraction (Ag-SPE). Originally developed by Christie,¹²⁹ this technique is commercially available and is based on the immobilization of silver ions as counter ions onto a SCX SPE cartridge.¹³⁰ After the loading of the sample onto the cartridge, FAMEs can be fractionated on the basis of degree of unsaturation, and cis and trans isomers can be resolved using different solvent mixtures in appropriate sequence. According to this procedure trans-monoenes are eluted with SFAs, while cis-monoenes are eluted with dienes. The technical paper¹³⁰ showed promising results, but the current limitation of this procedure is the lack of published and validated data regarding the recovery of FAME fractions and its application to different food matrices.

13.4.5 Silver Ion High-Performance Liquid Chromatography

Silver ion high-performance liquid chromatography (Ag-HPLC) initially suffered problems related to the development of stable columns with controlled silver content and, as a consequence, a shelf life that ensured reproducible separations. The first approach used for the preparation of the column was to impregnate HPLC grade silica gel with silver nitrate and pack it into the column.¹³¹ Unfortunately, this procedure requires much skill and practice, and a major problem is that silver

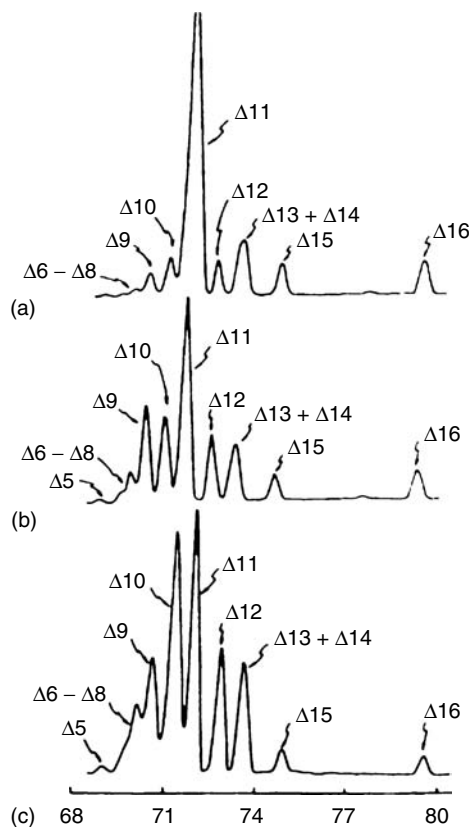


Figure 13.6 Chromatograms of the *trans*-18:1 acid isopropyl esters isolated from: A, beef tallow; B, human milk lipids; C, partially hydrogenated soybean oil. Analyses were done on a 100-m × 0.25 mm i.d. CP Sil 88 fused-silica capillary column (Chrompack, Meddelburg, The Netherlands) operated at 160°C with helium as a carrier gas (inlet pressure, 180 kPa). Identification of individual isomers was by comparison with synthetic compounds. (Reproduced from Wolff, R.L. and Bayard, C.C., *J. Am. Oil Chem. Soc.*, 72 (10), 1197, 1995. With permission.)

ions bleed continuously from the column into the mobile phase, potentially causing damage to the HPLC system due to its corrosive power. Furthermore, silver ions could contaminate the lipid fractions when Ag-HPLC is used in preparative mode prior to GC analysis.

More recently, a more practical approach to the preparation of Ag-HPLC columns has been to bind silver ions to a prepacked cation exchange column containing chemically bonded alkyl benzenesulphonic acid groups, as described by Christie.¹³² These columns are reported to be stable for long periods of time without leaching of silver ions. Nevertheless, many factors may influence their stability, such as the mobile phase composition, the storage conditions, and the purity of the samples injected. A commercial, ready-to-use silver ion column of the same type (Chromspher Lipids) is now available from Varian. This fact has increased the use and the significance of Ag-HPLC in separation of *cis* and *trans* isomers of fatty acids in recent years.

Theoretically, almost every type of detector can be used in Ag-HPLC detection of fatty acids. Practically, spectrophotometric (ultraviolet [UV]), evaporative light scattering (ELSD), and mass

spectrometric detectors are the most widely used. Fatty acids can be detected by UV detection at 205–210 nm, or they can be derivatized with appropriate reagents to obtain derivatives with stronger UV absorption at 245 nm, such as phenethyl, phenacyl, or *p*-methoxyphenacyl esters.¹³³ UV detectors are readily available, nondestructive, and fractions can be collected for further GC analysis. However, in the absence of derivatization of fatty acids, the choice of solvents is restricted to those with cut-offs below 220 nm. ELSD does not restrict the choice of the solvent, but it is destructive. To overcome this drawback, a stream-splitter could be inserted between the column and the detector to enable the collection of fractions.¹³⁴ Mass spectrometric detectors with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI)¹³⁵ have great potential in lipid analysis,¹³⁶ especially for triacylglycerol analysis.¹³⁷

The use of three major mobile phases predominates in Ag-HPLC: (i) based on toluene, (ii) based on a dichloromethane/dichloroethane mixture that was introduced by Christie and Breckenridge,¹³⁸ and (iii) based on hexane, usually modified by low amounts of acetonitrile.¹³⁹ The effects of mobile phase composition on the retention and resolution of isomers of fatty acid derivatives was investigated by Momchilova and Nikolova-Damyanova.¹⁴⁰ In this study the addition of acetonitrile, methanol, or isopropanol to hexane- or dichloromethane/dichloroethane-based mobile phases produced similar chromatographic profiles. As in the case of Ag-TLC, fatty acids can be separated according to the geometrical configuration, number, and, to a lesser extent, position of double bonds. Separation is based on the reversible formation of a weak charge-transfer complex between a silver ion and a double bond.¹⁴¹ The Ag-HPLC methodology and its application to fatty acids and other lipids have been thoroughly reviewed by Nikolova-Damyanova.¹⁴² Great contributions have been made in this field by the laboratories of Christie, Dobson et al.¹⁴² and Adlof.^{139,143} Christie and Breckenridge¹³⁸ analyzed geometrical isomers of fatty acid phenacyl esters with zero to three double bonds with UV detection at 242 nm, utilizing a Nucleosil 5SA column with silver ion loaded in the laboratory.¹³² The mobile phase was a dichloromethane/dichloroethane mixture (1:1, v/v) containing 0.5% acetonitrile. This method was applied to sheep adipose tissue, commercial margarines, and cooking fats.

A variety of isomeric fatty acid methyl esters were separated on a Chromspher Lipids™ HPLC column utilizing hexane/acetonitrile as the mobile phase and UV detection.¹³⁹ The *cis* and *trans* isomers of methyl linoleate (four isomers) and methyl α -linolenate (eight isomers) were resolved. Furthermore, 15 of the 16 possible isomers of the methyl arachidonate were resolved as well.

Adlof et al.¹⁴³ investigated the positional and geometrical isomer distribution of fatty acid methyl esters in partially hydrogenated vegetable oils by Ag-HPLC. They used a Chromspher column and UV detection at 206 nm, or alternatively RI detection or FID detection. Isocratic solvent conditions (0.15% acetonitrile in hexane) were used to separate saturates, *trans*-18:1, *cis*-18:1, and 18:2 (Figure 13.7). FAME fractions were collected and analyzed by GC. A further modification of the solvent system (0.08% acetonitrile in hexane) permitted the separation of $\Delta 14$, $\Delta 13$, $\Delta 12$, $\Delta 11$ -18:1 positional isomers (either *cis* and *trans*). The $\Delta 8$ and $\Delta 9$ isomers co-eluted and the $\Delta 10$ isomer was poorly resolved from the $\Delta 8 + \Delta 9$ peak. A typical chromatogram is presented in Figure 13.8. Ag-HPLC (UV) data agreed with results obtained by GC for *t*8-18:1 to *t*12-18:1, but less so for *t*13-18:1 and *t*14-18:1 isomers. Optimum resolution of the $\Delta 10$ isomer was achieved with the use of two Chromspher columns connected in series and by decreasing the sample amount to 0.5 μg or less. It is worth noting that the elution order of the 18:1 positional isomers on a silver ion column is the reverse of that obtained by GC on a cyanopropyl column.

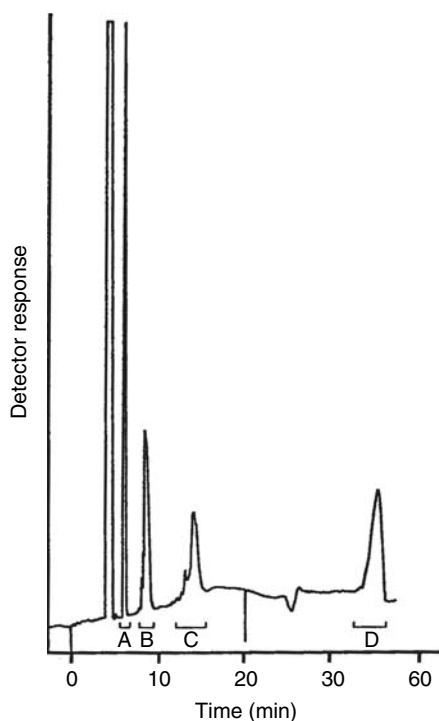


Figure 13.7 Analysis of partially hydrogenated vegetable oil fatty acid methyl esters by silver-ion high-performance liquid chromatography. Sample size 20 μg ; flow rate 1.0 mL/min; mobile phase 0.15% acetonitrile in hexane; RI detector. Fraction: A, saturates; B, *trans*-18:1; C, *cis*-18:1; D, 18:2. (Reproduced from Adlof, R., Copes, L., and Emken, E., *J. Am. Oil Chem. Soc.*, 72 (5), 571, 1995. With permission.)

In addition, Toschi et al.¹⁴⁴ were able to separate saturates, *trans*-18:1 isomers, and *cis*-18:1 isomers on a Chromspher lipids column or on a Spherisorb S5SCX column using dichloromethane/dichloroethane (50:50, v/v) with small amounts of acetonitrile (0.01–0.025%) as the mobile phase in less than 10 min. The combined saturated plus *trans*-monoene fractions were collected for analysis by GC and for comparison with the composition of the unfractionated sample. This method was applied to soybean and rapeseed oils with excellent results.

The complete separation of *cis* and *trans* isomers by Ag-HPLC can be used to provide quantitative data for positional isomers not separated by GC. However, Ag-HPLC is more limited than GC as a stand-alone method for the study of geometrical and positional fatty acid isomers in fats and oils. This technique represents a reliable and simple alternative to Ag-TLC for the pre-fractionation of isomers prior to GC analysis. Such a procedure has been recommended by AOCS for *trans* isomer analysis.¹⁴⁵

In the last decade, Ag-HPLC has found extensive application to the determination of CLA in milk and cheese samples,¹⁴⁶ in CLA preparations and biological specimens,¹⁴⁷ and in meat samples,^{148,149} using one to six columns connected in series.¹⁵⁰

Juaneda¹⁵¹ proposed a simple RP-HPLC method for the separation of *trans*-18:1 and *cis*-18:1 fractions as an alternative to Ag-HPLC, using two RP-HPLC columns (Kromasil-C₁₈, 250 \times 10 mm)

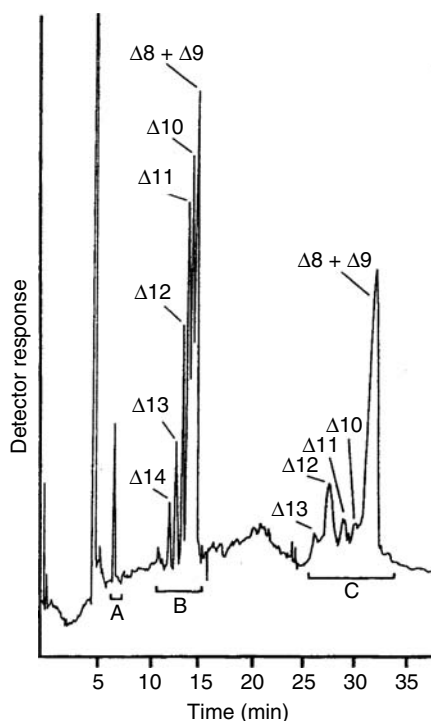


Figure 13.8 Analysis of partially hydrogenated vegetable oil 18:1 fatty acid methyl esters positional isomers by silver-ion high-performance liquid chromatography. Sample size, 0.4 μg ; flow rate, 1.0 mL/min; mobile phase, 0.08% acetonitrile in hexane; UV detection at 206 nm. Fraction: A, saturates; B, *trans*-18:1; C, *cis*-18:1. (Reproduced from Adlof, R., Copes, L., and Emken, E., *J. Am. Oil Chem. Soc.*, 72 (5), 571, 1995. With permission.)

connected in series. The *trans*- and *cis*-18:1 fatty acids as methyl esters were eluted in 35–40 min utilizing acetonitrile as the mobile phase at 4 mL/min. The purity of the isolated fractions was tested by GC-MS and GC-FTIR. The method was applied to milk FAMES with reliable results.

13.4.6 Mass Spectrometry of Fatty Acid Derivatives

GC, coupled to MS (GC-MS), is the most powerful technique for the structural analysis of fatty acid mixtures, in particular for the determination of double bond positions in isomeric fatty acids analysis.¹⁵²

Fatty acids are generally analyzed by GC as methyl ester derivatives. Unfortunately, the structural information obtained from the mass spectra of FAMES is frequently of limited value. For example, the position of the double bond in the aliphatic chain cannot be determined due to the bond migration that occurs during the ionization.¹⁵³

To overcome this problem, the analyst has two choices. One is the preparation of specific adducts with the double bonds that yield a specific fragmentation pattern.¹⁵⁴ The second approach is the derivatization of the fatty acid carboxyl group with a reagent containing a nitrogen atom.¹⁵⁵

When the derivatized fatty acid is ionized in the mass spectrometer, the nitrogen atom carries the charge and, consequently, double bond ionization and migration are minimized. Thus, the formation of characteristic fragment ions permits the localization of unsaturated bonds and other functional groups in the hydrocarbon chain.

The most common derivatives of this type used for the analysis of unsaturated fatty acids are picolinyl (3-hydroxymethylpyridinyl) esters¹⁵⁶ and 4,4-dimethyloxazoline (DMOX) derivatives.¹⁵⁷ It is worth considering that the mass spectra deriving from either picolinyl or DMOX derivatives do not provide information about the *cis/trans* configuration of the double bonds. These structural features need to be confirmed by an independent technique.

In the mass spectra of the DMOX derivatives, if a double bond is positioned between carbon n and carbon $n + 1$, then a mass interval of 12 amu between ions corresponding to fragments containing carbon $n - 1$ and carbon n is usually observed. In the case of the picolinyl esters, when a double bond is reached along the alkyl chain, a mass interval of 26 amu between ions corresponding to fragments containing $n - 1$ and $n + 1$ carbons is observed. These methods have been successfully applied to the analysis of *trans*-18:1 positional isomers in ruminant fat and in PVHO by Mossoba et al.¹⁵⁸ and by Aro et al.⁹⁷

Recently Van Pelt and Brenna¹⁵⁹ presented a GC-MS/MS (ion trap) method for determining the location of double bonds in PUFA methyl esters. This procedure is based on the chemical ionization of neutral FAMES in the gas phase with acetonitrile, called by authors “covalent adduct chemical ionization” (CACI). Briefly, acetonitrile under chemical ionization conditions in an ion trap mass spectrometer self-reacts to form (1-methyleneimino)-1-ethenylum (MIE, m/z 54), which reacts with double bonds of polyunsaturated FAMES to yield a series of covalent product ions, all appearing at $(M + 54)^+$. Collisional dissociations of these ions yield diagnostic fragments, permitting unambiguous localization of double bonds.¹⁶⁰ Interestingly, this method has also been used in the analysis of positional and geometrical isomers of CLA.¹⁶¹

13.4.7 Infrared Spectroscopy

Infrared (IR) spectroscopy is the simplest method for *trans* fat determination in oils and fats. This procedure provides the quantitation of *trans* fat in a pure oil or fat by measurement of the absorbance of the 996 cm^{-1} band. The lower limit of *trans* quantitation of fat is usually 5% (as % of total fat). The major drawback of this method is that it does not permit the identification of the single *trans* fatty acids. An overview of IR methodologies has recently been published by Mossoba et al.¹⁶²

13.5 Occurrence of Omega-3 and Trans Fatty Acids in Meat and Poultry Products

The fatty acid composition of meats and meat products differs considerably among animal species. In general, lipids from ruminant meat are composed of approximately 45–55% SFAs, 40–50% MUFAs, and about 5% PUFAs, with an $n-6/n-3$ ratio between 2 and 5, depending on the consumed diet. Fats from pork products are composed of approximately 35–40% SFAs, 40–50% MUFAs, and 10% PUFAs, with a highly variable $n-6/n-3$ ratio in the range of 4–10. Fatty acids

from chicken broiler and turkey meats are characterized approximately by 30–40% SFAs, 30–40% MUFAs, and 30% PUFAs, with an n-6/n-3 ratio of 10–20. On a percentage basis, the amount of total SFAs in raw meats is beef > lamb > pork > poultry; the amount of total MUFAs is beef and pork > lamb > poultry; the amount of total PUFAs is poultry > pork > lamb > beef.¹⁶³ The main difference in fatty acid composition among species is related to the percentage of 18:2n-6, which is higher in pork and chicken meats as compared to ruminant meats. This is obviously due to the high content of this fatty acid in the cereal-based diets consumed by these animals. In contrast, ruminant meat has relatively higher 18:3n-3 and lower 18:2n-6.

It is well established that Western human diets, with the advent of large-scale production of vegetable oils, have become deficient in n-3 fatty acids and rich in n-6 fatty acids, causing high values of the n-6/n-3 ratio in the diet and promoting the pathogenesis of many diseases.²³ Consequently, in recent years many researchers have studied strategies for increasing the content of beneficial polyunsaturated n-3 fatty acids in intramuscular fat from ruminants,^{164,165} pork,^{166–168} and poultry^{169,170} and have reviewed the contribution of meat sources to the dietary intake by humans of long-chain n-3 PUFAs.^{164,171} Data collected from the literature on n-3 fatty acid content of raw and processed meat and poultry products are presented in Table 13.3.

Increasing the n-3 content in meats can be achieved by including in the diet linseed oil, fresh grass, or grass silage (rich in 18:3n-3) or fish oil and fish meal (rich in EPA and DHA, depending on the source). Diets richer in 18:3n-3 result in an increased percentage of 18:3n-3 and EPA in the meat, while in most cases no effects on the content of DHA were observed. Increasing the amount of DHA was mainly obtained when marine products were included in the animal diet.

The increase of intake of linseed oil or fish oil by ruminants can also lead to increased concentration of Δ 11-18:1 and CLA in intramuscular fat, up to 4.5 and 0.63% on a percentage basis, respectively.^{164,172} In most studies, an increased n-3 PUFAs content in meat was accompanied by a decreased n-6 proportion and consequently a more favorable n-6/n-3 ratio. However, there is some concern regarding the shorter shelf life and lower organoleptic quality of n-3 enriched meats, which seem to show decreased oxidative stability and unpaired flavor.^{168,169} It is important to note that the recommended n-6/n-3 ratio in the human diet varies from 1 to 4, depending on which disease would be prevented.²³

The available information on trans fatty acid content of meat and meat products from different animal species is summarized in Table 13.2. It is worth considering the limitations of such data with regard to the following aspects. All data are presented as percentage of total fatty acids, without coupling these values with the fat content of different food items; thus, they are of limited value in estimating the contribution of meats to the intake of trans fatty acids in humans. When presented on an absolute basis in original articles (i.e., mg/100 g food), data were converted into percentages, when possible, to permit better comparison.

As described in Section 13.4.2, the different resolution capability of the various analytical methods leads to wide variations in the number of isomers detected and in quantitative results among laboratories. The trans monoenes predominate in literature references. Several authors reported the value of trans fatty acids as “*trans*-18:1,” without any information on the position of the double bond. In these cases it is assumed that vaccenic acid is the predominant trans fatty acid. In other cases the Δ 11-18:1 is referenced, but when 30- or 60-m cyanopropyl columns were used for GC analysis a probable overestimation of this fatty acid due to isomer co-elutions could occur. Data on CLAs content of meat and meat products are intentionally omitted.

Table 13.2 Trans Fatty Acids in Meat and Poultry Products

<i>Animal</i> ^a	<i>Food Items</i>	<i>Trans-18:1</i> ^b (wt%)	<i>Trans-18:2</i> ^c (wt%)	<i>Total Trans</i> (wt%)	<i>References</i>	
	Sausage (4)	1.41 ± 0.26	0.21 ± 0.02	1.62 ± 0.28	173	
	Souk (fermented sausage) (4)	1.75 ± 0.30	0.26 ± 0.14	2.01 ± 0.51		
	Salami (5)	0.46 ± 0.43	0.22 ± 0.07	0.68 ± 0.64		
	Pastrami (4)	0.55 ± 0.52	0.06 ± 0	0.61 ± 0.59		
	Beef ham (2)	1.17 ± 0.15	0.22 ± 0.02	1.40 ± 0.26		
	Adana kebab (4)	0.76 ± 0.68	0.32 ± 0.13	1.08 ± 0.34		
	Meat doner (Gyro) (4)	1.50 ± 0.17	0.36 ± 0.06	1.86 ± 1.04		
	Meatball, frozen (6)	1.21 ± 0.73	0.29 ± 0.02	1.50 ± 0.61		
	Beef burger, fried (3)	2.08 ± 0.08	0.24 ± 0.04	2.33 ± 0.10		
	Chicken ham (2)	0.33 ± 0.04	0.09 ± 0.02	0.42 ± 0.006		
	Chicken doner (3)	1.54 ± 0.81	0.23 ± 0.22	1.77 ± 0.93		
Bovine	IM fat	5.21 ± 0.28				174
	IT fat	10.70 ± 0.28				
	SC fat	8.89 ± 0.28				
Sheep	IM fat	2.80–3.96 ^d			175	
Bovine	IM fat			1.28 ± 1.01 ^e	98	
	SC fat			9.15 ± 3.09 ^e		
Beef	Rump	2.5	0.5		176	
	Round	1.9	0.4			
	Topside/silverside	3.4	0.4			
	Blade	2.1	0.6			
	Meat fat pooled	3.4	0.9			
Veal	Leg steak	1.9	0.4			
	Cutlet	2.1	0.4			
	Stir fry/diced	2.2	0.4			
	Meat fat pooled	5.9	1.1			
Lamb	Leg pooled	2.6	0.8			
	Loin	3.0	0.7			
	Forequarter cuts-pooled	2.7	1.0			
	Meat fat pooled	6.0	2.0			
Mutton	Leg	3.0	0.7			
	Casserole	2.7	1.1			
	Meat fat pooled		2.1			
	Fermented sausages	0.17–0.23		1.07–1.22		
	Breaden chicken (cooked)	27.4			177	
	Meat patty	6.8			178	
Bison	ST	0.52				
Elk	ST	0.45				
	LD	0.29				
	SS	0.36			179	
Chicken	Breast	0.36				

Table 13.2 (Continued)

<i>Animal^a</i>	<i>Food Items</i>	<i>Trans-18:1^b</i> (wt%)	<i>Trans-18:2^c</i> (wt%)	<i>Total Trans</i> (wt%)	<i>References</i>
Bovine	IM fat	1.3–7.00		2.78–9.52	96
Lamb/ Mutton	IM fat	2.92–6.70		4.32–9.19	
Pork	IM fat	0.17–1.60		0.19–2.23	
Chicken	IM fat	0.16–1.03		0.38–1.71	
Elk	Meat, raw	1.31	0.32	2.15	
Moose	Steak, leg	0.85	0.16	1.70	
Reindeer	Meat, raw	1.10	0.18	2.19	
	Meat, leg	1.03	0.09	1.72	
Turkey	Raw with skin	0.24–0.63	0.01–0.06	0.31–0.76	
	Flesh and skin	0.26	0.02	0.57	
	Raw	0.47	0.09	0.60	
	Roasted meat	0.50	0.25	1.16	
	Minced meat	0.78	0.15	1.11	
	Whole	0.27–0.84	0.22	0.73–1.27	
Duck	Flesh and skin, raw	0.62	0.04	0.67	
	Roasted	0.23	0.05	0.33	
Rabbit	Unprepared	0.36	0.09	0.61	
	Roasted	0.34	0.15	0.63	
	Raw	0.25	0.09	0.61	
Horse	Minced meat	0.19	0.05	0.45	
	Sausages	0.25–3.53		0.25–4.86	
Beef	Thick flank	0.8–4.5			180
	Outside round	0.3–3.4			
	Chuck	1.3–6.6			
	Rump	0.4–3.3			
	Brisket	1.4–3.8			
Veal	Thick flank	3.7–13.7			
	Outside round	0.7–3.9			
	Chuck	0.8–14.0			
	Rump	0.7–3.0			
	Brisket	1.6–10.9			
Lamb	Shoulder	1.9–7.8			
	Leg	1.3–8.9			
	Loin	1.0–10.5			
	Meat patty	3.09			181
	Luncheon meat	5.42			
Beef	IM fat	2.75 ± 1.28			182
Lamb	IM fat	4.67 ± 1.67			
Beef	Meat (40)			3.8 ± 0.8	119
	Sausages (40)			0.7 ± 0.5	
	Hamburger (50)			4.1 ± 0.7	
Bovine	Beef meat (10)			1.95 ± 0.94	183
	Beef tallow (2)			4.6	
	Meat patty			3.5	184

(Continued)

Table 13.2 (Continued)

<i>Animal</i> ^a	<i>Food Items</i>	<i>Trans-18:1</i> ^b (wt%)	<i>Trans-18:2</i> ^c (wt%)	<i>Total Trans</i> (wt%)	<i>References</i>
Turkey	Ground turkey	1.6–4.3	0.3–0.8		185
	Turkey composites	2.1–3.2	0.2–0.5		
Beef (4)				2.73 ± 0.56	186
Veal (3)				1.37 ± 0.42	
Lamb (3)				7.53 ± 1.14	
Mutton (3)				9.30 ± 1.21	
	Sausages (22)			0.68 ± 0.67	
Pork	Filet			0.2	
	Bacon			0.4	
	Ham, cooked			0.2	
	Ham, smoked			0.5	
Poultry	Rooster			0.5	
	Duck			0.5	
	Turkey			1.4	
	Wild pigeon			0.2	
	Sausages			1.7 (0.6–6.4)	
Bovine (45)	Beef meat			8.5 ± 2.7	188
Pork (35)	Pork meat			0.6 ± 1.0	
	Meat products (46)			0.5 ± 0.3	
Bovine	14 Retail cuts, lean portion raw (269)			3.20	189
Pork	7 Retail cuts raw			0.2	190
	cooked			0.3	
Bovine	Raw (1)			4.58	191
	Cooked (1)			5.00	
	Liver lipid (1)			0.91	
	Strained beef liver (1)			1.90	
	Strained lamb broth (1)			7.57	

^a In some cases the animal species of origin of processed food is unknown.

^b Where not specified the position of the double bond is unknown.

^c Values do not include the CLA content.

^d $\Sigma t-18:1 = t10-18:1 + t11-18:1$.

^e Total trans = $t-16:1 + t9-18:1 + t11-18:1 + t,t-18:2$.

Note: IM: Intramuscular, IT: Intermuscular, SC: Subcutaneous, ST: *Semitendinosus* muscle, LD: *Longissimus dorsi* muscle, SS: *supraspinatus* muscle.

Table 13.3 Omega-3 Fatty Acids in Meat and Poultry Products

<i>Animal</i>	<i>Food Items</i>	$\Sigma n-3$ (wt%)	<i>n-3 Fatty Acids Detected</i>	<i>Diet</i>	<i>n-6/n-3</i>	<i>References</i>
Pig	LD	0.41–0.54	18:3		17.05–23.01	193
Pig	LD	0.29–0.39	18:3, 20:3, 20:5, 22:5, 22:6			194
Pig	LD	3.32	18:3, 20:5, 22:5, 22:6	Tallow, soybean oil	7.3	195
Pig	LD	4.28–4.34	18:3, 20:5, 22:5, 22:6	Rapeseed oil, fish oil	4.5–4.6	
Pig	LD	0.72	18:3, 20:3	Tallow		196
Pig	LD	0.73	18:3, 20:3	Corn oil		
Pig	LD	1.00	18:3, 20:3	Rapeseed oil		
Pig	Salami	0.77–1.34	18:3, 20:5, 22:6		7.83–10.73	87
Pig	Muscle (loin steak)	2.4	18:3,20:3, 20:4, 20:5, 22:5, 22:6			182
Pig	Salami	1.27	18:3, 20:5, 22:5, 22:6	Maize, rice bran	16.80	197
Pig	Salami	0.56	18:3, 20:5, 22:5, 22:6	Maize	18.85	
Pig	Sausage	2.13–2.48	18:3, 20:5, 22:5, 22:6	Tallow, soybean oil	7.8	195
Pig	Sausage	3.12–3.44	18:3, 20:5, 22:5, 22:6	Rapeseed oil, fish oil	4.6–5.0	
Pig	Parma ham	0.97	18:3, 20:3	Standard		198
Pig	Parma ham	0.83	18:3, 20:3	Corn oil		
Pig	Parma ham	1.15	18:3, 20:3	Rapeseed oil		
Pig	Iberian ham	0.58	18:3			199
Pig	Lard	0.42	18:3		22.38	89
Bovine	Muscle (loin steak)	1.56	18:3, 20:3, 20:4, 20:5, 22:5, 22:6			182
Bovine	LD	2.54	18:3, 20:5, 22:5, 22:6	Grass	1.2	200
Bovine	LD	1.38	18:3, 20:5, 22:5, 22:6	Concentrate	8.9	
Bovine	LD	1.81	18:3, 20:5, 22:5, 22:6	Grass		201
Bovine	LD	0.77	18:3, 20:5, 22:5, 22:6	Concentrate		
Bovine	TB	3.67	18:3,20:3, 20:4, 20:5, 22:5, 22:6	Grass		

(Continued)

Table 13.3 (Continued)

<i>Animal</i>	<i>Food Items</i>	$\Sigma n-3$ (wt%)	<i>n-3 Fatty Acids Detected</i>	<i>Diet</i>	<i>n-6/n-3</i>	<i>References</i>
Bovine	TB	1.92	18:3, 20:4, 20:5, 22:5, 22:6	Concentrate		
Bovine	GB	3.90	18:3, 20:3, 20:4, 20:5, 22:5, 22:6	Grass		
Bovine	GB	1.72	18:3, 20:4, 20:5, 22:5, 22:6	Concentrate		
Bovine	GM	4.26	18:3, 20:3, 20:4, 20:5, 22:5, 22:6	Grass		
Bovine	GM	1.61	18:3, 20:4, 20:5, 22:5, 22:6	Concentrate		
Bovine	LT	1.68	—		10.45	174
Bovine	SC	0.34	—		10.08	
Bovine	LT	0.34	18:3, 20:5, 22:5, 22:6	Standard	9.81	202
Bovine	LT	0.71	18:3, 20:5, 22:5, 22:6	Fish oil	3.92	
Bovine	Muscle (loin steak)	1.03	18:3, 20:5, 22:6			182
Bovine	Leg muscle	0.40	18:3, 20:5			203
Bovine	Cured meat (bresaola)	0.68	18:3, 20:5, 22:6			88
Bovine	LD	2.9	18:3, 18:4, 20:3, 20:5, 22:5, 22:6	Range-raised	1.95	179
Bovine	LD	0.64	18:3, 18:4, 20:3, 20:5, 22:5, 22:6	Feedlot	6.38	
Chicken	White meat (breast)	1.19	18:3, 18:4, 20:3, 20:5, 22:5, 22:6		18.5	
Chicken	White meat (breast)	19.8	18:3, 20:5, 22:5, 22:6	Cod liver oil		169
Chicken	White meat (breast)	18.3	18:3, 20:5, 22:5, 22:6	Linseed oil		
Chicken	White meat (breast)	6.8	18:3, 20:5, 22:5, 22:6	Standard		
Chicken	White meat (breast)	3.2	18:3, 20:5, 22:6	Standard		170
Chicken	Dark meat (leg)	2.2	18:3, 20:5, 22:6	Standard		

Table 13.3 (Continued)

<i>Animal</i>	<i>Food Items</i>	$\Sigma n-3$ (wt%)	<i>n-3 Fatty Acids Detected</i>	<i>Diet</i>	<i>n-6/n-3</i>	<i>References</i>
Chicken	Skin	1.2	18:3, 20:5, 22:6	Standard		
Chicken	White meat (breast)	5.0–5.8	18:3, 20:5, 22:6	Fish meal		
Chicken	Dark meat (leg)	3.2	18:3, 20:5, 22:6	Fish meal		
Turkey	White meat (breast)	7.5–8.1	18:3, 20:5, 22:6	Fish meal		
Turkey	Dark meat (leg)	5.2–5.4	18:3, 20:5, 22:6	Fish meal		
Turkey	Leg muscle	1.50	18:3, 18:4, 20:3, 20:5, 22:5, 22:6			203
Turkey	Hamburger	0.8	18:3, 20:5, 22:6		27	204
Turkey	Frankfurter	1.3	18:3, 20:5, 22:6		21	
Turkey	Smoked ham	1.2	18:3, 20:5, 22:6		23	
Lamb	Muscle (steak loin)	2.50	18:3, 20:5, 22:5, 22:6			182
Lamb	LD	0.51	18:3	Rapeseed meal		205
Lamb	LD	0.54	18:3	Rapeseed–soybean meal		
Lamb	LD	0.60	18:3	Soybean meal		
Lamb	TB	0.56	18:3	Rapeseed meal		
Lamb	TB	0.60	18:3	Rapeseed–soybean meal		
Lamb	TB	0.64	18:3	Soybean meal		
Lamb	SM	0.58	18:3	Rapeseed meal		
Lamb	SM	0.59	18:3	Rapeseed–soybean meal		
Lamb	SM	0.67	18:3	Soybean meal		
Sheep	Muscle (early lamb)	3.30	18:3, 20:5, 22:5, 22:6		1.36	206
Sheep	Muscle (Merino)	1.96	18:3, 20:5, 22:5, 22:6		6.45	
Sheep	Muscle (Aragonesa)	3.22	18:3, 20:5, 22:5, 22:6		3.91	
Sheep	Muscle (Welsh Mountain)	3.51	18:3, 20:5, 22:5, 22:6		0.99	
Goat	Thigh	4.22	18:3, 20:5, 22:5, 22:6		1.69	207

(Continued)

Table 13.3 (Continued)

Animal	Food Items	$\Sigma n-3$ (wt%)	<i>n-3 Fatty Acids</i>		<i>n-6/n-3</i>	References
			Detected	Diet		
Goat	Rib-LD	1.20	18:3			208
Goat	BF	2.18	18:3			209
Goat	LT	0.71	18:3			210
Boar	Cured meat	1.70	18:3, 20:5, 22:6			88
Horse	Cured meat	5.49	18:3, 20:5, 22:6			88
Bison	LD	5.35	18:3, 18:4, 20:3, 20:5, 22:5, 22:6	Range-raised	1.94	179
Bison	LD	1.51	18:3, 18:4, 20:3, 20:5, 22:5, 22:6	Feedlot	5.73	
Elk	LD	5.00	18:3, 18:4, 20:3, 20:5, 22:5, 22:6	Free-ranging	2.84	
Ostrich	Leg muscle	2.60	18:3, 18:4, 20:3, 20:5, 22:5, 22:6			203

Note: LD: *Longissimus dorsi*, LT: *Longissimus thoracis*, SM: semimembranous, TB: *Triceps brachii*, GB: *Gluteobiceps*, GM: *Gluteus medius*, SC: subcutaneous adipose tissue.

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Chapter 14

Methods to Measure the Antioxidant Capacity of Meat Products

Volker Böhm and Lars Müller

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14.1 Introduction

Antioxidants have always been of interest for food chemists because they prevent rancidity. They have become of interest to biologists and clinicians due to their ability to protect the human body against damage by reactive oxygen species (ROS). Antioxidants are more than chain-breaking inhibitors of lipid peroxidation. Halliwell et al. [1] defined antioxidants as “any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate.” The shelf life of meat and meat products largely depends on the stability of their fat portions. The addition of natural spices (e.g., rosemary, sage, paprika), extracts of spices, essential oils, mixtures of spicy extracts, and ascorbic acid or tocopherols and synthetic antioxidants can inhibit fat breakdown [2,3]. However, minor components of meat, such as vitamin E (tocopherols, tocotrienols), conjugated linoleic acids, selenium, glutathione, carnosine, anserin, carnitine, and enzymes with antioxidant activity (glutathione peroxidase, superoxide dismutase [SOD], and catalase [CAT]), also influence the stability of meat products [4–7].

This chapter illustrates the existing methods to determine the antioxidant activity of hydrophilic and lipophilic antioxidants and most of the few assay comparisons.

14.2 Methods to Measure the Antioxidant Capacity

14.2.1 Assays for Detecting Thiobarbituric Acid-Reactive Substances

The assays to measure thiobarbituric acid-reactive substances (TBARS) are often used to determine lipid peroxidation. Malondialdehyde (MDA), which is formed as a product of lipid peroxidation, forms with thiobarbituric acid (TBA) a pink pigment with an absorbance maximum at 532 nm [8]. Many other compounds, like alkanals, proteins, sucrose, and urea, can also react with TBA to color molecules that interfere with the assay. Therefore, the TBARS reaction is nonspecific.

In the TBARS studies of Ruiz et al., high β -carotene concentrations proved to be prooxidative under decrease of tocopherol content [9]. The results of the TBARS studies conducted by Djenane et al. with fresh beefsteaks showed that the combination of carnosine with ascorbic acid provided the best antioxidative protection with regard to meat deterioration [10]. Carnitine was significantly less effective than any other antioxidant in delaying meat oxidation [10]. Sanchez-Escalante et al. [11] analyzed the effects of typical meat spices as natural antioxidants.

The TBA values demonstrated that the addition of ground peppers—both sweet and hot—to beef patties delayed and inhibited very significantly the oxidation of both myoglobin and lipid [12]. Capsaicin and lycopene also showed a significant antioxidative effect. Rosemary, oregano, and borage inhibited myoglobin oxidation and color fading [13]. Bedinghaus and Ockerman [14] already demonstrated the antioxidant activity of Maillard reaction products (MRP) in ground pork patties. The MRP, prepared by heating three individual reducing sugars (glucose, xylose, and dihydroxyacetone) under reflux with five amino acids (arg, his, leu, lys, and trp), added to fresh patties (3% v/w) and cooked until interior temperature had reached 68°C. The measured TBA values clarified that the most effective MRP was xylose-arginine when compared with the control [14]. Morrissey and Tichivangana [15] demonstrated that the addition of nitrite (20 mg/kg) caused a significant inhibition of lipid peroxidation, measured by the TBA test, in a cooked muscle system. Nitrite (50 mg/kg) resulted in a highly significant reduction in TBA values.

To advance the assay, column chromatographic methods (high-performance liquid chromatography [HPLC], gas chromatography) were developed to separate the MDA from other compounds prior to measurement. First, Jardine et al. [16] investigated the main TBARS reaction products via liquid chromatography-mass spectrometry (LC-MS) method. As model lipid they used linoleic acid, which was oxidized by cupric ions. They verified that the formation of the pink pigment happens by condensation of TBA and MDA in a 2:1 molar ratio.

14.2.2 Assays to Measure Hydrophilic Antioxidants

14.2.2.1 Trolox Equivalent Antioxidant Capacity Assay

The system is based on the application of an enzyme with peroxidase activity (e.g., horseradish peroxidase) which oxidizes 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in the presence of hydrogen peroxide to $ABTS^{\bullet+}$, first investigated by Childs and Bardsley [17]. The current version of the trolox equivalent antioxidant capacity (TEAC) assay, which has been widely used in the last decades for determination of antioxidant activity, was developed by Miller et al. [18]. They used the peroxidase activity of metmyoglobin. Antioxidant reductants and hydrogen donors quench the absorbance of the resulting long-lived radical cation $ABTS^{\bullet+}$ in relation to their antioxidant capacity. Trolox, a water-soluble vitamin E analog acts as calibration standard. Miller et al. [18] measured the absorbance of the bluegreen colored radical cation at 734 nm exactly 6 min after starting oxidation, whereas other laboratories measured the lag phase until the formation of $ABTS^{\bullet+}$ started [19,20]. A critical point of this assay is the overlay of two mechanisms—the delay of the radical formation and the radical quenching ability of the antioxidant. Some antioxidants are able to delay the generation of $ABTS^{\bullet+}$, others quench the generated radical cation and some are able to react in both ways [21].

Today, a couple of modified TEAC assays exist in which a preformed $ABTS^{\bullet+}$ is used. Miller et al. [22] prepared the radical cation with manganese dioxide followed by membrane filtration. Re et al. [23] formed the colored molecule with potassium peroxodisulfate in darkness within 12–16 h prior to use. The method with MnO_2 as oxidant has the potential to form the $ABTS^{\bullet}$ and the $ABTS^{\bullet+}$, whereas the method with $K_2S_2O_8$ only produces the $ABTS^{\bullet+}$. After diluting the $ABTS^{\bullet+}$ solution to an absorbance of 0.70 ± 0.02 at 734 nm and mixing with the samples, the absorbance was taken [23,24].

Gatellier et al. [6] already published a study that showed that the diet finishing mode—pasture or mixed diet—had no significant effect on the antioxidant status, measured as TEAC values, of bovine meat (*M. longissimus dorsi*).

14.2.2.2 2,2-Diphenyl-1-Picrylhydrazyl Assay

The stable, commercially available radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) has an absorbance maximum around 515 nm, which decreases upon reaction with antiradical compounds.

The DPPH• and the antioxidant, both solved in methanol, are mixed [25]. The decrease of absorbance is measured in constant time intervals until the reaction reaches a plateau phase. Trolox is used for calibration. The antiradical activity is defined as the amount of antioxidant which is necessary to decrease the basal DPPH• concentration to 50% (efficient concentration = EC₅₀ (mol/L AO/mol/L DPPH•)). The antiradical power is calculated as 1/EC₅₀ [25].

Koleva et al. [26] developed a HPLC method with on-line DPPH detection. After separation of the compounds and ultraviolet (UV)-detection at 254 nm, a postcolumn reaction with ethanolic DPPH• solution occurred in which the decrease of absorbance was detected photometrically as negative peak at 517 nm [26]. Recently, a high-throughput DPPH• method was presented by Cheng et al. [27]. The decrease of absorbance was measured in 96 well microplates in a plate reader every minute up to 1.5 h and calculated as the area under the curve.

The studies of the antioxidant activity of egg-yolk protein hydrolysates, measured with the DPPH• assay by Sakanaka and Tachibana [28], showed that it is a free radical scavenger, particularly of the peroxy radical, which is the major propagator of the oxidation of fat chains. When incorporated into beef and fatty tuna homogenates, egg-yolk protein hydrolysates effectively inhibited lipid peroxidation in both homogenates.

14.2.2.3 β -Carotene Bleaching Assay

The β -carotene bleaching assay was developed by Marco in 1968 [29]. The method is based on the combined oxidation of β -carotene and linoleic acid, whereas degradation products of linoleic acid oxidize β -carotene. This bleaching of the orange colored carotenoid is measured spectrophotometrically at 450 nm [29].

Taga et al. [30] modified the assay by using a solution of β -carotene in chloroform with an absorbance between 0.60 and 0.90 at 470 nm. This solution was mixed with linoleic acid and Tween 40 as solubility enhancer. After removal of chloroform by using a rotary evaporator at 40°C, slowly adding oxygenated distilled water to build an emulsion, the antioxidant solution was added and the absorbance was measured continuously in cuvettes heated to 50°C until the absorbance of the blank sample without antioxidant was about 0.03.

Eminagaoglu et al. [31] used the β -carotene bleaching assay to analyze the essential oil and the methanolic extracts of different species of savory on their antioxidant activity. But also animal originated beastlike compounds, for example, casein calcium peptides, showed a strong antioxidant activity with the β -carotene bleaching test [32].

In 1984, Bors et al. [33] published a rapid test similar to the β -carotene bleaching assay, which used the bleaching of the carotenoids crocin, and canthaxanthin.

14.2.2.4 Superoxide Radical Scavenging Activity Assay

This assay is based on the scavenging activity of meat extracts against superoxide radicals, which were generated *in vitro* by xanthine oxidase (XOD) at 37°C. After the XOD reaction is stopped with sodium dodecyl sulfate (SDS), the scavenging capacity is determined by using nitro-blue tetrazolium (NBT) reduction. In this method, O₂^{•-} reduces the yellow dye (NBT²⁺) to a blue-colored formazan, which is measured spectrophotometrically at 560 nm. Antioxidants were able to inhibit the formation of the blue formazan NBT [34,35]. The results were calculated as the percentage inhibition.

Sakanaka et al. [36] used this method to analyze the oxygen scavenging activity of egg-yolk protein hydrolysates and their effect on lipid peroxidation. Their results suggested that egg-yolk protein hydrolysates are good sources of natural antioxidants.

14.2.2.5 Total Radical-Trapping Antioxidant Parameter Assay/ Oxygen-Radical Absorbance Capacity Assay

Wayner et al. first published the total radical-trapping antioxidant parameter (TRAP) in which 2,2'-azobis-(2-amidinopropane hydrochloride) (ABAP=AAPH) decomposes thermally and yields peroxy radicals in a constant rate. The period of inhibited oxygen uptake by plasma antioxidants was measured using an oxygen electrode. Trolox was used as standard like in the oxygen-radical absorbance capacity (ORAC) assay a few years later [37].

The (total) ORAC used by Cao et al. [38] is based on the fluorescence degradation of the fluorescent protein B-phycoerythrin (B-PE) from *Porphyridium cruentum* by peroxy radicals built by decomposition of ABAP at 37°C. In intervals of 5 min, fluorescence was measured at an emission wavelength of 565 nm and an excitation wavelength of 540 nm. The ORAC value defines the net protection area under the fluorescence curve (AUC) of B-PE quenched by the antioxidant [38]. Wayner et al. [37] and Ghiselli et al. [39] calculated the TRAP/ORAC value by measuring the lag time to avoid interactions from plasma or lipids whereas Cao et al. [38] measured the fluorescence until reaction was completed. Measuring the ORAC value by using a fluorescent protein showed problems with the oxidative damage of the protein.

Ou et al. [40] described an improved version of the ORAC assay by using fluorescein instead of B-PE as fluorescent probe. In comparison with the B-PE method these ORAC values were higher due to the lower photostability of B-PE compared to fluorescein. The high cost of B-PE was another disadvantage [40]. This optimized version of the ORAC assay became very popular due to the low cost and the possibility to determine the hydrophilic chain-breaking antioxidant capacity against peroxy radicals. Huang et al. [41] developed this version to a high-throughput one in 96 well microplates on a multichannel system with a microplate fluorescence reader.

Glazer [42] analyzed the protective role of creatinine with the TRAP system and showed that creatinine did not protect ascorbic acid against peroxy radicals and cupric ions.

14.2.2.6 Photochemiluminescence Assay

In the photochemiluminescence (PCL) assay the sensitive detection by using chemiluminescence (CL) takes place after photochemical generation of free radicals. The reaction is initialized by optical excitation of a photosensitizer *S* resulting in the generation of the superoxide radical $O_2^{\bullet-}$ [43]:



Luminol, a chemoluminescent reagent for detection, acted to visualize the free radicals. This substance has abilities as photosensitizer as well as oxygen radical detection reagent [43].

Vichi et al. [44] analyzed the antioxidant activity of lard stabilized with extracts of sage or oreganum by using the photoluminescence analysis. In comparison with the control lard the samples with extracts (5 µg extract/g lard) showed higher radical scavenging capacities. However, pigs fed with feed additives did not show a higher antioxidant activity than control samples [44].

14.2.2.7 *Chemiluminescence*

To evaluate the antioxidant capability of certain antioxidants, quenching of CL is also used. Ashida et al. [45] published a method using methyl linoleate hydroperoxide (MLHPO) and luminol/cytochrome *c* to initiate CL. MLHPO reacts with cytochrome *c* to an oxyradical (MLO[•]), which generated CL by oxidation of luminol. Added antioxidants in the system scavenge free radicals and CL intensity decreases. When all antioxidants are consumed, the light emission restores. The lag phase that accrued is directly related to the concentration of antioxidants in the reaction mixture [45]. Robinson et al. [46] added an enhancer phenol (e.g., *p*-iodophenol) to produce a longer and more stable light emission with higher intensity. Hydrogen peroxide was used as oxidant. An online HPLC-CL method based on the luminol CL was published by Dapkevicius et al. [47].

The CL studies of Mozdzan et al. [48] showed that carnosine, a β -alanyl-L-histidine dipeptide, which is found at relatively high concentrations in skeletal muscles and brain, has a significant antioxidant activity especially in the presence of transition metals. Nagatsuka et al. [49] analyzed a typical Japanese gelatin gel, so-called “Nikogori,” among others with a modified CL assay with AAPH as radical generator. The data showed a high antioxidative level of Nikogori gelatin gel from chicken wing meat and beef shin meat.

14.2.2.8 *Ferric Reducing Ability of Plasma/Ferric Reducing Antioxidant Power Assay*

A simple measurement of the reducing ability of antioxidants is given with the ferric reducing ability of plasma/ferric reducing antioxidant power (FRAP) assay, first described by Benzie and Strain in 1996 [50]. In comparison to many other test systems it does not need any radical, because only the reducing activity and not the radical quenching ability is determined. After mixing the FRAP reagent, composed of ferric chloride, 2,4,6-tripyridyl-*s*-triazine (TPTZ) in hydrochloric acid and acetate buffer (pH 3.6), the reading of absorbance at 593 nm is started immediately up to 8 min [50]. Some years later, a few changes made it possible to use a microplate reader [51]. The reducing capability is calculated using the absorbance difference between sample and blank. A ferrous standard solution acted as standard.

Benzie and Strain [52] also published a method called ferric reducing/antioxidant power and ascorbic acid concentration to determine the total antioxidant power, the concentration of ascorbic acid, and the relative contribution of ascorbic acid to the antioxidant power simultaneously. Care should be taken if FRAP values of antioxidants are measured in alcoholic media. Pulido et al. discovered differences in comparison with FRAP values determined in water [53].

Bower et al. [54] measured FRAP values from beef jerky products with added raisins. A formulation with 15% raisins had antioxidant values more than 600% higher than the control samples without raisins. The high antioxidant level may prevent lipid peroxidation, but the raisins can lead to off-flavor during processing [54].

14.2.2.9 *Linoleic Acid Oxidation Assay/Ferric Thiocyanate Method*

This simple redox assay is based on work of Mitsuda et al. [55], modified by Chen et al. [56]. After mixing the extracts, dissolved in phosphate buffered saline (PBS), with linoleic acid, dissolved in ethanol, the reaction mixture is stored at 60°C in the dark for a defined time.

In several intervals aliquots of the mixture are added to a solution of ammonium thiocyanate and ferrous chloride—dissolved in 3.5% HCl—and the absorbance of the colored solution is measured at 500 nm after 3 min.

The assay was used by Sakanaka et al. [36] and they showed that egg-yolk protein hydrolysates could be suitable as natural antioxidants to prevent oxidation of polyunsaturated fatty acids and related food ingredients.

14.2.2.10 Hydroxyl Radical Scavenging Activity Assay

The assay is based on the oxidative degradation of 2-deoxyribose to MDA by hydroxyl radicals (OH^\bullet) formed in the Fenton reaction. The Fenton reaction between Fe^{2+} and hydrogen peroxide generates hydroxyl radicals which degrade 2-deoxyribose to MDA. The reaction takes place in PBS and is started by adding H_2O_2 . Added antioxidants can inhibit the decomposition. After an incubation period of 4 h the reaction is stopped by adding trichloroacetic acid and TBA. The mixture is boiled for 10 min followed by cooling to room temperature. The measured absorbance at 520 nm is used to evaluate the hydroxyl radical scavenging ability. The results are given in percentage inhibition of 2-deoxyribose by hydroxyl radicals [57].

By using a modified version of this assay Lee and Hendricks [58] analyzed the effect of L-carnosine on the antioxidant status of ground beef homogenates. The study showed that this dipeptide prevented oxidation and could be useful to increase the shelf life of meat products. Chung [59] studied antioxidative abilities of garlic compounds (allyl cysteine, alliin, allicine, and allyl disulfide) with this assay and published that these substances exhibit different protective effects against free radical damage.

14.2.2.11 Electron Spin Resonance/Electron Paramagnetic Resonance Assay

The inhibition of the free radical process' initiation due to antioxidants can be determined by using the electron spin resonance (ESR) or electron paramagnetic resonance spin trapping assay. After formation of hydroxyl radicals by homolytical cleavage of hydrogen peroxide by UV light, or by the Fenton reaction, antioxidants decrease the ESR signal depending on their activity.

Carlsen et al. [60,61] used this technique to determine the antioxidative action of pork. The water-soluble protein fraction had the highest antioxidative potential compared to salt-soluble and insoluble fractions. Heat-treated minced meat showed prooxidative effects to lipid peroxidation assigned to myoglobin and hemoglobin derivatives. Different extracts of Parma ham, an Italian dry-cured ham, were analyzed by Adamsen et al. [62] using ESR spectroscopy to evaluate their efficiencies as scavengers of free radicals. The heme moiety of pigments in aqueous phosphate extract showed antioxidative properties. The more lipophilic pigment, extracted with acetone/water, had the most significant effect and the tests to inhibit lipid peroxidation in cooked pork showed that the pigments protected α -tocopherol against degradation [62]. High-pressure treatment is known to induce lipid peroxidation. Bragagnolo et al. [63] studied the potential of rosemary in cooked and in high-pressure treated (600 MPa, 10 min) minced chicken breast by ESR. Adding rosemary to the product before high-pressure treatment is effective in protecting against formation of "pressed-over-flavor" upon subsequent cooking.

14.2.2.12 *Electrochemical Assay*

The electrochemical assay is based on a flow injection system (FIS) equipped with an electrochemical detector with a glassy carbon working electrode which works amperometrically at a potential of 0.5 V [64].

The FIS is based on the amperometry and uses the measurement of the oxidation and reduction of an electroactive compound at the working electrode by applying a continuous potential. The determined current is related to the electrochemical reaction rate. The electrochemical assay was successfully used for a direct, fast, and reliable checking of the antioxidant capacity in lipophilic food extracts, which is a great advantage compared to most other exclusively hydrophilic methods.

Bortolomeazzi et al. [65] analyzed the antioxidant capacities of main phenolic compounds present in wood smoke used in the food industry. In addition to DPPH• scavenging and crocin bleaching assay, a cyclic voltammetric experiment was conducted. All three methods, although based on different approaches, showed high antioxidant activities of wood smoke phenols. But the effect of these compounds on the oxidative stability of smoked foods needs further investigations [65].

14.2.2.13 *Ferrous Oxidation Xylenol Orange Assay*

Nourooz-Zadeh et al. [66] used the ferrous oxidation xylenol orange (FOX) assay to measure lipid hydroperoxides (ROOHs) in low-density lipoprotein (LDL). The assay is based on the oxidation of ferrous ions to ferric ions in the presence of various oxidant species (e.g., hydroperoxides [H₂O₂]) in acidic medium. Xylenol orange, *o*-cresolsulfonphthalein-3,3-bis (methyliminodiacetic acid sodium salt), forms a blue-purple colored complex with the ferric ion, being measurable at 560 nm. The color intensity, which is measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. Hydrogen peroxide was used for calibration. The use of butylated hydroxytoluene (BHT) during the incubation was necessary to prevent lipid chain oxidation during ferrous oxidation. With the help of triphenylphosphine the measurement of plasma ROOHs is possible [66]. The advantage of the FOX assay was that the kinetics of the reaction were independent of the chemical structure of ROOHs [67]. The easy, stable, reliable, sensitive, and cost-saving procedure made the method possible for fully automated use and to measure total oxidant status [68].

Grau et al. [69] used the FOX assay to measure the lipid hydroperoxides in raw and cooked dark chicken meat.

14.2.3 *Assays to Measure Lipophilic Antioxidants*

A high number of assays exist to measure antioxidant activity based on aqueous radical generators and on hydrophilic markers. It is not possible to determine the antioxidant activity of lipidic compounds and of lipophilic ingredients of plasma such as carotenoids with these methods. But recently a few mainly spectrophotometric and fluorescence assays were developed.

14.2.3.1 *Trolox Equivalent Antioxidant Capacity Assay*

The basic principles of the TEAC assay are explained in Section 14.2.2.1. Miller et al. [24] published a TEAC version to measure the antioxidant activity of lipophilic antioxidants especially carotenoids

with a preformed $\text{ABTS}^{\bullet+}$ prepared in aqueous solution by using manganese dioxide. The $\text{ABTS}^{\bullet+}$ solution was diluted with PBS to an absorbance of 0.70 ± 0.02 at 734 nm. The carotenoids were dissolved in acetone and diluted in hexane/acetone 90:10. After mixing both solutions, the mixture was vortexed for a defined time and the absorbance at 734 nm was taken. Trolox was used for standard calibration. Böhm et al. [70] modified the lipophilic TEAC slightly. They dissolved the carotenoids in hexane. After mixing the solutions a centrifugation of the mixture at 14,000 rpm for 1 min was followed by measuring the absorbance of the lower hydrophilic bluegreen-colored layer at 734 nm exactly 2 min after starting the mixing [70]. In 2002, Cano et al. published an on-line HPLC method to measure both hydrophilic and lipophilic TEAC values [71].

The major lipophilic antioxidants in tomatoes were phenolics, flavonoids, and lycopene [72,73].

The lipophilic TEAC assay only determines the radical quenching capacity of the antioxidant compound. Actually, the lipophilic TEAC was often used to measure the antioxidant capacity of plant extracts, but scarcely in meat products.

14.2.3.2 Lipophilic Oxygen Radical Absorbance Capacity Assay

Aldini et al. [74] developed a fluorimetric method, called lipophilic oxygen radical absorbance capacity (L-ORAC), in which 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) acted as lipid generator and 4,4-difluoro-5-(4-phenyl-1,3-butanieryl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY^{581/591}) as lipophilic fluorescent probe to measure the oxidation of the lipidic compartments of plasma. The decrease in red fluorescence ($\lambda_{\text{ex}} = 580$ nm, $\lambda_{\text{em}} = 600$ nm) was used to measure the degradation of BODIPY and the increase in green fluorescence ($\lambda_{\text{ex}} = 500$ nm, $\lambda_{\text{em}} = 520$ nm) to determine the formation of oxidation products [74]. Li et al. [75] and Nicolescu et al. [76] analyzed the products of oxidation and nitroxidation between BODIPY and ROS or reactive nitrogen oxide species (RNOS) with LC-MS.

In 2006, Kwak et al. [77] presented a microplate-based oxygen radical absorbance capacity assay to analyze lipophilic compartments in plasma. They carried out the assay in DMSO:butyronitrile 9:1 (v/v), used BHT to create a standard calibration and calculated the ORAC values using the green fluorescence curves.

This assay had entered in analysis of molecular biology and was also used to determine the antioxidant nutrients in both the hydrophilic and lipophilic compartments of plasma [74,78,79]. The L-ORAC could also become important for detecting antioxidant status in meat and fat.

14.2.3.3 Oxygen Radical Absorbance Capacity with Randomly Methylated β -Cyclodextrin

Huang et al. enlarged the hydrophilic ORAC assay (Section 14.2.2.5) to lipophilic antioxidants [80]. They used randomly methylated β -cyclodextrin (RMCD) as solubility enhancer for lipid soluble substances. Vitamin E and other lipophilic compounds were dissolved in 7 percent RMCD (w/v), dissolved in acetone-water 50:50 (v/v) [80].

Bangalore et al. [81] were able to show a correlation between concentration of lycopene (extracted from Lyco-O-Mato 6% extract) and the ORAC values measured in 0.4–1.6% RMCD.

This assay could be useful to determine the oxygen radical scavenging capacity of lipophilic additives of meat products to evaluate the effect of carotenoids on the oxidative status of meat and fat.

14.2.4 Rancimat Method

The Rancimat method is based on the reaction of a fat sample with an air stream at temperatures between 50 and 220°C. The volatile content, mainly formic acid, is transferred with the air stream in a water-filled bin whose conductivity is measured continuously. The recorded oxidation curves show an inflexion point, the so-called time of induction, which provides a basis to assess the oxidative stability of the sample. This method was developed as fully automated version to detect the induction time of fats and oils and replaced the high elaborate active oxygen method (AOM; AOCS Cd 12-57), which was used at that time. The established method was affiliated into many national and international engineering standards, for example, ISO 6886 and AOCS Cd 12b-92.

The Rancimat method was often used to analyze fat, meat, and meat products [82]. Gerhardt et al. [83] used the Rancimat method to analyze the influence of spices on the stability of pork fat. Almost all spices examined had an antioxidative effect which varied in intensity. The Rancimat method is still used. El-Sayed et al. [84] showed that frying frozen prefried chicken meat expresses more deteriorative effects than the fresh meat throughout the frying process. Rižnar et al. [85] analyzed the antioxidant activity of rosemary extract in chicken frankfurters and showed that the addition of rosemary ingredients, mainly carnosic acid, is useful for the food industry.

14.2.5 Measurement of Activity of Antioxidative Enzymes

In meat, the essential antioxidant enzymes are also implicated to decrease the oxidation of unsaturated fatty acids. SOD, CAT, and glutathione peroxidase (GSHPx) constitute the primary mechanism for protecting cells from oxidative damage [86]. SOD scavenges superoxide anion by forming hydrogen peroxide and CAT safely decomposes H_2O_2 to H_2O and O_2^{*-} . GSHPx can decompose both hydrogen peroxide and lipoperoxides formed during lipid oxidation. These antioxidant enzymes were relatively stable in meat during refrigerated storage in beef and turkey [87,88]. Thus, they can offer a protection against free radical damage for some time postmortem [88].

All antioxidant enzymes activities were measured directly in fresh or frozen meat [6]. The enzymes were prepared from muscle cells by homogenizing them in PBS, centrifuged at 1000 g [89] and the protein concentration was determined by the biuret method [90].

The method for measuring the total SOD activity (Cu-Zn SOD and Mn SOD) is based on the procedures used by McCord and Fridovich [91], modified by Weser et al. [92]. Marklund and Marklund (1974) [93] published an enhanced version by using inhibition of pyrogallol autoxidation in a basic medium (pH 8.2). The rate of pyrogallol autoxidation in presence of a defined volume of muscle extract was compared to a blank by measuring the increase of absorbance at 340 nm during 2 min. The SOD activity was calculated in units of SOD activity to inhibit the pyrogallol autoxidation by 50% [6].

The CAT activity is measured by the rate of disappearance of hydrogen peroxide at 240 nm following the method of Aebi [94]. The meat extract, diluted in PBS, was mixed with hydrogen peroxide and the decomposition of H_2O_2 was recorded during 8 min. The CAT activity was expressed as nanomole decomposed H_2O_2 per minute and milligrams of protein [94].

GSHPx activity was analyzed with the GSH reduction coupled to a NADPH oxidation by glutathione reductase by Agergaard and Jensen [95] in bovine and porcine whole blood. In this assay the meat extract was added and the rate of NADPH oxidation was measured at 366 nm and the GSHPx activity was calculated as nanomole oxidized NADPH per minute and milligram of protein.

The antioxidant enzymes glutathione peroxidase (GSHPx) and CAT were affected by both the thermal process and the antibiotic residues (enrofloxacin and ciprofloxacin) in cooked meat [96]. Carreras et al. did not find differences in SOD activity in all samples investigated, supporting the theory that this enzyme could play a major role in preventing lipid oxidation of cooked meat [96].

Mercier et al. [97] evaluated the effect of finishing diet (pasture or mixed-diet) of Charolais cows on lipid and protein oxidation in beef homogenates. The different diets had opposite effects on SOD and GSHPx activities. Pasture-finishing mode of animals induced increasing SOD activity but decreasing GSHPx activity. No significant effect of diet was noted on CAT activity.

In addition to the listed methods, a high number of scarcely used assays to determine antioxidant capacity exists. The spectrophotometric cupric reducing antioxidant capacity (CUPRAC) assay that uses a copper(II)-neocuproine reagent [98–100], the *N,N*-dimethyl-*p*-phenylenediamine (DMPD) assay, which is based on bleaching of a DMPD radical cation (DMPD^{•+}) [101], the fluorimetric peroxy radical scavenging capacity (PSC) assay that uses 2',7'-dichlorofluorescein diacetate (DCFH-DA) [102,103], and the assay that uses inhibitory effects of free-radical scavengers on the oscillations of the Briggs–Rauscher reaction [104] belong to them. The Cu²⁺-mediated LDL oxidation assay, first performed by Esterbauer et al. [105,106], was often used to measure antioxidant activity of pure chemicals, for example, carotenoids and plant extracts like tea but not to analyze meat or fat.

14.3 Comparative Studies

The higher number of various test systems to measure antioxidant activity require a comparison of the results of these different assays. Indeed, until now only a negligible number of comparative studies exist. But most of the authors published data of more than one assay to make it easier to compare results with other research groups.

Prior and Cao [107] compared different analytical methods to measure total antioxidant status in serum and concluded that no single measurement of antioxidant status is going to be sufficient and that a “battery” of methods will be necessary to adequately assess oxidative stress in biological systems. Huang et al. summarized a large number of assays discussing the chemical principles behind the reactions. In that summary, most assays cited in this chapter, for example, ORAC/TRAP, TEAC, FRAP, and DPPH, were compared [108].

Not long ago Schlesier et al. [20] analyzed the often used reference substances gallic acid, ascorbic acid, uric acid, and the vitamin E analog Trolox on their antioxidant activity by using FRAP, TEAC, TRAP, DPPH, and PCL assays. In all test systems gallic acid, as representative of the group of polyphenols, showed the highest antioxidant activity compared to the other compounds.

Descalzo et al. [109] analyzed the antioxidant status of fresh meat from pasture and grain-fed cattle. The FRAP levels of nonenzymatic antioxidants of meat produced on pasture were about 50% higher than those of meat from grain-fed animals. However, no differences were found on the ability of the tissue homogenates to reduce ABTS^{•+}. The odor profile determined by electronic nose analysis indicated a significant linear correlation between a set of sensors and the FRAP values [109].

The studies of Berk et al. [110] showed that vitamin E (α -tocopherole acetate) supply significantly increased the vitamin E content of serum and liver of fattening pigs. The values determined by TBARS assay and Rancimat method clarified that vitamin E content of pork decreased depending on time of frozen storage. But storing time had no significant influence on vitamin E content of backfat.

Most of the assays can only determine the antioxidant activity in micromolar range needing minutes to hours. Only the PCL assay is suitable for analyses in the nanomolar range.

14.4 Conclusions

Today, the antioxidant activity of meat compounds can be analyzed by using many methods in all kinds of matrices. The TBARS assay is often used to determine the lipid peroxidation, even if the reaction is nonspecific. To analyze the lipid peroxidation with the LDL oxidation assay, which uses the oxidative modification of human LDL, is also possible.

The fully automated Rancimat method has often been used in past and present. During the last 15 years a lot of methods using radicals have been developed. In addition to the often used TEAC assay, using the green-colored ABTS radical cation, the DPPH assay, and the DMPD assay are further test systems using spectrophotometry. The bleaching of colored carotenoids, for example, β -carotene and crocin, is also frequently used.

The TRAP assay and the ORAC assay are two similar fluorimetric test systems, using fluorescein or R-/B-PE as fluorescent probe, and are applicable for hydrophilic and lipophilic compounds (L-ORAC with RMCD). The lipophilic ORAC (L-ORAC) uses certain BODIPY derivatives as fluorescent probe and has the ability to determine the antioxidant capacity of strong nonpolar compounds, for example, carotenoids.

PCL, a highly sensitive test system, combines the photochemical generation of free radicals with the sensitive chemoluminescence detection. FRAP assay, FOX assay, ESR spin trapping assay, and electrochemical assays are further possibilities to quantify the antioxidative activity by determining the reductive ability of substances.

Over the years, the tendency increased to publish antioxidant capacity measured with more than only one test system to allow a comparison between the data and the results of other investigations. Comparison studies clearly showed differences from test to test. For example, the TEAC values of galangin and chrysin, two flavonoids that differ in only one OH group, were comparable but galangin was a much better antioxidant in the TBARS assay [111]. It is strongly recommended to use more than one assay to determine the antioxidant potential of food extracts or other samples. However, a ranking within each assay is possible. The problem of transferring the *in vitro* results to the human organism is still waiting for solutions.

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Chapter 15

Vitamins

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15.1 Introduction

Vitamin content in processed meat and poultry (PMP) derives from naturally available vitamins—PMP being a good source of B vitamins; thiamine, riboflavin, and niacin are probably the most important in the group [1]. Vitamins E and C are added to PMP due to their reducing properties as antioxidants. Losses during processing can modify the vitamin content of PMP. In many cases, the fortified levels of vitamins are high in comparison to natural levels [2].

PMP is a good source of vitamins, although vitamin content depends on the nature of the raw materials used and on the other ingredients. PMP includes not only the animal skeletal muscles but also their glands and organs (tongue, liver heart, kidneys, brain, and so on); in the case of sausages, intestine sausage casings; fat in tallow; and lard and blood. The main sources of meat are cattle (beef), calves (veal), hogs (hams, pork, and bacon), sheep (mouton), and young sheep (lamb) [3].

PMP can be a good source of vitamins, though any applied processing treatment can affect the vitamin content. Therefore, the availability of methodologies for monitoring the quality (technological indicators) of the product and for evaluating their nutritional value is of interest.

15.2 Fat-Soluble Vitamins

15.2.1 Extraction

Analysis of fat-soluble vitamins in foods and specifically in PMP requires a complex sample preparation before vitamin determination via liquid chromatography (LC).

Hot saponification with ethanolic KOH solution containing an antioxidant is one of the most widely used procedures for extracting vitamins A, E, D, and carotenoids from PMP to disrupt the matrix, release the free form from esters, hydrolyze triacylglycerols to glycerol, remove fats and other interfering substances, and produce soaps of free fatty acids. Vitamin K cannot be extracted by saponification because of its instability in alkaline media. In PMP, saponification has been applied to tocopherols (T) and tocotrienols (T₃) [4], retinol/ β -carotene [5], retinol and α -T [6–9], and retinol determination [10–13]. After saponification, the unsaponifiable fraction has been extracted with hexane, isooctane, and petroleum-diisopropylether (see Table 15.1). Washing the hexane extract with 60% (v/v) sulfuric acid facilitates a short high-performance liquid chromatography (HPLC) analytical time by removing interfering compounds with longer retention times than α -T [13].

Liquid-liquid direct extraction using solvents such as hexane, ethanol, acetone, methanol, tetrahydrofuran, and petroleum ether is a simpler method of sample preparation. In PMP, this procedure has been applied primarily to vitamin K determination, using 2-propanol or methanol as a protein denaturing solvent and hexane or chloroform as the organic solvent to extract the vitamin (see Table 15.2). Two different extraction solvents for vitamin K determination in PMP (see Table 15.2) have been compared [19]. No statistically significant differences have been found between 2-propanol-hexane and chloroform-methanol in terms of extraction efficiency. The analyzed contents in bovine liver of phyloquinone as well as of MK-4, MK-8, and MK-9 were similar with both solvents. However, the recovery of K₁₍₂₅₎ was significantly better ($p < 0.02$) when 2-propanol-hexane was used. On the other hand, the ratios of both phyloquinone and menaquinones to K₁₍₂₅₎ were similar in the first and second extractions using both methods, indicating that the extractability of endogenous K vitamins was similar to that of the added standard. In addition, extraction with 2-propanol-hexane was shown to be reproducible and easy to perform.

Table 15.1 Fat-Soluble Vitamin Determination by HPLC in Processed Meat and Poultry Using Saponification

Vitamin	Food	Extraction	HPLC Parameters	Reference
T: α -T, β -T, γ -T. T ₃ : α -T ₃ , β -T ₃	Liver, heart, kidney, and tongue of different species; sausage, ham, cured and canned products	Fat samples + AA + water. 99.5% ethanol 20 min. 50% KOH, room temperature, overnight. Extraction: <i>n</i> -hexane, washed. Evaporation. Dissolution <i>n</i> -hexane with 1% butylated hydroxytoluene (BHT).	LiChrosorb Si 60 (25 × 0.4 cm, 5 μ m) 37°C. <i>n</i> -Hexane:diisopropyl ether 93:7. 2.1 mL/min. Fluorescence detection.	4,14
α -T	Freeze-dried liver	Ethanol + 1% pyrogallol 1% w/v. KOH 70°C 7 min with agitation. Extraction: petroleum-diisopropyl ether (3 + 1) shaking 5 min. Washed water. Centrifugation 180 g 10 min. 50:50 EDTA: AA. Ethanol vortex, 70°C 10 min, vortex 5 min. KOH vortex 78°C 20 min, vortex 10 min. Washed water. Extraction: iso-octane (\times 2) vortex 1 min. Evaporation: 65°C/N ₂ . Dissolution: iso-octane.	Rad-PAK cartridge C-18 (5 μ m). MetOH. 1 mL/min. Fluorescence detection: 295/330 nm.	6
	Beef liver, muscle, and subcutaneous fat	—	Silica column (15 × 0.39 cm, 5 μ m) 15°C. Tetrahydrofuran (THFu): iso-octane 4:96. i.v.: 20 μ L. Fluorescence detection: 296/325 nm.	7
	Beef burgers and chicken frankfurters	—	Lichrosorb (25 × 0.4 cm). Isocratic: hexane:isopropyl alcohol 99:1. 1 mL/min. i.v.: 20 μ L. Fluorescence detection: 290/330 nm.	11
	Pork tissue, liver, and fat	10% AA + KOH + MetOH water bath 80°C 10 min, vortex 5 s, water bath 20 min. Extraction: hexane 5 min/60% H ₂ SO ₄ 60% 2 min.	Watman Partisil PXS 10/25 ODS. Hexane:propane-2-ol 99:1. 1.6 mL/min. i.v.: 40 μ L. Fluorescence detection: 295/325.	12,13
	Cured pork sausage, uncured restructured pork roast	AA + 11% KOH in ethanol, water bath 80°C 15 min shaking 200 rpm. Extraction in ice: iso-octane 2 min vortex.	Waters Resolve Guard-Pak Silica. Waters Resolve Silica (15 × 0.39 cm, 5 μ m). 15°C. Iso-octane:THF 96:4. 1 mL/min. i.v.:30 μ L. Fluorescence detection: 296/325 nm.	9

(Continued)

Table 15.1 (Continued)

Vitamin	Food	Extraction	HPLC Parameters	Reference
Retinol and β -carotene simultaneously	Beef, mutton, chicken burger/frankfurter/heart/liver/thigh	KOH + ethanol electric heating 30 min. Extraction: hexane ($\times 4$). Water washed to free alkali. Dried Na_2SO_4 . Evaporation: water bath. Dissolution: hexane; in HPLC: mobile phase.	Open-column chromatography: Glass column alumina packed (aluminium oxide 90, neutral, activity grade I deactivated with water). β -carotene: 1 st . Elution: 4% acetone in hexane. Evaporated: water bath + N_2 . Redissolution: hexane. Detection: 450 nm. Retinol: 2 nd . Elution: 15% acetone in hexane. <i>Idem</i> β -carotene. Detection: 325 nm. HPLC: μ bondapak C_{18} (30×0.39 cm, 10 μm). Waters Guard-PAK pre-column. ACN:MetOH:ethylacetate 88:10:2. 2 mL/min. i.v.: 50–100 μL . Detection: β -carotene 436 nm, retinol 313 nm. C18 Nova Pak. Retinol: MetOH:water 95:5. 325 nm. Carotenoids: MetOH:THFu 90:10. 450 nm. ODS-2 (15×0.4 cm, 5 μm). THFu: ACN 10:90. 0.8 mL/min. i.v.: 10 μL . Retinol: 325 nm. Tocopherol: Fluorescence 292/330 nm.	5
Retinol, α - and β -carotene	Chicken and lamb chops cooked	Retinol: 9% alcoholic KOH + 10% AA. Extraction: petroleum ether with 10% brine solution. Concentration and dissolution: MetOH. Carotenoids: 941.15 AOAC		15
Retinol and α -T	Cooked sausages: lunch, chopped (pork, beef, and turkey), vitamined chopped, Sicilian mortadella	Pyrogallol + 13 N KOH, ethanol, N_2 45°C 20 min refluxing. Centrifugation 3000 rpm 5 min. Extraction: <i>n</i> -hexane ($\times 3$). Concentration: 40°C vacuum. Dissolution: ethanol.		16
Ergocalciferol (I5) and cholecalciferol	Liver	10% AA + aqueous 50% KOH + 99% ethanol N_2 30 s room temperature, overnight, stirring. Straight phase HPLC: μ -Porasil (30×0.39 cm, 10 μm). Silica guard column. Gradient elution <i>n</i> -hexane:2-propanol:	Zorbax ODS and Vydac 201 TP54 connected with thin capillary tube. MetOH:water 96:4. 1 mL/min. i.v.: 50–80 μL . Detection: 2654 nm.	17, 18

<p>Extraction: petroleum ether:diethyl ether (1:1) (×2, 2 min). Washed water until pH neutral, dried Na₂SO₄. Evaporation. Dissolution: <i>n</i>-hexane.</p>	<p>14 min: 98:8:1.2 15 min: 94:2:5:8 15 min: 85:15 10 min: 98:8:1.2 1 mL/min. i.v.: 0.5–1 mL. Fraction collection time: 9–14 min. Evaporated N₂. Dissolution: 100–200 µL 7% water in MeOH. RP-HPLC: ODS Vydac 201 TP54 (5 µm, 25 × 0.46 cm). C18 guard column. Methanol: water 93:7. 1 mL/min. i.v.: 30–180 µL. Evaporation N₂. Dissolution in 4% water in MeOH.</p>	<p>25-OH-D₂ (1S) and 25-OH-D₃</p>	<p>Spherisorb S5 NH₂ Phase Sep (25 × 0.46 cm, 5 µm) Guard column silica µPorasil. Hexane:2-propanol 97:3. 1 mL/min. i.v.: 35–80 µL. Detection: 264 nm.</p>
<p>Straight phase HPLC: <i>idem</i> above except fraction collection time 22–31 min and dissolution 100–300 µL 13% water in MeOH.</p>	<p>RP-HPLC: <i>idem</i> above except MeOH:water 83:17, i.v.: 55–220 µL. Fraction collection: 14–25. Dissolution: 100–120 µL <i>n</i>-hexane or 0.5% 2-propanol in <i>n</i>-hexane.</p>		

Table 15.2 Determination of Vitamin K by HPLC in Processed Meat and Poultry

Vitamin	Food	Extraction	Purification	HPLC Parameters	Reference
K ₁ , K ₂ ; MK-4, MK-6, MK-7, MK-9, MK-10, MK-13	Animal liver, spleen, kidney, heart, muscle	Homogenization: 66% 2-propanol. Extraction: hexane 5 min. Centrifugation: 800 g, 5 min. Evaporation: reduced pressure, room temperature. Dissolution: hexane, shaking 1 min.	SPE silica: Cleaned: <i>n</i> -hexane: diethyl ether 96:4, hexane, washed: <i>n</i> -hexane, eluted: <i>n</i> -hexane:diethyl ether 96:4. Evaporation: reduced pressure, room temperature. Dissolution: hexane, shaking 1 min. Tin-layer chromatography: Silica Gel 60 F ₂₅₄ (20 × 20 × 0.025 cm), cleaned: chloroform, developed: petroleum:diethyl ether 85:15, 20 min. ca. 12 cm, dried air room temperature: 5 min, UV detection: 254 nm, extraction: chloroform 5 min, centrifugation: 800 g, 5 min. Evaporation: reduced pressure, room temperature. Dissolution: ethanol, shaking 1 min.	Nucleosil C18 (15 × 0.46 cm, 5 μm). 92.5 or 97.5 % ethanol with 0.25% sodium perchlorate. 1 mL/min. Coulometric reduction: two porous graphite electrodes in series. Potential upstream and downstream electrode +0.25 and -0.55 V, guard cell potential -1.0 V. Fluorescence detection: 320/430 nm.	21
K ₁ , K ₂ ; MK-4 to MK-10	Pig liver, bovine liver; beef meat, roast; pork meat, chop; chicken meat, leg, and thigh	IS (K ₁₍₂₅₎) + 2-propanol 100°C, 5 min. 2-propanol 2 min + <i>n</i> -hexane 2 min + water. Centrifugation: 1500 g, 5 min. Evaporation and dissolution: hexane.	HPLC: Porasil (30 × 0.39 cm, 10 μm). Hexane with 1% diethyl ether. 1.5 mL/min. i.v.: 500 mL. Evaporation. Redissolution: mobile phase HPLC.	HPLC: Vydac 201 TP54 column (25 × 0.46 cm, 5 μm). MeOH: ethanol (with 10 mM ZnCl ₂ , 5 mM sodium acetate, 5 mM acetic acid) 83:17. 0.8 mL/min in 8 min and 1.5 mL/min in 4 min, maintained for 28 min. i.v.: 50 μL. Postcolumn reduction: reactor (5 × 0.21 cm), Zn metal to fluorescent hydroquinone derivatives. Fluorescence detection: 238/425 nm.	19

<p>Chloroform-MetOH + IS (K₁₍₂₅₎) + 2-propanol 100°C, 5 min. 2-propanol 2 min. Centrifugation: 2000 g, 5 min.</p>	<p>Confirmation with HPLC-MS: ion trap mass, positive ion atmospheric pressure chemical ionization (APCI). Drying gas (N₂): T^a 350°C, 5 L/min, APCI interface T^a 00°C. Nebulizer gas (N₂): 50 psi. Voltage corona discharge needle: 1.8 kV. Scan range: 150–900 m/z.</p>	<p>20</p>
<p>Chloroform:methanol 2 min. Evaporation. Ethanol. Evaporation. Dissolution: hexane.</p>	<p>MK4, MK5: trap drive 4L capillary exit and skimmer voltages 122 and 44 V. MK6 to MK10: trap drive 5L capillary exit and skimmer voltages 110 and 36 V.</p>	<p>20</p>
<p>K₁, MK4, dK Fast food: Bacon and sausage with or without other foods (egg, cheese)</p>	<p>Water + IS (K₁₍₂₅₎) + 2-propanol:hexane 3:2, shaking 3 min, sonication 60 s, shaking 3 min. Centrifugation: 1800, 5 min. Evaporation: Speed Vac. Dissolution: hexane.</p>	<p>20</p>
<p>Trans- and cis-K₁ Frankfurters, sausages pork, sausages beef, paté pork</p>	<p>SPE: 500 mg Bond Elut silica. Washed: hexane:diethyl ether 95.5:3.5 and hexane, eluted: hexane:diethyl ether 95.5:3.5. Evaporation. Reconstituted: methylene chloride + MetOH with 10 mM ZnCl₂, 5 mM acetic acid, and 5 mM sodium acetate. Centrifugation: 1800 g, 5 min.</p>	<p>22</p>
<p>Lipase + 0.2 M phosphate buffer 0.2 M 37°C 2 h (pH 7.6–8.2). After cooled ethanol + K₂CO₃ + hexane, shaking 30 min. Evaporation: N₂. Dissolution: MetOH.</p>	<p>Gradient elution: 11.5 min: 90:10, 0.6 mL/min 11.5 min: 70:30, 0.8 mL/min 19.5 min: 90:10, 0.8 mL/min 23.5 min: 90:10, 0.6 mL/min Postcolumn reduction: reactor (2 × 50 mm), Zn metal. Fluorescence detection: 244/430 nm. YMC C30 (25 × 0.46 cm, 5 μm). Postcolumn reduction: reactor (20 × 3 cm) Zn metal to fluorescent hydroquinone derivatives. Anhydrous sodium acetate + ZnCl₂ + glacial acetic acid + MetOH + dichloromethane. 1–1.5 mL/min. i.v.: 20–50 μL. Fluorescence detection: 243/430 nm.</p>	<p>22</p>

Note: dK: dihydrophyloquinone, K₁: phyloquinone, K₂: menaquinone-n, MK.

Taking into account all these facts, extraction with 2-propanol-hexane was recommended for routine analysis of vitamin K in meat products. Later, the same solvents have been used by Ferreira et al. [20] to measure vitamin K in the U.S. food supply, including PMP (bacon, sausage; see Table 15.2).

The aforementioned classical methods are characterized by high consumption of organic solvents and the long time required for analysis [23,24]. To obtain more information on general aspects and the application of these methods to fat-soluble vitamin determination, we recommend the review carried out by Ye and Eitenmiller [25]. As an alternative to these classical methods, and offering numerous advantages that will be discussed later on, supercritical fluid extraction (SFE) has been applied to fat-soluble vitamin determination in PMP.

15.2.1.1 *Supercritical Fluid Extraction in Fat-Soluble Vitamin Determination in Processed Meat and Poultry*

Both modalities of SFE—solvent collection and solid-phase trap SFE—constitute an alternative to the classical methods of fat-soluble vitamin (A, E, D, and β -carotene) determination in PMP, as has been reported in recent publications (see Table 15.3). The advantages of SFE include minimal consumption of organic solvents (environmental protection), the exclusion of oxygen, reduction of thermal treatment, lower economic cost, shorter extraction times, and less laborious processing, since these procedures can be automatized. This results in lower labor costs with considerably higher sample throughputs and potentially higher selectivity than conventional extraction methods [23,24,26–28].

In several studies, SFE has been compared to conventional methods. Thus, Berg et al. [26] applied two similar methodologies in two different automatic SFE instruments, using solvent collection and solid-phase trapping for vitamin A and E determination in minced meat and liver paste (see Table 15.3). Good agreement between fat-vitamin contents was obtained using conventional extraction and SFE. Important divergence between conventional extraction and SFE was only seen in samples having very low content of both vitamins [26,28].

Following SFE, some authors perform and optimize saponification of the extracts obtained with SFE [26,28]. It is necessary to carefully select the time and amount of reagent required for saponification, because during this process lipids compete in the hydrolysis with vitamin esters (retinol esters and tocopherol esters) [26]. The advantage of saponifying after SFE is the same as when saponification is used in conventional vitamin analysis, that is, to facilitate determination, because vitamin esters are hydrolyzed to their mother compounds and free fatty acids are released from triacylglycerols. The fact that in HPLC separation free fatty acids elute close to the solvent front, well separated from vitamins, considerably simplifies the procedure [26,27].

Saponification after SFE has in some cases been replaced by enzymatic hydrolysis to allow simultaneous extraction and hydrolysis of fat and vitamin esters. If this step is performed under supercritical conditions, the enzyme must be able to withstand high pressure and elevated temperature. Lipases are one class of enzymes of interest in this respect. Accordingly, an online SFE/enzymatic hydrolysis procedure using immobilized lipase has been developed for the determination of vitamin A in meat products (in minced pork and beef, and in low- and high-fat liver paste) [27]. Several lipases have been tested; among them, Novozyme 435 (*Candida antarctica* type B) has shown the highest activity toward retinyl palmitate/retinyl ester (fat-soluble vitamin ester). The investigated lipases did not show activity with either alpha-tocopheryl acetate or with other tocopheryl esters, leading to a slightly more complex final analysis when vitamin E must also be determined.

Table 15.3 Fat-Soluble Vitamin Determination by HPLC in Processed Meat and Poultry Using SFE

Vitamin	Food	Extraction	HPLC parameters	Reference
A, β -carotene	Calf liver	Liquid-liquid extraction: anhydrous Na ₂ SO ₄ , MeOH-chloroform (4:1) (\times 3). Evaporation: N ₂ . Redissolution: HPLC solvent. SFE: SH + sample. SFE with solvent collection: static extraction (1 min) + dynamic extraction (40 min; 2 mL/min, 80°C), CO ₂ (31 mPA), restrictor temperature (60°C) 1.5 mL/min, hexane.	ODS C18 (8 \times 4.6 cm, 3 μ m) ACN-MeOH-tetrahydrofuran-ammonium acetate-BHT. i.v.: 30 μ L. Vitamins A: 325 nm. β -carotene: 452 nm.	23
A, E	Minced meat, liver paste	SH + MeOH + (sample + SH + AA) + SH. SFE—solid-phase trap (ODS): static step (5 min, 365 bar) + three dynamic steps (1.25 and 2 \times 20 min) supercritical CO ₂ (0.8 g/mL) with 4% MeOH, extraction temperature (80°C), nozzle temperature (85°C), trap temperature (90°C), 4% MeOH as modifier. Flow rate 1 mL/min. Trap elution conditions: hexane:dichloromethane (1:1), 2 mL/min, trap temperature (40°C), nozzle temperature (45°C). SFE—solvent collection: static extraction (15 min; 365 bar CO ₂ density 0.8 g/mL; 5% MeOH added to CO ₂) + dynamic step (60 min; flow rate 1 mL/min), extraction temperature (80°C), restrictor temperature (85°C), collection temperature (5°C), collection solvent (ethanol:di-isopropyl ether (1:1) + palmitoyl AA). Saponification: evaporated extracts from SFE (solvent or solid-phase trap collection) + ethanol (with AA) + aqueous 50% KOH + Ar or N ₂ . Incubation: 30 min 40°C, shaking: 10 min 40°C. Extraction: cold water, petroleum ether, organic phase washing with water. Evaporation. Dissolution: Ethanol. European Standard Method (CEN, 1997): Saponification and liquid-liquid extraction.	MetOH:water 96:4. 1 mL/min. i.v.: 20 μ L. UV detection: Lichrospher RP-18 (25 \times 0.4 cm, 5 μ m) guard column Lichrospher RP-18 (0.4 \times 0.4 cm, 5 μ m). Retinol: 325 nm, α -tocopherol: 295 nm. Fluorescence detection: Lichrospher RP-60 (25 \times 0.4 cm, 5 μ m) guard column Lichrospher RP-60 (0.4 \times 0.4 cm, 5 μ m). Retinol: 325/475 nm, α -tocopherol: 295/330 nm.	26

(Continued)

Table 15.3 (Continued)

Vitamin	Food	Extraction	HPLC parameters	Reference
A, E	Minced pork and beef meat, and low- and high-fat liver paste	Online SFE/enzymatic hydrolysis Silica based hydromatrix (SH) + enzyme + SH + (sample + SH) + 5% water and 0.1% BHT in ethanol + SH. SFE with solvent collection: CO ₂ modified with 5 vol% ethanol. Collection in 0.1% BHT in ethanol. Evaporation: N ₂ . Dissolution: ethanol.	Lichrospher RP-18 (25 × 0.4 cm, 5 μm). MetOH:water 98:2.1 mL/min. i.v.: 20 μL. UV detection: vitamin A (all- <i>trans</i> -retinol) and retinyl palmitate 325 nm, α-tocopheryl acetate 284 nm. Fluorescence detection: nonsterified tocopherols 294/330 nm.	27
A (all- <i>trans</i> -retinol), E, β-carotene and cholecalciferol	Minced meat (pork) and liver paste	a) SFE with solvent collection or solid-phase trap + saponification. SH + MetOH + (sample + SH + AA) + SH. SFE with solvent collection: static extraction (15 min; CO ₂ density 0.8 g/mL; 0.5% MetOH and 95% CO ₂) + dynamic step (60 min; flow rate 1 mL/min), extraction temperature (80°C), restrictor temperature (85°C), collection temperature (5°C), collection solvent (ethanol:diisopropyl ether 1:1). Extraction time 75 min. β-Carotene: (i) <i>Nonpolar extraction step</i> : static extraction (5 min) + dynamic step (40 min; flow rate 1 mL/min) CO ₂ density 0.95 g/mL. extraction temperature (40°C), restrictor temperature (40°C), collection temperature (5°C) collection solvent (dichloromethane:hexane 1:1). (ii) <i>Polar extraction step</i> : static extraction (5 min) + dynamic step (40 min; flow rate 2 mL/min) CO ₂ density 0.88 g/m with 5% MetOH as modifier, extraction temperature (80°C), restrictor temperature (80°C), same condition for collection. Extraction time 90 min.	Vitamin A, E, and D: Spherisorb C ₁₈ (250 × 0.46 cm, 5μm) and guard column (7.5 × 0.21 cm). Methanol: water 90:10. i.v.: 50 μL. UV detection: vitamin A 325 nm; D 265 nm; E 295 nm. Fluorescence detection: vitamin A 325/440 nm; vitamin E 295/310 nm. β-carotene: C-18 nonaqueous mobil phase with ammonium acetate, MetOH, ACN, and dichloromethane. Detection: 453 nm. Two isocratic chromatographic systems (25–30 min) or gradient isocratic chromatographic system (60 min).	28

SFE with solid-phase trap (C18): static step (5 min) + two dynamic steps (1.25 and 40 min) supercritical CO₂ (0.8 g/mL) with 4% MeOH, extraction temperature (80°C), flow rate 1 mL/min, trap temperature (90°C).

β-carotene: static step (5 min) + two dynamic steps (1.25 and 40 min) puresupercritical CO₂ + two polar dynamic steps (1.25 and 40 min) supercritical CO₂ with 4% MeOH, same the extraction parameters.

Trap elution conditions: hexane:dichloromethane 1:1, trap temperature (40°C), total elution volume (6–10 mL) and time (75–130 min).

Saponification: Evaporation: extracts from SFE (solvent o solid-phase trap collection) + ethanol (with AA) + 50% aqueous KOH + Ar or N₂.

Incubation: 30 min 40°C, shaking: 10 min 40°C.

Extraction: cold water + petroleum ether, organic phase washing with water. Evaporation. Dissolution: ethanol. Incubation: 30 min 40°C, shaking: 10 min 40°C. Extraction: cold water + petroleum ether, organic phase washing with water. Evaporation. Dissolution: ethanol.

b) European standard method (CEN, 1997): Saponification and liquid–liquid extraction.

In this case, all tocopherols and tocopheryl esters must be determined, because vitamin E content has to be calculated using the concentration values for the different compounds plus their vitamin E activities. Fortunately, in most natural food products, only tocopherol itself (and in a few cases alpha-tocopheryl acetate) makes a significant contribution to the vitamin E content.

The SFE method jointly with enzymatic hydrolysis is faster, more automated, and consumes smaller amounts of organic solvents than the methods based on liquid-liquid extraction (conventional method), or SFE using offline saponification for vitamin A and E determination, because no additional saponification or cleanup steps are needed. As a comparison, using conventional techniques, the extraction of six samples requires about 9 h of manual work, whereas some 24 samples can be run and handled using SFE/enzymatic hydrolysis with about the same demand in terms of manual work. Moreover, SFE/enzymatic hydrolysis consumes several times less (20–25-fold) organic solvent. The use of integrated enzymatic hydrolysis/alcoholysis with SFE combined with HPLC offers an analytical procedure that is gentle on the easily degradable vitamins [27].

A collaborative study carried out within a European Union project (SMT4-CT96-2089), in which SFE-based methodologies were developed and applied to the determination of vitamin A (*all-trans*-retinol), vitamin E (tocopherols), β -carotene, and vitamin D in seven different processed foods, including liver paste and minced meat (see Table 15.3), has been published [28]. The emphasis is placed on the approaches used to validate the developed methodology, although important experiences of a general nature made during the development stage by the different partners of the project are also discussed. Validation includes an internal cross-validation between laboratories with experience in SFE and vitamin determination, several ruggedness tests employing different types and models of equipment and involving personnel with varying levels of experience in vitamin determination, and an intercomparison including participants with less experience in this type of determination.

The use of solid-phase trapping in samples containing large amounts of interfering substances involves an inherent risk of breakthrough losses of analytes because of the limiting capacity of the trapping material. In processed foods such as liver paste, fat-soluble vitamins often occur together with high concentrations of triacylglycerols, which may be simultaneously extracted. To avoid the risk of vitamin breakthrough losses, a fractionated extraction \pm elution step has been developed. This procedure, in conjunction with a rather high trap temperature (90°C) to prevent condensation of the modifier on the trap, resulted in quantitative recoveries [28].

It is very important to carry out saponification, as far as possible, without exposure to light and air, and in the presence of an antioxidant. In the subsequent extraction step, water should be cold to prevent emulsion formation, thereby making the phase separation easier. As long as the vitamins are dissolved in the sample fat, they are not especially sensitive to oxidation. Therefore, running extraction without antioxidants in the extraction cell leads to somewhat but not drastically decreased values. The entire saponification procedure takes about 90 min, though more than one sample can be treated simultaneously. With access to automated evaporation systems, the manpower time needed for each sample, when running six samples simultaneously, is about 15 min. Since saponification is performed after the relatively selective SFE procedure, only 40°C and a short time (30 min) are needed to achieve quantitative hydrolysis compared with conventional methodology, in which 80°C is applied. This greatly reduces the risk of isomerization, leading to recoveries of close to 100% [28].

With access to an autosampler for the HPLC system, which makes overnight running possible, SFE becomes the step that determines the sample throughput. With an automated SFE system, approximately 12 samples can be processed over 24 h, that is, at least twice the number achievable with a conventional extraction method [28].

In the analytical procedures, several important steps must be taken into account. One concerns sample preparation, which includes several steps including weighing, mixing, and careful filling of the extraction cell to avoid the formation of channels. During these relatively tedious operations, it is important to avoid sunlight as much as possible. It is also necessary to protect vitamins with an antioxidant during the different steps of the analytical procedure. Another important point is that in supercritical carbon dioxide, a small percentage of methanol (4–5% v/v) is needed to quantitatively extract the vitamins (except for β -carotene) from the food samples. Although the solubility of the vitamin in pure supercritical carbon dioxide is sufficient, the addition of methanol or ethanol to the extraction cell facilitates extraction of the analyte from the food matrix. When a polar modifier is not used, the adsorption of vitamins onto the sample matrix and water adsorbing material (here Hydromatrix) makes the transport of analytes through the extraction cell very slow. This is especially true for vitamin E. A third finding is that the addition of 2 mL of methanol during sample preparation step, in the extraction cell, is necessary, and when using a solid-phase trap, a fractionated extraction \pm elution procedure is required to prevent breakthrough losses [28].

SFE is well suited for extracting fat-soluble vitamins from food products, although validation work is required to establish the accuracy and precision of the procedure. The sample size is only 0.5–2.0 g compared with 10 ± 100 g in conventional vitamin analysis. Detection limits obtained using the SFE approach are similar to those achieved by conventional solvent extraction, allowing the determination of vitamins at concentrations of 0.1 mg g^{-1} (0.1 ppm). This methodology is quite general and it should be possible to extend it to other fat-soluble vitamins and processed foods as well [28].

Lower relative standard deviations are usually found for solid as compared to liquid trap SFE, and the detection limits for the SFE method using optimized conditions are 0.002 and 0.05 mg/100 g for vitamin A and vitamin E, respectively. These limits are mainly obtained by HPLC. Similar detection limits have been found using ultraviolet (UV)-detection and the relatively old fluorescence detector [26].

It has been suggested that SFE requires an organic modifier to improve recoveries of vitamin A from some animal tissues [23]. Another feature is that a water-adsorbing material must be introduced in the extraction cell to remove the excessive amounts of water present in many processed foods. Besides this, the addition of antioxidants to the different steps in the analytical procedure should be noted [28].

A review has been done on fat-soluble vitamin analysis by SFE and supercritical fluid chromatography (SFC). The review includes a brief description of suitable supercritical media as well as basic theory on SFE and SFC processes (study of parameters affecting the process). Furthermore, guidelines are provided for optimizing the extraction and separation parameters to facilitate successful development of the method. Finally, applications using SFE and SFC in the enrichment and final determination of fat-soluble vitamins in meat and other products are reviewed [24].

15.2.2 Purification

In PMP, purification has been applied only to extracts of vitamins K and D from previously saponified samples (see Tables 15.1 and 15.2). Nonpolar components such as triacylglycerols have to be removed from sample extracts. Samples with low fat content (<10%) are most effectively and easily purified with the semipreparative HPLC. Because of marked differences in the polarity

of different vitamin K forms, effective removal of nonpolar components with a C₁₈ cartridge is not possible without losses in the recoveries of long-chain menaquinones. Owing to the complexity of the sample matrix, the purification capacity of silica cartridges alone is insufficient, even when large cartridges (up to 2 g) are used. An efficient purification of the bovine liver extract was achieved by silica solid-phase extraction (SPE) and semipreparative HPLC purification together. SPE was, however, omitted during development of the analytical method, because its repeatability was poor. For routine determinations of fat-soluble vitamins in animal products with high fat content, lipase hydrolysis followed by semipreparative HPLC as the purification method is the procedure of choice [19].

Silica SPE has been used in vitamin K determination to purify animal tissue and fast food including PMP, though the methodology has not been exhaustively validated [20,21].

15.2.3 *Liquid Chromatography*

During the late 1970s, HPLC became widely used for fat-soluble vitamin analysis, mainly because of its ability to effect rapid separation, nondestruction of the sample and, more importantly, the good resolution achieved [5].

Beta-carotene and retinol have been determined simultaneously, with two fixed-wavelength detectors in series connected in a single chromatographic run, in various food groups, including PMP [5]. Reverse phase (RP)-HPLC has been compared with the Association of Official of Analytical Chemists (AOAC) open-column (alumina) chromatographic method (see Table 15.1). The AOAC method was found to yield significantly higher retinol content in the studied foods (ratio retinol AOAC/HPLC in PMP: 0.8–12) due to the presence of other pigments that gave falsely elevated absorbance readings. Although there were no statistically significant differences in β -carotene contents obtained by both methods (HPLC and AOAC), more foods (chicken heart, liver, thigh, and mutton) with higher values were obtained by the latter method. The authors indicate that other pigments, and possibly other carotenoids, were present in the eluate, and that for some of the studied foods (chicken heart, ratio β -carotene AOAC/HPLC 15), the higher values may not be of much nutritional significance, because for most of these foods, carotene values were low—less than 10 μg per 100 g of edible portion.

Fluorescence detection offers higher selectivity and sensitivity for all-*trans*-retinol and especially for tocopherols as compared with UV detection, but the separation power of the column and the vitamin contents are usually high enough to permit UV detection to be used [28].

Although reverse-phase HPLC with electrochemical (EC) or fluorimetric detection after postcolumn reduction provides enough sensitivity and specificity for phyloquinone analysis, much progress will have to be made in analytical methods before reliable data on menaquinones in animal products are produced. Their reliable identification is difficult and must be confirmed with at least two detection systems. Much effort is also required for the extraction and purification steps because of the complexity of animal food matrices. There are only a few studies on the determination of menaquinones, although special attention has been focused on the detection and separation of K vitamins. Koivu-Tikkanen et al. [19] used fluorescence detection instead of the EC used in other studies, because with an EC detector efficient separation of different K vitamins was not possible in a reasonable time. A fluorescence detector permits both gradient elution and the change of flow rate during analysis, so it offers more alternatives for achieving better and more practical separation of different vitamin K forms. In addition, the fluorescence detector is more sensitive than EC.

15.2.4 Gas Chromatography

A procedure including lipid extraction, saponification, solid-phase clean-up, and capillary gas chromatography (cGC) has been reported for the determination of retinol (vitamin A) and α -tocopherol (vitamin E) in poultry tissues. Retinol and α -tocopherol were determined separately by cGC–flame ionization detection using a fused-silica open tubular capillary column (30 m \times 0.25 mm) coated with 5% phenylmethylsilicone and with a film thickness of 0.25 μ m. Solvent extraction followed by saponification was sufficient to provide a purified extract which was directly analyzed for retinol by cGC in the solvent venting mode. However, to accurately determine α -tocopherol by cGC, further purification of the extract by SPE was necessary. A silica SPE column was used to remove interfering cholesterol from the extract. α -T was analyzed in its derivatized form. Absolute and relative recoveries from spiked samples were obtained ranging from 80–95% for both compounds. 5α -Cholestane and α -tocopheryl acetate were used as internal standards (IS). The low recoveries obtained for α -tocopherol suggest that some α -tocopherol was degraded during the derivatization step. The reproducibility of the procedure was quite satisfactory since the relative standard deviation values (10%), evaluated for retinol and α -tocopherol, reflect the total procedure from the extraction to the cGC analysis step. 5α -cholestane and α -tocopheryl acetate (IS) presented similar absolute recoveries. The use of retinyl acetate as IS for retinol analysis was not possible because it must be added after the saponification step. The procedure is reproducible and allows the detection of concentrations as low as 1 mg/g [8].

15.2.5 Supercritical Fluid Chromatography

SFC permits the separation of vitamins from compounds of widely different polarities and molecular masses, and eliminates the need for derivatization in fat-soluble vitamin determination. In theory, and most often also in practice, it is faster than LC, due to the higher mass transfer rate in the chromatographic process [24]. However, no applications of this methodology to PMP have been found.

15.3 Water-Soluble Vitamins

Many of the endogenous water-soluble vitamins in meat products are bound to protein. Therefore, acid and enzyme hydrolysis is needed to release the vitamins to be measured. Microbiological methods are used for the determination of some B vitamins because of their sensitivity. However, these methods are tedious and time consuming.

HPLC is the most widely used technique for determining natural and fortified levels of water-soluble vitamins in meat products.

In Tables 15.4 through 15.6, determinations of water-soluble vitamins in PMP are summarized.

15.3.1 Thiamine (Vitamin B₁)

Thiamine is present in all animal tissues and therefore in all natural unprocessed animal foods. Lean pork, heart, kidney, and liver are good dietary sources of thiamine. Total thiamine is the sum of thiamine, thiamine monophosphate, thiamine pyrophosphate, and thiamine triphosphate. The latter is the principal form in animal products.

Table 15.4 Thiamine (B₁) and Riboflavin (B₂) Determination by HPLC in Processed Meat and Poultry

Vitamin	Food	Extraction	Purification	HPLC Parameters	Reference
B ₁	Pig's liver (CRM)	0.055 M HCl autoclaved 121°C 4 h. 2.5 M sodium acetate to pH 4.5. Papain and diastase 37°C 18 h. Heat 100°C 5 min. Filtered.		LC 18 (25 × 0.46 cm, 5 μm). 25°C. MetOH phosphate buffer (0.05 M sodium phosphate dibasic heptahydrate + 85% orthophosphoric acid, pH 3.55) 10:90. 1 mL/min. i.v. 20 μL. UV-Vis diode array detection: 280–288 nm.	29
B ₁	Cooked sausages: lunch, chopped (pork, beef, and turkey) vitamined chopped, Sicilian mortadella	0.1 N HCl stirred autoclaved 120°C 20 min. 2.5 M sodium acetate to pH 4–4.5. 6% clara-diaastase (α-amylase, cellulose, invertase, peptidase, phosphatase, and sulfatase) 50°C 3 h. Protein precipitate trichloroacetic acid 90°C 15 min. Water to 100 mL. Filtered. Oxidation to thiochrome: 1% potassium ferricyanide in 15% aqueous NaOH.	C ₁₈ Sep-Pak cartridge. Activated: MetOH:water 2:2; MetOH:5 mM phosphate buffer pH 7.5:95. Elution: MetOH.	Spherisorb C ₈ (25 × 0.4 cm, 5 μm). 35°C. Phosphate buffer (5 mM, pH 7): ACN 70:30. 0.65 mL/min. i.v. 10 μL. Fluorescence detection: 360/430 nm.	30
B ₂				Spherisorb ODS-2 (25 × 0.4 cm, 5 μm). 35°C. Heptanesulfonic acid (5 mM pH 2.7):acetonitrile 75:25. 0.6 mL/min. i.v. 10 μL. Fluorescence detection: 227/520 nm.	31
B ₂	Raw and cooked beef liver, fast food hamburgers	Comparison of manual and robotic extraction: MetOH + methylene chloride + IS Homogenization 15,000 rpm 75 s. 100 mM citrate-phosphate buffer pH 5.5. Homogeneization 15,000 rpm 30 s. Centrifugation (manual extraction: 40,000 g 4°C 10 min. Robotic extraction: 712 g ambient temperature 10 min).		Two PLRP-S columns in series (Polymer) (15 × 0.46 cm + 25 × 0.46 cm, 5 μm macroporous polystyrenedivinylbenzene resin 100 Å pore size). PLRP-S guard column (0.5 × 0.3 cm). 40°C.	32, 33, 34 ^a

Mobile phase gradient: ACN:0.1% sodium azide 10 mM citrate-phosphate buffer, pH 5.5:0 min 3:97
 43 min 6:94
 51-70 min 14:86
 80-90 min 3:97
 Flow rate: 0-43 min 1.2 mL/min;
 43-80 min 1 mL/min; 90 1.2 mL/min (convex gradient)
 Fluorescence detection: 450/522 nm.
 Lichrospher 100 RP18 (25 × 0.4 cm).
 MeOH:water:acetic acid (with 0.005 M heptane sulfonic acid) 34:65:1. 1 mL/min. i.v.: 100 µL.
 Fluorescence detection.

0.1 N HCl autoclaved 121°C 30 min. 2 M sodium acetate to pH 4-4.5. 6% clara-diasase (α -amylase, cellulose, invertase, peptidase, phosphatase, and sulphatase) 50°C 3 h. Heating 97°C 10 min. Water to 100 mL. Centrifugated 4000 rpm (2800 g) 25 min. Cooling and filtered (Whatman No. 40 filters).

Thiamine: AOAC 942.23 36
 Riboflavin: AOAC 970.65 37
 Spherisorb silica (50 × 0.21cm, 20 µm). CH₃Cl:MeOH 90:10.
 Thiamine: 1 mL/min. i.v.: 10-20 µL.
 Riboflavin: 0.8 mL/min i.v.:5 -10 µL.
 Fluorescence detection: Thiamine excitation 367 nm. Riboflavin excitation 270 nm.

Beef, pork, and chicken (grilled, fried, roasted, cooked)
 Meat products
 Ground beef, cooked wieners, bologna, and canned pork
 0.1 N HCl autoclaved 121°C 30 min. 2 M sodium acetate to pH 4-4.5. Takadiastase and papain 42-45°C 2.5-3 h. Proteins precipitated with 50% thricloroacetic. Heating 5 min water bath 100°C. Cooling and filtered (Whatman No. 40 filters).
 Riboflavin was converting to lumiflavin: pH 10-12 (15% NaOH), UV lamp 30 min. Glacial acetic acid. Extraction CH₃Cl. Extract dried (Na₂SO₄).

(Continued)

Table 15.4 (Continued)

Vitamin	Food	Extraction	Purification	HPLC Parameters	Reference
		Thiamine was oxidized to thiochrome with 1% alkaline potassium ferricyanide and extracted with isobutyl alcohol.			
	Lyophilized pig's liver (reference material)	0.1 M HCl 100°C water bath or autoclave 121°C 30 min. 2.5 M sodium acetate to adjusted pH 4.5. Papaina, 1% glutathione, acid phosphatase, and α -amylase 37°C 18 h. Thiamine was converted to thiochrome.		Lichrospher 100 RP 18 (15 × 0.4 cm, 5 μ m); guard column RP 18 (0.4 × 0.4 cm, 5 μ m). MetOH:0.05 M sodium acetate 30:70. 1 mL/min. i.v.: 20 μ l. Fluorescence detection: Thiamine 366/435 nm. Riboflavin: 422/522 nm.	38
	Lyophilized pig's liver (reference material), steam-cooked meat roll products	0.1 M HCl 100°C water bath 15 min. Homogenization 1 min 9000 rpm. 100°C water bath 45 min. 2 M sodium acetate to adjusted pH 4.3–4.7. Several enzyme formulations were assayed (takadiastase \pm papain or acid phosphatase \pm papain) 37°C 18 h. Proteins precipitated with 50% thricloroacetic. Heating 10 min water bath 100°C. Cooling and filtered (Whatman No. 40 filters). Thiamine was converted to thiochrome with 1% potassium ferricyanide in 15% NaOH and extracted with isobutyl alcohol.		Inertsil 5 ODS-2 Chrompack (20 × 0.3 cm) Thiamine: MeOH:water 40:60. 0.4 mL/min. i.v.: 20 μ l. Fluorescence detection: 366/434 nm. Riboflavin: MeOH:water 80:20. 0.3 mL/min. Fluorescence detection: 450/510 nm.	32

Ham and liver	0.01 M HCl autoclaved 121°C 30 min. Sodium acetate to adjusted pH 4.5. Takadiastase, claradiastase, and papain 37°C, 16–18 h. Filtered, pH adjusted 6.5, second filtration.	Clean-up columns: Nucleosil C ₁₈ cartridge 500 mg. Precondition: MetOH with 5 mM sodium heptane-sulfonate. Washed: water:MetOH 80:20 with 0.005 M sodium heptane-sulfonate. Elution: water:MetOH (50:50) with 0.005 M sodium heptane-sulfonate.	Nucleosil C ₁₈ (15 × 0.46 cm, 3 μm) and Nucleosil C ₁₈ guard column (2 × 0.46 cm 10 μm) 45°C. 0.01M potassium dihydrogenphosphate buffer (pH 3): ACN 84:16 containing 5 mM sodium heptanesulfonate (ratio 85:15 for liver samples). i.v. 50 μL. UV detection: 254 nm. Thiamine: 0.47 g PIC [®] B6 Waters in 500 mL 85% methanol + 1 mL glacial acetic acid (pH 6.0 adjusted with 5% Na HCO ₃). Fluorescence detection: 360/435 nm. Riboflavin: 45% MetOH in water with 0.005 m PIC [®] B7 Waters. Fluorescence detection: 360/525 nm.
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^a A simultaneous HPLC separation of TRF, RF, FMN, FAD has been developed.

Table 15.5 Water-Soluble Vitamin Determination by HPLC in Processed Meat and Poultry

Vitamin	Food	Extraction	Purification	HPLC Parameters	Reference
B ₅	Canned meat product (CRM)	Sample + IS Hopantenic acid autoclaved 103°C 20 min.		LC-MS C ₁₈ (15 × 0.3 cm, 3µm) Water and 0.025% trifluoroacetic acid (pH 2.6); ACN. Gradient elution: 0–3.5 min 100:0, 3.5–11 min 75:25, 11–19 min 65:35, 19–20 min 90:10, 20–26 min 100:0. 0.5 mL/min. i.v. 20 µL. MS with positive ESI and selected ion monitored (SIM) mode. 140 V fragmentor voltage, 10 L/min drying gas flow, 350°C drying gas temperature, 30 psig nebulizer pressure, 4500 V capillary voltage. Monitor <i>m/z</i> 220 (vitamin B ₅), 234. Lichrospher 100 RP 18 endcapped column RP 18 (0.4 × 0.4 cm, 5 µm). MetOH:phosphate buffer (33 mM, pH 2.5). Gradient elution: Pantotenic acid: MetOH gradient 0–10% 25 min, 8 min 90:10; final 0:100. 1 mL/min.	41
B ₅	Pig liver	<i>Pantotenic acid</i> : Tris buffer (pH 8). Shaking 5 min, filled to 50 mL and centrifuged 11500 rpm 10 min. <i>Total B₅</i> : Acetate buffer (pH 4.5) + pepsin. Incubation 50°C 3h (pH 8) + Tris buffer (pH 8) + alkaline phosphatase + pantheinase. Incubation 20 °C 18 h. Filled to 50 mL and centrifuged 11,500 rpm 10 min.	SPE: Chromafix 400-SB (SAE); 400-SA (SCE)		42
				Total B ₅ : MetOH gradient 10–45 % 7 min, 11 min 45:55, final 90:10. Postcolumn derivatization: 200 mM NaOH + 1 mM OPA + 1.6 mM 3-MPA. Fluorescence detection: 345/455 nm.	

B ₆ (PM, PMP, PN, PL, PLP simultaneously)	Chicken (raw and fried)	Sulfosalicylic acid + IS (3hydroxypyridine) 10 min in ice. CH ₂ Cl ₂ mixed 5 min. Centrifugation: 1800 g, 10 min 4°C.	Chemimert LC83 (4 cm × 1 cm) packed with Dowex AG 2-X8 (200–400 mesh). Elution: 0.1 N HCl.	Ion exchange (diethylaminoethyl groups bound to G5000 power white) (7.5 × 0.75 cm). Guard column Bio-Gel HPTt (5 × 0.4 cm) NaCl + glycine buffer (pH 9.8). i.v. 3 µL. Postcolumn reagent: Na ₂ HPO ₄ (pH 4.9). Fluorescence detection: PM, PMP, PLP (330/400 nm). PN and PL (310/400 nm). Lichrospher 100RP 18 endcapped C ₁₈ (25 × 0.4 cm, 5 µm).	43
B ₆	Pig's liver (CRM)	Sodium acetate (pH 4.5) + glyoxylic acid + ferrous sulfate + papain + phosphatase + α-amylase 18 h 37°C. Water to 100 mL and filtered. Filtered + NaOH + sodium brohydride shaken. Glacial acetic acid. Filtered: cellulose acetate, 0.5 µm. Metaphosphoric acid stirring 2 min room temperature. Water to 100 mL. Centrifuged 5 min 3000 rpm. Filtered.		MetOH:sodium acetate. 30:70. 1 mL/min. i.v.: 20 µL. Fluorescence detection 290/395 nm.	38
B ₆ (PM, PMP, PN, PL, PLP)	Cooked sausages: lunch, chopped (pork, beef and turkey) vitamed chopped, Sicilian mortadella Pork meat products	HCl + IS (4-deoxyriodixine) shaking 30 min 100°C. pH (4–4.5) + takadiastase shaking 3h 45°C. Trichloroacetic acid 5 min 100°C. Water to 100 mL. Trichloroacetic acid shaking. Centrifuged 10 min 3000 rpm (2×). Acid extracts centrifuged 5 min 4000 rpm and 5 min (-20°C) elimination fat. Centrifuged.		Hypersil BDS C ₁₈ (10 × 0.46 cm, 5 µm). Potassium phosphate buffer (pH 3.2):ACN. 99:1. 35°C. i.v.: 10 µL. Fluorescence detection: 290/395 nm.	44
B ₆	Boiled ham			Spherisorb ODS C ₁₈ (25 × 0.4 cm, 5 µm). 30°C. 0.01 M H ₂ SO ₄ . 1 mL/min. i.v.: 20 µL. Fluorescence detection: 290/395 nm.	36, 45
B ₆				Luna C ₈ (25 × 0.46 cm, 5 µm). (Octanesulfonic acid 5 mM–0.5% triethylamine – 2.45% acetic acid – 15% MetOH):ACN 90:10. 1 mL/min. i.v.: 20 µL.	46

(Continued)

Table 15.5 (Continued)

Vitamin	Food	Extraction	Purification	HPLC Parameters	Reference
THF, 5-MTHF, 5-HCO-THF, FA and folate total	Liver products	Extraction with γ -carboxy peptidase (pH 4.9) in a water bath 2 h, 37°C.	SAE cartridges (Quaternary amine). Elution: 0.01M phosphate buffer (10% NaCl + 1% AA) (pH 4.5)	Shandon Hypersil ODS (15 × 0.46 cm, 3 μ m). Gradient ACN and 30 mM phosphate buffer (pH 2.2). 0.8 mL/min. Fluorescence detection: THF, 5-MTHF, 5-HCO-THF: 290/356 nm. FA: 290 nm.	47
Biotin	Beef liver, chicken liver, pork chop	Sample: water (1:4) + HCl 100 °C, 120 min. Centrifugation: 550 g 10 min. 10 M NaOH (pH 2.5).		Avidin-binding assay: C ₁₈ . i.v.: 0.5 mL Retention time determined by radiolabeled biotin.	48
Niacin	Beef, pork, and lamb heat processed	HCl 121°C 30 min. 2 mM sodium acetate to adjusted pH 4–4.5. Takadiastase and papain 42–45°C, 2.5–3 h. Proteins precipitated with 50% Trichloroacetic acid. Water bath 100°C, 10 min. Cooling and filtered (Whatman No. 40).		Alltech C ₁₀ 10- μ m. 0.02 M phosphate buffer: MeOH 70:30 (pH 7). 1 mL/min. i.v. = 10–20 μ L. UV detection: 254 nm.	40
Dry-cured meat		HCl + MetOH 121°C 30 min. Trichloroacetic acid. Water to 100 mL. Filtered through filter paper and cellulose acetate filter (0.45 μ m).		Ion exchange: OmniPac PCX 500 microbore column (25 × 0.2 cm) 5% ACN 140 mM, formic acid 15 mM sodium formate. 250 μ L/min. 40°C. v.i.: 10 μ L.	49
Cooked sausages: lunch, chopped (pork, beef and turkey) vitamined chopped, Sicilian mortadella		Sample + water stirring 2 min. Complete volume 100 mL. Centrifugation 3000 rpm 5 min. Protein precipitation: Filtered extract + saturated Zn ₂ SO ₄ + 1N NaOH. Filtered.		UV detection (262 nm)/MS detection (ESI+), 50 V, 350°C, SIM. Spherisorb ODS (25 × 0.4 cm, 5 μ m). 35°C. Heptanesulphonic acid solution (5 mM, pH 3.3): ACN 75:25. 0.65 mL/min. i.v.: 10 μ L. UV detection: 261 nm.	50

	Raw and cooked meats	HCl 121°C, 30 min.	51
AA, DHA	Hot dog (raw, boiled, fried)	Sample + citric acid. Elimination of fat by hexane. Centrifugation 1200 g 5 min.	52
AA, DHA, IAA, DHIA	Meat based food products, chopped ham	8% <i>m</i> -phosphoric acid/acetic acid. Shaking vortex. Centrifugation.	53
AA, IAA	Ham, canned ham, canned sausage, paté de foie	Sample + HPO ₃ . Polytroed and centrifuged.	54
AA	Cooked sausages	Sample + 5% <i>m</i> -phosphoric acid 5%.	55
AA	Meat products	Ground sample + <i>m</i> -phosphoric acid.	56
AA, IAA	Processed meat	Ground sample + <i>m</i> -phosphoric acid. Filtered.	57

C₁₈ column (25 × 0.46 cm).

ACN: Potassium dihydrogen phosphate buffer (pH 7) + 0.01 M sodium exansulfonate. 12.5:87.5. 1 mL/min. 45 °C. i.v.: 50 µL. UV detection: 254 nm.

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Total AA (AA+DHAA) and IAA (IAA+DHIAA): 500 µL supernatant + H₂PO₄ + homocysteine. AA and/or IAA: 500 µL supernatant + H₂PO₄. Amperometric detection.

Spherisorb NH₂ cartridge (25 × 0.4 cm). 0.02 M potassium phosphate buffer (pH 3.6):ACN 40:60. UV detection: 248 nm.

RP-HPLC ion-pairing C₁₈. Trioctilmethylammonium chloride/ MetOH (pH 5.05).

UV detection: 255 nm. RP-HPLC ion-pairing C₁₈. 1,5-dimethylhexylamine. UV detection: 254 nm.

Table 15.6 Vitamin B₁₂ Determination

<i>Vitamin</i>	<i>Food</i>	<i>Extraction</i>	<i>Method Determination</i>	<i>Reference</i>
B ₁₂	Bovine muscle (raw and cooked)	0.1 M acetate buffer (pH 4.6) + papain. 1% cyanide 60°C 1 h.	Radioassay (Quantaphase B ₁₂ , Bio Rad Laboratories).	58
B ₁₂	Meat products	Disodium phosphate + anhydrous citric acid + sodium metabisulfite 121°C 10 min. Centrifugation.	Microbiological (<i>Lactobacillus leichmannii</i>). Turbidity (% T): 540–660 nm.	36
B ₁₂	Beef liver	0.5 M acetate buffer (pH 4.8) + KCN 98°C 30 min, in the dark. Centrifugation: 10,000 g 10 min.	Chemiluminescence B ₁₂ analyzer and microbiological method (<i>Lactobacillus leichmannii</i> ATCC 7830). Turbidity (% T): 600 nm.	59

Acid hydrolysis by boiling or autoclaving is used to release the vitamins from the food matrix. In thiamine determination, an enzymatic hydrolysis is used to release it from phosphate esters. Various conditions of acidity (mainly HCl 0.1 M) and enzymes (takadiastase, papain, pepsin, clara-diaastase, acid phosphatase, and amylases) have been proposed, and several methods for the determination of thiamine in meat products have been reported [60,30].

Ndaw et al. [38] have studied the use of different enzymes in the extraction of vitamins B₁, B₂, and B₆ from meat and pig liver before their HPLC determination; they propose the use of a mixture of α -amylase, papain, and acid phosphate that allows the release in a single step of the different forms (phosphorylated and protein-bound) of these vitamins.

Conversion of thiamine and phosphate esters to their fluorescent thiochromes continues to be the almost universal choice for detection. The fluorescence of thiochrome coupled to HPLC provides an analytical system with a detection limit in the low picogram range [61].

Although HPLC is the method of choice, excellent analytical data can be obtained using microbiological or manual thiochrome procedures (AOAC Official Method 942.23) [62]. An intercomparison study has been done on the determination of vitamin B₁ in different foods, among them lyophilized pig liver, applying HPLC (normal and reversed phase) methods, manual fluorimetry methods, and microbiological assays (MBAs) with *L. fermentum* and *L. viridescens* [60]. The authors indicate that neither the type of HPLC column (normal or reversed phase) nor the type of reaction (pre- or postcolumn) affects the results. MBAs tended to yield higher contents compared to those from HPLC methods for pig liver. This was also found in an earlier intercomparison [63]. It is not clear whether these higher values result from the presence of additional factors stimulating (nonspecific) growth of the organism (i.e., positive drift) or from “bound” thiamine unavailable for HPLC analysis. This apparent difference between methods requires some further comparative study.

In a comparison between thiamine determination in pork and lean beef by capillary electrophoresis (CE) and HPLC, the values obtained were similar. It is reported that the CE method, which is economical and ecologically sound, constitutes an interesting alternative tool to the HPLC method [64].

15.3.2 Riboflavin (Vitamin B₂)

Riboflavin functions metabolically as the essential component of two flavine coenzymes, flavine mononucleotide (FMN) and flavine adenine dinucleotide (FAD), which act as intermediaries in electron transfer in biological oxidation-reduction reactions. Animal protein sources such as meats are good sources of riboflavin. Acidification and enzyme hydrolytic treatment guarantee the complete hydrolysis of FAD and FMN to riboflavin. One of the most important problems in riboflavin analysis is the lability of flavines to light and alkaline conditions, so all analytical procedures have to be performed avoiding both factors [65].

Since thiamine and riboflavin are compatible for combined extraction, traditional extraction methodology used for chemical or microbiological analyses has been applied to the combined or simultaneous analysis of these vitamins. The extraction procedures generally applied in the determination of total thiamine and riboflavin by HPLC involve hot acid digestion to release free thiamine and riboflavin and their phosphate esters from their association with proteins, followed by enzymatic hydrolysis of the phosphate esters to complete the release. Generally, the acid hydrolysis step is carried out by heating the foodstuff with 0.1 M HCl, and commercial diastases (which have mixed amylase, protease, and phosphatase activity) are used to accomplish enzymatic hydrolysis [32].

Owing to the lability of flavines to light and to alkaline or extremely acidic pH values, special nonhydrolytic extraction conditions are required to quantify riboflavin and its coenzymes (FMN and FAD). The coenzymes are also susceptible to hydrolysis below pH 5.0. This requires carrying out the extraction and analysis of the individual vitamers between pH 5.0 and 7.0 and under subdued light conditions [66]. A nondegradative extraction, with methylene chloride, methanol, and a citrate-phosphate buffer (pH 5.5) and simultaneous quantitation of riboflavin, FMN, and FAD, has been proposed by Russell and Vanderslice [33], and applied to beef liver, cooked beef steak, and hamburgers. In a later study, the authors [67] developed a robotic HPLC determination of riboflavin, FMN, and FAD in different food products (among them raw and cooked beef liver and cooked beef steak). The robotic method compares favorably with manual extraction, including determinations on samples known to contain degradative enzymes. The robotic method generally produces slightly higher results than the manual method, which is indicative of less degradation and interconversion of the individual vitamers during extraction. The robotic extraction is faster than its manual counterpart and allows operation in the complete absence of light.

Simultaneous HPLC with UV detection determination of thiamine and riboflavin in liver, offering the advantage of not requiring the pre- or postcolumn derivatization needed in thiamine determination with fluorescence detection, has been described [39]. The method is applicable to liver samples, because the levels of thiamine and riboflavin in these products are high enough to be detected by UV.

Even though numerous methods have been reported for the determination of riboflavin content in foods, RP-HPLC has been recommended, employing a UV detector or a fluorescence detector because of the strong UV light absorption of flavines and their intense fluorescence [31].

An intercomparison study has been done on the determination of vitamin B₂ in several foods, among them lyophilized pig liver, using RP-HPLC methods and MBAs with *L. rhamnosus* and *Enterococcus faecalis* [60].

The official AOAC fluorimetric method for determining riboflavin (970.65) [62] includes oxidation of the organic matter in the sample extract with KMnO₄, followed by elimination of the oxidant agent excess with H₂O₂. A comparison between the determination of total riboflavin

in cooked sausages by ion-pair RP-HPLC and the official AOAC method, including sample oxidation, showed the destructive influence of the oxidation step, while its elimination increased the sensitivity [31].

15.3.3 Vitamin B₆

Vitamin B₆ is the generic descriptor for 3-hydroxy-2-methylpyridine derivatives having the biological activity of pyridoxine. It comprises three chemically, metabolically, and functionally related forms (or vitamers): pyridoxine or pyridoxol (PN), pyridoxal (PL), and pyridoxamine (PM). Pyridoxamine phosphate (PMPh) and pyridoxal phosphate (PLP) are the main vitamin B₆ vitamers in meat. HPLC techniques are the most common in analyzing the various vitamers of B₆ (fluorescence detection). In the quantification of the different vitamers, sulfosalicylic or metaphosphoric acid have been used to precipitate protein in chicken [43] and cooked sausages [44], respectively. Determination of total B₆ requires hydrolysis: acid (HCl or H₂SO₄) and enzymatic (phosphatase, or diastase, or an enzyme mixture of α -amylase, papain, acid phosphatase) [38].

The official microbiological method (AOAC 961.15 [62]) allows the determination of PN, PL, and PM by using an anion exchange resin and *Saccharomyces uvarum*.

The existence of different vitamin B₆ vitamers in complex matrices makes analysis difficult. Therefore, it is not surprising that different HPLC procedures have been the techniques of choice.

Separation of vitamin B₆ vitamers is well suited to ion exchange, RP-HPLC, or ion-pair RP-HPLC because of their pH-dependent ionic nature [44]. RP-HPLC with coulometric EC detection has been developed to determine PM, PL, and pyridoxine, and applied to pig liver (CRM 487). The method is rapid (17 min) and sensitive (limits of quantification for PM, PL, and pyridoxine of 2.1, 2.01, and 0.99 ng/mL, respectively) [29].

A possible interconversion among vitamers due to the extraction and analytical procedure, resulting in apparent losses and changes in the B₆ vitamer composition, cannot be ruled out. This is the reason why identification of the vitamin B₆ vitamers by HPLC has been shown to be a source of error [60].

15.3.4 Vitamin B₁₂ (Cyanocobalamin)

The predominant forms of cobalamin present in animal tissues include hydroxocobalamin and the two coenzyme forms, methylcobalamin and adenosylcobalamin.

Meat and animal organ tissues, especially liver, are excellent dietary sources of vitamin B₁₂, with cobalamin originating from intestinal flora and coprophagia in the case of herbivorous animals. Ruminant meat is an important source of vitamin B₁₂ for human nutrition, accounting for approximately two-thirds of the daily intake of vitamin B₁₂ by humans. In addition, the chemical forms of vitamin B₁₂ present in meat are the biologically active forms, which are of direct importance to human nutrition [58].

The release of vitamin B₁₂ from proteins is generally obtained by autoclaving the food sample (121°C) or by heating at 100°C. Protease treatment (pepsin) has also been recommended. The addition of sodium cyanide during sample treatment prior to quantification converts the native vitamin forms into dicyanocobalamin. Vitamin B₁₂ compounds are extracted in a phosphate buffer containing a reducing agent (metabisulfite or ascorbic acid [AA]) to protect the cobalamins throughout the extraction [29].

Generally, for the routine analysis of vitamin B₁₂, a microbiological method (AOAC official method 952.20 [62]) has been used. However, this kind of methods is tedious, time consuming, and technically difficult.

HPLC methods for determination of B₁₂ have been reported but are less sensitive than the microbiological methods [59]. However, RP-HPLC with coulometric EC detection without prior transformation of cobalamins to cyanocobalamin that is both rapid (17 min) and sensitive (limit of quantification 0.11 ng/mL) has been applied to pig liver (CRM 487) [29].

The radioisotope dilution assay (RIDA), the most specific B₁₂-binding protein assay, has been clinically used for the routine assay of human serum B₁₂, but has also been used for the determination of B₁₂ content in liver and sausages [68] and cooked meat [58]. This method offers two advantages over the microbiological method: It is faster, requiring only half as long to perform the analysis, and it is more sensitive (detection limits of 1 µg/kg versus 2 µg/kg for the microbiological method) [68]. The correlation coefficient between the values obtained by the microbiological method and RIDA is excellent ($r = 0.983$) [69]. The RIDA method, however, requires radioisotope facilities and apparatuses, and also raises the problem of using a radioisotope.

The application of a fully automated chemiluminescence analyzer for the determination of vitamin B₁₂ in serum has been used for vitamin B₁₂ determination in foods, including beef liver, and compared to the microbiological method. The coefficient correlation for the two methods is 0.99. The microbiological method is time consuming (2 or 3 days) and requires a sterile technique and a well-trained full-time technician. The chemiluminescence method is simpler, quicker (180 samples analyzed per hour), easy to run (fully automated), and more selective and reproducible than the microbiological method. However, the sensitivity of the microbiological method (0.01–0.2 µg/L) is much higher than that of the chemiluminescence method (0.05–0.2 µg/L) [59].

Biomolecular interaction analysis (BIA), a biosensor-based technique that involves the continuous, nonlabeled monitoring of sensor-bound, ligand-analyte interaction via surface plasmon resonance (SPR) has been applied to vitamin B₁₂ determination in foods (among them sheep liver). The method was compared with reference MBA and radioisotope protein-binding analysis (RPBA), also described as the RIDA method. The correlation coefficient for BIA-MBA was $r = 0.9922$. RPBA estimations were low, with an overall bias of approximately 8% relative to MBA [70].

15.3.5 Niacin (Vitamin B₃)

Niacin is the generic term for two vitamers, nicotinic acid and nicotinamide. In meat it is primarily present in the nicotinamide form. Liver, heart, kidney, and lean meat constitute rich sources of niacin; it is present in bound (nicotin adenin dinucleotide [NAD] and nicotin adenin dinucleotide phosphate [NADP]) and free forms.

In the determination of niacin in foods, several extraction procedures have been used. The terms “total” and “free” niacin are defined by the extraction methods used in the analysis. Determination of total niacin in foods requires a hydrolysis procedure. Owing to the relative stability of the niacin vitamers, either acid or alkaline hydrolysis can be used to convert nicotinamide to nicotinic acid for quantitation [71]. Usually, after hydrolysis a sample clean-up step is done to eliminate interferences before HPLC analysis and/or derivatization, improving selectivity and sensitivity.

A microbiological method (*Lactobacillus plantarum*, AOAC 944.13 [62]) is used for niacin determination. This method is costly, laborious, and time consuming (72 h incubation) [72].

Colorimetric methods using cyanogen bromide and sulfanilic acid (AOAC 961.14 and 981.16 [62]) have conventionally been used for the estimation of niacin in foods. These methods offer the advantage of allowing automatization by a flow system, though with the disadvantage of the use of cyanogen bromide—a noxious and unstable reagent.

RP-HPLC with UV detection has been used for determining niacin in meat products [73,74]. However, simultaneous determination of nicotinic acid and nicotinamide is difficult due to the different basicity and polarity of the two vitamers and/or interference problems. The detection of these vitamers requires the use of ion-pair reagents; nicotinic acid and nicotinamide have been simultaneously determined in cooked sausages by RP-HPLC with UV detection and ion-pair reagents [50].

Problems of selectivity and repeatability in niacin determination by ion-pair or RP methods are due to interferences derived from the standard hydrolysis procedure—interferences that can be reduced or minimized by time-consuming sample clean-up or derivatization procedures.

Cation-exchange separation and UV detection at 262 nm allows the simultaneous separation of nicotinic acid and nicotinamide from meat and meat products. The method includes rapid sample preparation without the need of any sample clean-up procedure, and has been validated by impact chemical–mass spectrometry (IC-MS) [49].

An alternative to HPLC methods for niacin determination is CE. An alkaline or acid digestion has been used to release niacin from the food matrix (cooked meats and canned ham) followed by SPE cleanup and determination by CE. This technique is preferred to HPLC, since it produces cleaner traces, is faster, and is also more cost effective [73,74].

15.3.6 *Pantothenic Acid (Vitamin B₅)*

Vitamin B₅ (pantothenic acid) exists in foodstuffs in its free form, as well as bound in coenzyme A (CoA) and acyl carrier protein. The determination of total vitamin B₅ content therefore requires the release of the vitamin from its bound forms. Neither acid nor alkaline hydrolysis can be used since pantothenic acid is degraded by such treatments. The only practicable alternative is enzymatic hydrolysis.

A microbiological method based on the turbidimetric growth of *Lactobacillus plantarum* response has been approved by the Association of Analytical Communities (AOAC 945.74 [62]) as the official method for vitamin B₅ determination in food products. Although this method has been successfully applied to the determination of vitamin B₅ in complex samples where the matrix components could interfere with the growth of *Lactobacillus plantarum*, its selectivity is limited. In addition, the method is time consuming; 2–3 days are needed from sample reception to the reporting of results [41].

Radioimmunoassays and enzyme immunoassays offer very high selectivity; however, from a practical point of view, these methods have disadvantages (the use of radioisotopes and scintillation counting for radioimmunoassay, and the acquisition of noncommercially available antisera for indirect enzyme immunoassay). Moreover, nothing can guarantee their specificity owing to the possibility of cross-reactions, most particularly in the food matrices.

CE and chromatographic methods, while much more specific than the above mentioned methods, have been subject to very little development owing to the particular physical properties of pantothenic acid: This molecule, of very low volatility and non-fluorescent, absorbs very weakly in the UV region, and only at wavelengths shorter than 210 nm [42].

A fluorimetric determination of pantothenic acid in foods by LC with postcolumn derivation has been applied to pig liver; different treatments of the sample (absence of enzyme hydrolysis,

alkaline phosphatase-pantetheinase, or pepsin and alkaline phosphatase-pantetheinase) allow the determination of free pantothenic acid, free and bound pantothenic acid, or CoA and total vitamin B₅ [42].

15.3.7 Folate

Folate exists in several chemical forms in liver, its main storage organ, as well as other animal tissues.

HPLC has been applied to the quantification of the main folate forms, tetrahydrofolate (THF) and 5-methyltetrahydrofolate (5-MTHF), in raw and processed liver products. THF was the predominating form in fresh pig and beef livers and in beef kidneys, but in frozen chicken liver the predominant vitamer was 5-MTHF [47].

An intercomparative study on folate analysis in food (including pig liver) by HPLC showed 5-MTHF to be the most successfully determined by all laboratories, whereas little or no agreement was found for the other folate vitamers. It was suggested that possible reasons for the variability in results between laboratories included the poor stability of folates during extraction unless proper protective measures were taken, and variability in deconjugation. Poor quality and stability of commercial standards can cause systematic errors in quantitation. Although particular attention was given to peak identification, peak impurities, misidentification, and unsuitable extraction were also among the most probable sources of error [75].

15.3.8 Biotin

A microbiological method (*Lactobacillus plantarum*) or photometry/fluorimetry (based on the formation of a complex with avidin or streptavidin) are generally complex to implement and do not distinguish *d*-biotin from its inactive analogues. HPLC appeared particularly well suited for this isolation. A satisfactory detection limit could only be obtained after pre- or postcolumn conversion of *d*-biotin into fluorescent derivative. Enzymatic hydrolysis, followed by postcolumn derivatization by avidin-fluorescein 5-isothiocyanate (FITC) and fluorimetric detection of the complex obtained, has been applied to biotin determination in beef liver. The method offers good recovery ($90 \pm 106\%$), satisfactory repeatability (coefficient of variation less than 7%), and a very low detection limit (0.005 µg/g) [76].

Total biotin content in food products (among them beef and chicken liver and pork chop) have been determined using acid hydrolysis and the HPLC/avidin-binding assay; biotin was separated from its inactive form with detection limits of approximately 0.001 ng biotin [48].

15.3.9 Vitamin C

Vitamin C exists naturally as two biologically active vitamers, L-AA and dehydroascorbic acid (DHAA), and is often bound to proteins in food. Erythorbic acid (Era), also referred to as D-isoascorbic acid (IAA), is one of the stereoisomers of AA. Owing to its reducing properties, IAA is widely used as an antioxidant in meat products to stabilize nitrate and nitrite and to accelerate the curing process.

Metaphosphoric acid is the most commonly used vitamin C extractant because it prevents hydrolysis of the lactone ring and inhibits oxidation. To minimize sample degradation during

preparation, purging of samples with inert gases such as nitrogen or argon, rapid freezing and storage of sample at -70°C , and minimizing exposure to light have been proposed [77].

The spectroscopic methods cannot differentiate between AA and IAA.

HPLC with UV detection has been used to determine Era/IAA and AA in canned or processed meat products [57].

The same technique but with EC detection has been applied to processed meat for the quantification of AA, DHA, IAA, and dehydroisoascorbic acid (DHIAA). The limit of detection was 0.2 mg/100 g [54].

15.4 Loss of B Group Vitamins Due to Processing

Cooking conditions strongly affect the content of B vitamins in meat, due to their high solubility and thermal instability. Irrespective of the cooking method and equipment used, losses of B vitamins during cooking are highly variable, being dependent on the temperature-time combination used, the type of meat and its initial vitamin content, moisture loss, pH, sample size, and water-holding capacity.

Thiamine has been shown to be the most unstable when heated and therefore is still regarded as the indicator vitamin during thermal processing, because it is both heat-labile and water-soluble. A regression equation has been developed for predicting thiamine retention in meats according to the thermal conductivity, cooking temperature, mass of sample, and cooking time involved [78].

Losses of riboflavin during thermal processing are negligible in most foods. Niacin is probably the most stable of the B-vitamins [46].

Studies carried out on the retention of B vitamins from PMP are reported below. The high variability in published retention values can be ascribed to the many factors affecting it.

Retentions of thiamine in the range of 85–97% have been reported in connective heating of meat loaves depending on the air speed temperature and humidity used [79].

In chicken breast and thigh muscle cooking in a fan-assisted oven (45 min at 190°C), retentions of 79 and 86% for thiamine and pantothenic acid, respectively, are mentioned [80].

The study of the effect of different heat-processing methods (rotating hot air, charbroiling) on riboflavin, niacin, and thiamine in beef, pork, and lamb show the heat stability of niacin; the decrease in riboflavin content in beef in all assayed heating procedures; and the stability of thiamine in lamb and its decrease in beef (charbroiling) and pork (rotating hot air, charbroiling, and deep-fat frying) [40].

The effect of rotating hot air, charbroiling, and deep fat frying on thiamine, riboflavin, and B₆ vitamin content in beef, lamb, and pork has been compared. While no differences due to the heating method have been found, the end-point of cooking had a significant effect, particularly for thiamine and riboflavin in beef loin steaks. The rare product retained 71% of thiamine and 72% of riboflavin, compared to 50 and 55% in the well-done product [81].

In cooked meats (beef, veal, lamb, horse, ostrich, sirloin, pork, chicken, turkey, and rabbit), thiamine is undoubtedly the most susceptible to thermal degradation. Indeed, after cooking, thiamine was found to be undetectable in most of the samples; riboflavin, generally most stable to heat, showed retention between 20 and 58%. Less severe losses in retention of between 30 and 51% occurred with niacin content [51].

A comparison of vitamin B₆ content in raw chicken and in fast food fried chicken showed that deep fat frying produces losses of 6.5% in the vitamin B₆ content. The breading and batter may assist by trapping the liquid and therefore decreasing loss of the water-soluble vitamins [43].

Microwave cooking resulted in significantly greater retention of vitamin B₆ in poultry than roasting in a conventional oven. Thiamine content was higher in broilers cooked in a microwave cooker when compared to an electric oven, with no difference between an 800 and a 1600 W microwave cooker [82].

Similarly, in pork and chicken meat, microwave-heated samples showed retentions as high as 85.6–96% for thiamine and 59.9–80.9% for vitamin B₆, whereas in conventional roasted samples 48–96% of thiamine and 21.6–48.5 of vitamin B₆ were retained. Vitamin retention demonstrates clearly that heating of muscle tissues with microwaves is less destructive to heat-sensitive vitamins than conventional roasting [83].

In the case of vitamin B₁₂, the influence of cooking on its content is associated with two additive phenomena: on the one hand, loss of water and lipids, and on the other hand the destruction of vitamin B₁₂—which depends mainly on the temperature and, second, on the duration of the application of heat. The risk of loss in vitamin B₁₂ linked to cooking is estimated when the content is expressed on a lipid-free dry matter basis: −25% chuck braised and −5.5% for deep fat fried rump steak. Roasting rump steak and cooking rib steak did not seem to induce significant losses of vitamin B₁₂ [58].

A recent study has evaluated the retention of different vitamins (retinol, thiamine, riboflavin, and niacin) in chicken and lamb chops according to the procedure used in cooking (earth oven, microwave, and oven roast cooking). Losses of retinol can be associated with destruction by heat and loss into the melted fat leaching out into the drippings. Thiamine is the most heat-labile vitamin, while riboflavin and niacin are relatively stable vitamins. Losses of riboflavin and niacin can be attributed mostly to leaching losses into the drippings. As reported above, microwave oven cooking—which requires shorter heating times—tended to retain higher amounts of vitamins [15].

Abbreviations

AA	ascorbic acid
CAN	acetonitrile
AOAC	Association of Official of Analytical Chemists
APCI	atmospheric pressure chemical ionization
BIA-SPR	biomolecular interaction analysis–surface plasmon resonance
BHT	butylated hydroxytoluene
CE	capillary electrophoresis
cGC	capillary gas chromatography
CoA	coenzyme A
CRM	certified reference material
DHAA	dehydroascorbic acid
DHIAA	dehydroisoascorbic acid
EC	electrochemical
Era	erythorbic acid
ESI	electrospray ionization
FA	folic acid
FAD	flavin adenine dinucleotide
FITC	fluorescein 5-isothiocyanate
FMN	flavin mononucleotide

5-HCO-THF	5-formyltetrahydrofolic acid
IC-MS	inject chemical-mass spectrometry
i.v.	injection volume
IS	Internal Standard
IAA	isoascorbic acid
HPLC	high-performance liquid chromatography
LC	liquid chromatography
MS	mass spectrometry
MetOH	methanol
MBA	microbiological assay
MPA	mercaptpropionic acid
5-MTHF	5-methyltetrahydrofolate
NAD	nicotin adenin dinucleotide
NADP	nicotin adenin dinucleotide phosphate
OPA	orthophthaldehyde
PMP	processed meat and poultry
PN	pyridoxine or pyridoxol
PL	pyridoxal
PM	pyridoxamine
PMPH	pyridoxamine phosphate
PLP	pyridoxal phosphate
RIDA	radioisotope dilution assay
RF	riboflavin
RPBA	radiosotope protein-binding analysis
RP-HPLC	reverse phase-high performance liquid chromatography
SAE	strong anion exchange
SCE	strong cation exchange
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SH	silica-based hydromatrix
SIM	selected ion monitored
SPE	solid-phase extraction
T	tocopherol
T ₃	tocotrienol
THF	tetrahydrofolate
THFu	tetrahydrofuran
TRF	total riboflavin
UV	ultraviolet

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Chapter 16

Minerals and Trace Elements in Meat Products

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16.1 Introduction

Recently nutrition professionals have focused their attention on the macronutrients directly related to the diseases most prevalent in our society, passing over other nutrients, such as minerals and trace elements [1].

The main source of minerals and trace elements is through dietary intake; therefore, it is necessary to know what foods provide the greatest contributions. In general, meat products are foods of frequent and traditional consumption, not only because of their pleasant sensory characteristics, but also because of their easy purchase, preparation, and conservation, particularly considering that the evolution of eating habits has led to less time dedicated to cooking.

In addition to consumer lifestyle concerns, understanding the mineral composition of meat products is an important nutritional and analytical topic. Mineral components are present in this food matrix at a very small level, and the matrix often includes organic constituents. This last factor is especially important because of the analytical difficulties in developing a reproducible, repeatable, and reliable method for analyzing food composition.

Rapid, accurate, and precise analytical methods are necessary to supply correct data for labeling and for food composition databases of minerals and trace elements, which are of interest to consumers as well as to nutritionists. In terms of classic methods for analysis of minerals, titrimetric, colorimetric, gravimetric, and ion-selective electrode procedures are most often used. Nowadays, these methods are widely and routinely utilized for macromineral analysis due to the available experience of most laboratory staff. Therefore, reference analytical methods focusing on individual quantification described for specific foods are available from the Official Methods of Analysis of Association of Official Analytical Chemists (AOAC International) [2]. Related official methods [3] and other reports that include slight modifications to minimize interference are also available [4].

However, these traditional methods for mineral analysis are currently available only for a very limited number of macronutrients, such as sodium, potassium, phosphorous, calcium, and magnesium, which occur at high concentrations that are not difficult to determine. Minor or trace elements can be divided into essential (iron, zinc, copper, manganese, selenium, and chromium) and potentially toxic trace elements (aluminum, cadmium, lead, and others), predominantly at low and ultralow concentrations, respectively. Their analysis requires a high level of analytical expertise because of the high susceptibility to matrix interference at low levels, insufficient recoveries, deficient detection limits, and related methodological difficulties [5,6].

Moreover, other factors must be considered in choosing an analytical method, including cost and time of analysis, number of samples, and multielement capability. In this respect, atomic spectroscopic methods are often sufficient, at a modest cost and with an appropriately trained and experienced analyst, to meet most analytical requirements of meat products laboratories that must control the mineral content in raw material and end product.

The following sections are intended to provide the methodological information required to carry out reliable analytical determination of minerals and trace elements in meat products with widely used optical techniques. Emphasis will be placed on the control of contamination sources,

exhibited by the analytical blank. Special attention will be directed to stages of sample preparation and mineralization, and also to the proper use of reference materials in the verification of the data provided by the methodology used. An analytical method for the determination of selected macrominerals and trace elements in meat products, using absorption and emission atomic spectrometry, will be described. And finally, a compilation of analytical data for essential and nonessential elements in a wide number of meat products will be provided.

16.2 General Considerations of Mineral and Trace Element Analysis in Meat Products

16.2.1 Sources of Error in Mineral and Trace Element Analysis

The most common difficulty in mineral and trace element analysis involves the degree of contamination found in the analytical laboratory, which has adverse effects regardless of the concentration level. The worst situation normally arises when analyzing very low concentrations of ubiquitous elements such as aluminum, iron, or zinc [7]. Nevertheless, food laboratories usually perform analysis at both minor and trace (mg/kg) levels. The ability to successfully guarantee reliable result depends on keeping the risk of contamination under control. This risk is evaluated by the blank reagent. Several independent sources could be considered, including the laboratory and working areas atmosphere, cleaning of laboratory ware, purity of chemicals, and good practice by the analyst.

In this respect, several reference studies on this subject have been published [8–13].

The priority infrastructure for implementing an effective contamination control strategy is a clean laboratory, which contributes only negligible amounts of impurities from airborne particulate. However, most laboratories lack this qualification, and therefore relatively simple precautions are adopted to reduce dramatically the sources of contamination. The simplest approach in the conventional laboratory is the use of filtered air enclosures, a typical clean evaporation system, and a laminar flow hood supplied with a high-efficiency particulate air (HEPA®; HEPA Corporation, Anaheim, CA) filter, to be used in all operations during the sampling process that entail risk of contamination. Other concrete aspects related to laboratory environment have been treated extensively in the literature [10,14–17]. In addition, Boyer and Horwitz [13] provide a checklist containing the steps for progressively upgrading a conventional laboratory to make it more suitable for mineral and trace element analysis. Using these techniques, the blank value can be reduced and a suitable degree of contamination achieved for determining the analytical specifications of most elements of interest in meat products [18].

In addition, the installation of a purification system, producing ultrapure water with a resistivity of 18 MΩ cm, is imperative. Two steps are generally considered necessary for the preparation of high-quality water. Preliminary purification by reverse osmosis, followed by an anion- and cation-exchange resins system, provides water quality suitable for general purpose use in the food laboratory [19,20].

Sampling and sample preparation with low contamination requires proper materials [8,9,21–25]. The general tendency in mineral analysis is the change from glassware, porcelain, and stainless steel wherever possible to the use of polymers, quartz, and pure metals. In this sense, low-density polyethylene or polypropylene are strongly recommended as container material for sample storage [26,27], in addition to polytetrafluoroethylene (PTFE; Teflon®, E.I. Du Pont de Nemours & Company, Wilmington, Delaware) and pure metals such as titanium or platinum, due to their purity and relative thermal stability [17,21]. Extra care should be taken in keeping these materials

properly clean. Initially a combined hydrochloric–nitric acid and ultrapure water cleaning procedure was recommended [21], although the comparison of other methodologies leads to a much simpler cleaning procedure, which consists of soaking with 10% nitric acid (v/v) for a period of 2 weeks before first usage, a routine 72-h leaching period, and subsequent rinsing three times with ultrapure water [24].

Homogenizers and blades should incorporate sheets made of titanium or another high-purity metallic alloy that does not cause contamination [9].

Chemicals are another critical source of contamination [27–29]. Inorganic acid purity is of particular importance, because of the relatively large amount of these acids required for sample dissolution and other chemical operations. In a more practical sense, sub-boiling distillation is revealed as the choice method of purification for mineral acids used in trace analysis of meat products, where commercial reagents are not adequate due either to a lack of purity or to high upper limit specifications [28,29].

The analyst may be an unexpected source of contamination. Human fingers, sweat, hair, and clothing have been established as the occasional origin of contamination [30,31]. The use of talc-free vinyl or polyethylene gloves provides a generally acceptable alternative to ensure reproducibility of blanks [32].

In short, most food laboratories are lacking in adequate infrastructure and therefore, mineral and trace element analysis of meat products is performed in conditions involving residual contamination. In the absence of a clean room and other specific infrastructure elements mentioned earlier, requirements might be met and the best possible working conditions achieved through the control of the cleaning material, the use of pure reagents, and proper laboratory practices.

16.2.2 Sampling and Sample Homogenization

Sampling design in the analysis of minerals and trace elements depends strongly on the purpose of the study, that is, whether it is to establish the element content in certain meat products according to dietary intake or to assess the quality control in its manufacture. Sampling of meat product studies is usually carried out at random by researchers, purchased in local markets and supermarkets in most cases, following a strict plan to achieve representative sampling [33–39].

Heterogeneous samples, as meat products, have to be homogenized by cutting, mixing, chopping, milling, grinding, mincing, or blending, since subsampling procedures require a truly representative sample of the acquired bulk material to avoid erroneous analysis. Precautions should be taken to avoid cross-contamination between samples. This usually entails dismantling mechanical titanium blenders between samples and carefully washing in 10% nitric acid solution all metal free parts that come into contact with the sample before reassembling [40].

Alternatively, cryogenic grinding may assist in this homogenization process where a high fat content is present. This sophisticated technique consists of a titanium mill or Teflon and quartz balls contained in an all-Teflon container [40,41]. Other successful homogenization methods for mineral analysis include the use of agate grinders and porcelain or glass mortars, and drying samples before reduction of particle size.

16.2.3 Sample Mineralization

Most mineral and trace element analytical techniques require a previous step of sample mineralization, carried out by dry ashing or wet decomposition. The destruction of organic material contained

in meat products serves several purposes: (i) to release minerals and trace elements from the complex matrix, (ii) to concentrate metals present at very low levels to bring them to a suitable concentration for analysis, and (iii) to destroy and dilute the matrix sufficiently so that the effect of matrix interference on the analysis will be minimized.

Mineralization of the sample is a decisive stage in the analytical process. A number of papers and reviews have been published on decomposition procedures for mineral and trace elements [5,13], concluding that the digestion method must be selected to suit the type of sample, the elements being determined, and finally, the analytical method. Much information can be found in the literature regarding advantages and disadvantages of frequently used applications of both types of ashing [11,42] with respect to losses, blank reagent, sample size, ashing time, degree of decomposition, and economical aspects [43].

The oldest and simplest method of dry ashing consists of heating in a muffle furnace in the presence of air at elevated temperatures to remove the organic constituents. In the open vessel method, the meat product sample is placed in a suitable crucible, made of silica or platinum, restricting the temperature at 450°C to minimize losses and volatilization of analytes such as lead, copper, zinc, and iron. A collaborative study showed no significant losses under these ashing conditions [44].

To a certain extent, dry ashing is suitable for analysis of several macrominerals (Na, K, P, Ca, Mg) and trace elements (Fe and Mn) in food matrices, provided auxiliary agents are added to enhance the decomposition of organic matter and to prevent ignition, which causes loss through smoke particles [43]. Apart from external contamination and retention by crucible material, disadvantages include long ashing times of several hours.

Wet decomposition with oxidizing acids is currently the most commonly used method for destruction of the organic matrix, carried out at atmospheric pressure in open systems or at higher pressures in a closed vessel by conductive or microwave heating.

Ternary acid (nitric, perchloric, and sulfuric acid) is the most popular reagent used for complete digestion of meat products, but safety concerns make use of perchloric acid unattractive. Conventional open acid mineralization is a quick method (normally 3–5 h), with reduced and controlled losses by volatilization, but it has practical drawbacks, most importantly the need to employ highly pure reagents, due to sensitivity to contamination. In this respect, Teflon or similar fluorated plastics are strongly recommended [40,42,43].

When carried out in closed systems with conductive heating, also called Parr bombs, the acid used can be heated to higher temperatures, resulting in higher oxidizing power as well as higher efficiency of sample digestion [45–47]. In addition, where a microwave oven is used, these advantages are even more pronounced, resulting in shorter times needed for food digestion. Volatile elements do not leak from the vessel. Toxic fumes released into the laboratory atmosphere are avoided. Smaller volumes of acids may be used, or high-purity nitric acid can be used alone, thereby reducing contamination of the sample. Würfels et al. [48–50] have proved the complete mineralization of different types of samples of animal and vegetal origin in PTFE closed vessels.

Modern microwave digestion systems monitor both pressure and temperature in the digestion vessels [51]. Both digestion time and microwave heating are programmed following a previously determined profile of temperature or pressure to digest successfully a relative high weight of dry sample, typically up to 0.75–1.0 g. Microwave acid digestion is widely used in food analysis [52]. Theoretical aspects of microwave sample preparation are addressed in depth by Kingston and Jassie [53], with a special emphasis on how microwaves interact with different material and information concerning applications of microwave digestion methods for elemental analysis.

In general, microwave digestion procedure is preferable for practical reasons [51]. It provides high sample throughput, while minimizing contamination and operator intervention. The microwave

energy can be readily controlled and programmed automatically, ensuring better reproducibility. These attributes provide an excellent opportunity for automatization and easy transference to other food analysis laboratories.

16.2.4 Analytical Techniques

The choice of the most appropriate instrumental method in the analysis of minerals and trace elements in meat products requires a clear understanding of the capabilities and limitations of the analytical techniques, particularly taking into consideration adequate sensitivity and detection limits, precision and minimization of interference problems, commercial availability of instrumentation, sample throughput, and economical cost.

From the analytical composition point of view, different minerals and trace elements found in meat products might be divided into two classes, depending on the frequency of analyses required in the meat laboratory. First, macrominerals (Na, K, P, Ca, and Mg) and several trace elements (essential [Fe, Zn, Cu, and Se] and nonessential [Cd, Pb, and Hg]) are of interest usually in quality control practices to estimate their safety and nutritional quality. Second, for other trace elements, among which are manganese, chromium, aluminum, and arsenic, data are less frequently needed.

Recommended suitable analytical methods, on the basis on the experience gained during international studies [1,5] or the analysis and certification of reference materials [54,55], are given in Table 16.1.

Although for calcium or phosphorous classical analytical methods such as gravimetry or volumetry are applicable, the final determination considered here focuses on faster instrumental methods, as mentioned earlier.

Mineral determination with the aid of spectrophotometry usually involves a selective separation and complexation with either an inorganic or an organic reagent, through formation of ion-associated chelate compounds. This method offers a suitable sensitivity to be approved for analysis of phosphorous (AOAC Official Method 991.27 or 960.29), manganese (921.02, 917.04), and copper (947.03) [2]; otherwise various older colorimetric methods have been superseded, falling into disuse (arsenic [973.33], cadmium [945.58], copper [960.40], lead [934.07], mercury [952.14], and zinc [944.09]) [2]. Although in principle this method could be applied to the analysis of a number of different elements in meat products, in practice its use appears to be confined to specific situations due to its time-consuming character.

Atomic spectrometry often meets the analytical requirements for final determination of minerals and trace elements in meat products, using a flame, an inductively coupled plasma, or a graphite furnace for atomization. Solutions are normally required to carry out the sample introduction. Therefore, meat product samples must be digested for effective sample handling, such as microwave closed PTFE vessel system as reported elsewhere.

Flame atomic absorption spectrometry (F-AAS) or flame atomic emission spectrometry (F-AES)—the latter limited to only a few alkali and earth alkaline elements, such as sodium, potassium, calcium, and magnesium—might potentially be used for all macromineral and certain trace determinations (Table 16.1). The equipment is reasonably inexpensive, easy to operate, and few well-defined interferences occur.

When the sensitivity and detection limits are the major limiting factor, graphite furnace atomic absorption spectrometry (GF-AAS) is the most common alternative. Regardless, measurement is usually automated; a trained staff must be familiar with the meat product matrix, so as to avoid interferences and optimize the electrothermal program to obtain precise and

Table 16.1 Suitable Analytical Techniques for Meat Products Laboratories

<i>Analyte</i>	<i>Instrumental Method</i>	
	<i>Unielemental</i>	<i>Multielemental</i>
Macromineral		
Sodium	F-AAS, F-AES	ICP-AES, NAA
Potassium	F-AAS, F-AES	ICP-AES, NAA
Phosphorous	SP	ICP-AES
Calcium	F-AAS, F-AES	ICP-AES, NAA
Magnesium	F-AAS, F-AES	ICP-AES
Essential trace elements		
Iron	F-AAS, SP	ICP-AES, NAA
Zinc	F-AAS, SP	ICP-AES, ASV, NAA
Copper	F-AAS, SP	ICP-AES, ASV, ICP-MS, NAA
Manganese	F-AAS, SP	ICP-AES, ICP-MS, NAA
Selenium	HG-AAS, GF-AAS	ICP-MS, NAA
Chromium	GF-AAS	ICP-MS
Potentially toxic trace elements		
Cadmium	GF-AAS	ASV, ICP-MS, NAA
Lead	GF-AAS	ASV, ICP-MS, NAA
Aluminum	GF-AAS	ICP-AES
Arsenic	HG-AAS, GF-AAS	ICP-MS
Mercury	CV-AAS, GF-AAS	ICP-MS, NAA

Note: F-AAS, flame atomic absorption spectrometry; HG-AAS, hydride-generation atomic absorption spectrometry; GF-AAS, graphite-furnace atomic absorption spectrometry; CV-AAS, cold vapor atomic absorption spectrometry; F-AES, flame atomic emission spectrometry; SP, spectrophotometry; ICP-AES, inductively coupled plasma atomic emission spectrometry; NAA, neutron activation analysis; ASV, anodic stripping voltammetry; and ICP-MS, inductively coupled plasma mass spectrometry.

accurate results. The development of effective background correction based on the Zeeman effect, in combination with the stabilized temperature platform technique and automated introduction, has made GF-AAS a much more effective technique for trace element analysis in meat products.

Vapor generation techniques, such as cold vapor AAS (CV-AAS) or hydride generation AAS (HG-AAS), can be used in the analysis of mercury (CV-AAS) and selenium and arsenic (HG-AAS) in meat products. In both cases, a gaseous generation stage acts as a cleanup and preconcentration step for the analyte, providing an analytical improvement when modern flow injection systems are used.

Globally, AAS is a versatile and powerful tool in food analysis, producing analytical results of high accuracy and desired precision. Unfortunately, this technique is rather slow to use because of single-element capability.

At this point, inductively coupled plasma atomic emission spectrometry (ICP-AES) either simultaneous or sequential, allows a multielement analysis together with a greater linear dynamic range and detection limits comparable with or superior to those of F-AAS. Likewise, ICP-AES

requires a great deal of operator experience to avoid spectral interference. Inductively coupled plasma mass spectrometry (ICP-MS) is currently the most sensitive and powerful technique for trace elements, where the wide dynamic range of analytes results in shorter analysis times. Both ICP-AES and ICP-MS have been used in the determination of nutritional and potentially toxic trace elements in different types of foods. However, the initial running and maintenance cost is relatively high; therefore, this technique has not seen widespread use in meat products laboratories, despite its high capacity for analysis, which has made it almost absolutely necessary in many applications of trace analysis in other research fields.

Anodic stripping voltammetry (ASV) has demonstrated a high potential regarding sensitivity and precision in both biological materials and aqueous samples. It is made attractive by the low limit of detection in a limited number of elements, specifically zinc, cadmium, lead, and copper, and by the relatively low cost of the instrument required. Nevertheless, electrochemical techniques demand total mineralization. The method requires the completion of any decomposition technique with the addition of perchloric acid and heating until dry. This drawback, together with the need to use a time-consuming standard addition method to eliminate the influence of the matrix on the results, has hampered the ordinary usage of ASV in elemental analysis of meat products.

Finally, neutron activation analysis (NAA) is nowadays the most powerful method for quantitative measurements of many elements in different fields, including food analysis. The main components of meat matrix form virtually no radioactive isotopes, so the method is highly sensitive for trace element analysis. Under appropriate experimental parameters, 65–70 elements may be determined simultaneously with a high degree of accuracy and precision. In spite of these advantages, the requirement of access to a nuclear reactor is a major difficulty.

A collection of selected literature for food samples determination from recent years is given by Aras and Ataman [56], including brief information on the analytical technique employed, sample handling, and other useful facts.

16.2.5 Quality Control

The aim of any mineral or elemental quantitative analysis is to obtain accurate and precise data. It is necessary to keep in mind that diverse measurements to assess accuracy will entail some uncertainty due to the imprecision of the procedure. The assessment of these parameters integrated into quality control has to be an integral part of any good analytical program. Thus, the quality of the resulting data depends on analytical standardization at all stages of analysis. Good laboratory practices in sampling and sample handling, as mentioned earlier, the utilization of well-characterized suitable standards (stock solutions, in-house standards, and reference materials), and finally, good quality assurance strategies are essential to provide a proper degree of confidence in the results obtained. Therefore, a method reagent blank, in-house internal standard, and reference material must be included to provide ongoing quality control information with each analytical batch of mineral and trace element quantification.

Certified reference materials are widely used to verify the performance of the applied methods, providing an unequivocal benchmark to demonstrate the credibility of the analytical results. The major problem in meat product analysis is to match sample matrix with an appropriate standard reference material. However, this fact is not a handicap at the present day, due to available reference materials and the wealth of information on matrix composition of these standards provided by producers and suppliers (Table 16.2).

Table 16.2 Meat Matrix Reference Materials

<i>Organism</i>	<i>Address</i>	<i>Reference Material</i>
National Institute of Standards and Technology (NIST)	Standard Reference Materials Program, 100 Bureau Drive, Gaithersburg, MD 20899-2322, USA; e-mail: srinfo@nist.gov	SRM 1577b bovine liver Certified values: Ag 0.039 mg/kg, Ca 116 mg/kg, Cd 0.50 mg/kg, Cl 0.278%, Cu 160 mg/kg, Fe 184 mg/kg, K 0.994%, Mg 601 mg/kg, Mn 10.5 mg/kg, Mo 3.5 mg/kg, Na 0.242%, P 1.10%, Pb 0.129 mg/kg, Rb 13.7 mg/kg, S 0.785%, Se 0.73 mg/kg, Sr 0.136 mg/kg, Zn 127 mg/kg; indicative values for Al, As, Br, Co, Hg, Sb, V
		SRM 1546 meat homogenate Certified values: Ca 323 mg/kg, Fe 11.4 mg/kg, Na 9990 mg/kg; indicative values: B, Cl, Cu, I, K, Mg, P, S, Zn
		RM 8414 bovine muscle powder Reference values: Al, As, B, Br, Ca, Cd, Cl, Co, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Mo, N, Na, Ni, P, Rb, S, Se, Sr, Zn; indicative values for Ba, Cs, F, Sb, V
Institute for Reference Materials and Measurements (IRMM)	Management Reference Materials (MRM) Unit, Retieseweg, B-2440 Geel, Belgium; e-mail: bcr.sales@irmm.jrc.be	CRM 184 bovine muscle Certified values: Cd 13 µg/kg, Cu 2.36 µg/kg, Fe 79 mg/kg, Hg 2.6 µg/kg, Mn 334 mg/kg, Pb 239 µg/kg, Se 183 µg/kg, Zn 166 mg/kg; indicative values for Ca, Cl, Cr, I, K, Mg, Na, Ni, P
		CRM 185R bovine liver Certified values: As 33.0 µg/kg, Cd 544 µg/kg, Cu 277 mg/kg, Mn 11.07 mg/kg, Pb 172 µg/kg, Se 1680 µg/kg, Zn 138.6 mg/kg; indicative values for Al, As, Br, Co, Hg, N, Sb, V
		CRM 384 pork muscle Certified values: K 15.5 g/kg, Mg 1.00 g/kg, Na 2.8 g/100 g, N (Kjeldahl) 13.7 g/100 g, fat 10.8 g/100 g, ash at 550°C 4.6 g/100 g; indicative values for Ca, Cl, P
LGC Promochem	Reference Material Production, Queens Road, Teddington, Middlesex TW11 0LY, United Kingdom; e-mail: uk@lgcpromochem.com	LGC 7000 beef/pork meat Certified values: Ca 253 mg/kg, K 1590 mg/kg, Na 1360 mg/kg, Zn 14.2 mg/kg, moisture 66.5 g/100 g, fat 12.4 g/100 g, ash 0.78 g/100 g, nitrogen 1.96 g/100 g; indicative values for Ca, Cl, Cr, I, K, Mg, Na, Ni, P
		LGC 7001 pork meat Certified values: Ca 319 mg/kg, K 1860 mg/kg, Na 1510 mg/kg, Zn 12.2 mg/kg, moisture 68.6 g/100 g, fat 7.8 g/100 g, ash 0.90 g/100 g, nitrogen 1.97 g/100 g; indicative values for Fe
		LGC 7002 pork/chicken meat Certified values: Ca 295 mg/kg, K 2 140 mg/kg, Na 19, 100 mg/kg, Zn 10.9 mg/kg, fat 12.1 g/100 g, nitrogen 1.91 g/100 g; indicative values for Cl, Fe

(Continued)

Table 16.2 (Continued)

<i>Organism</i>	<i>Address</i>	<i>Reference Material</i>
		NAMI-SMRD-2000 fresh meat Certified values: Ca 70.3 mg/kg, Fe 6.33 mg/kg, K 1859 mg/kg, Na 8533 mg/kg, P 1075 mg/kg, nitrogen 1.63 g/100 g
National Research Centre for Certified Reference Materials	Office of CRMs, No. 18, Bei San Huan Dong Lu, Hepingjie, 100013 Beijing, China; e-mail: nrccrm@public3.bta.net.cn	GBW 08551 pork liver Certified values: Al 0.044 µg/g, Ca 197 µg/g, Cd 0.067 µg/g, Cu 17.2 µg/g, Fe 0.105%, K 1.15%, Mg 747 µg/g, Mn 8.32 µg/g, Mo 3.8 µg/g, N 10.86%, Na 0.233%, Pb 0.54 µg/g, Se 0.94 µg/g, Zn 172 µg/g; indicative values for Co, Cr, P GBW 08552 pork muscle Certified values: Br 6.2 µg/g, Ca 147 µg/g, Cl 0.187%, Cu 3.88 µg/g, Fe 43.6 µg/g, K 0.813%, Mg 988 µg/g, Mn 0.48 µg/g, N 12.27%, Na 0.202%, P 0.813%, Rb 42.7 µg/g, Se 0.49 µg/g, Zn 94.2 µg/g; indicative values for Ba, Co, Cr, Cs, Hg, Mo, Pb, Sr
		NCS ZC71001 beef liver Certified values: Ca 189 µg/g, Cl 0.29%, Co 0.254 µg/g, Cu 91.6 µg/g, Fe 346 µg/g, K 1.05%, Mg 668 µg/g, Mn 8.92 µg/g, Mo 3.76 µg/g, Na 0.222%, P 1.30%, Rb 23.6 µg/g, Se 0.56 µg/g, Sr 0.53 µg/g; indicative values for Al, Ba, Br, Cd, F, Hg, Rb, Pb, S, Ti
		NCS ZC81001 pork muscle Certified values: Br 6.2 µg/g, Ca 147 µg/g, Cl 0.187%, Cu 3.88 µg/g, Fe 43.6 µg/g, K 1.4%, Mg 988 µg/g, Mn 0.48 µg/g, N 12.27%, Na 0.202%, P 0.813%, Rb 42.7 µg/g, Se 0.49 µg/g, Zn 94.2 µg/g; indicative values for Ba, Co, Cr, Cs, Hg, Mo, Pb, Sr
		NCS ZC73015 chicken Certified values: Br 0.016%, As 0.109 µg/g, B 0.76 µg/g, Ba 1.5 µg/g, Br 1.6 µg/g, Ca 0.022%, Ce 0.06 µg/g, Cl 0.153%, Cr 0.59 µg/g, Cs 0.070 µg/g, Cu 31.46 µg/g, Dy 1.1 µg/g, Fe 31 µg/g, Hg 3.1 µg/g, K 1.46%, La 0.024 µg/g, Li 0.034 µg/g, Mg 0.128%, Mn 1.65 µg/g, Mo 0.11 µg/g, N 3.8%, Na 0.47 µg/g, P 0.76%, Pb 0.07 µg/g, Rb 11.6 µg/g, S 0.25%, Se 0.11 µg/g, Sr 5.3 µg/g, Y 0.008 µg/g, Zn 34 µg/g
Swedish National Food Administration	Anders Staffas, NFA PT-Food Chemistry, Box 622, SE-751 26 Uppsala, Sweden; e-mail: anst@slv.se	PT25-K69-00 meat-based foodstuffs containing lean pork, water and flour Certified values: ash 2.66 g/100 g, moisture 68.8 g/100 g, fat 14.2 g/100 g, nitrogen 1.63 g/100 g, Na 8557 mg/kg, K 1865 mg/kg, Ca 76.3 mg/kg, Fe 5.38 mg/kg, P 1068 mg/kg PT32-K83-03 meat-based foodstuffs consisting of minced meat Certified values: ash 2.10 g/100 g, moisture 79.0 g/100 g, fat 7.15 g/100 g, nitrogen 1.61 g/100 g, Na 1360 mg/kg, K 2071 mg/kg, Ca 4706 mg/kg, Fe 3437 mg/kg, P 3437 mg/kg

16.3 Experimental Design for Mineral and Trace Element Analysis in Meat Products

16.3.1 Preliminary Remarks

In light of the aforementioned, systematic contamination is controlled by careful choice of equipment, reagents, and cleaning procedures. Thus the following points should be thoroughly taken into account to minimize external contamination: (i) Choose carefully the material employed in analysis according to the chemical properties. Teflon or other polymeric material is preferred in sample preparation; (ii) Perform all sample preparations wearing powder-free vinyl or polyethylene gloves; (iii) Acid-wash all utensils that come into contact with the sample and standards with 10% nitric acid for several days, soak, and rinse three times with ultrapure water; (iv) Store all clean material in clean, tightly lidded polyethylene or Plexiglas® (Röhm & Haas Company, Darmstadt, Germany) boxes; (v) Control the level of the analyte in water and other reagents used in the analytical determination. Use high-purity water with 18 MΩ cm resistance, purified by an ion-exchange system rather than distillation. Sub-boiling distillation is the most effective means of obtaining concentrated high-purity acids; and finally, (vi) Wherever possible carry out all sample preparation and preanalytical steps under clean air laminar flow hoods.

16.3.2 Sample Handling

16.3.2.1 General Principle

Meat product samples are dried in a drying oven with walls previously coated by means of a Teflon spray, until a constant weight is achieved. Later representative samples are homogenized in a food processor or blender modified with titanium blades, to reduce volume and to facilitate handling and conservation.

16.3.2.2 Equipment and Material

- Drying oven
- Mechanical blender with titanium blades
- Analytical balance
- Polyethylene bags with safety lock (8.5 × 13.5 cm)
- Polyethylene bags (26 × 36 cm)
- Talc-free vinyl gloves
- Plastic tray and cutlery
- Polyethylene beaker (250 mL)

16.3.2.3 Procedure

Unwrap the collected meat product and place it on a plastic tray covered by a polyethylene bag. With the assistance of plastic cutlery, eliminate inedible parts. Cut the food with a plastic knife into smaller portions to facilitate drying. Place the portions in a polyethylene beaker previously weighted in analytical balance. Dry at 105°C until constant weight is achieved. Grind the samples with a blender, in which original stainless steel blades have been replaced with blades of high-purity titanium. Finally, store the homogenized and dried samples in properly identified hermetically closed plastic bags under cryogenic conditions (−18°C) until the analysis.

16.3.3 Mineralization of Sample

16.3.3.1 General Principle

The organic matter of the sample is destroyed by sub-boiling nitric acid digestion in a closed Teflon vessel with microwave heating. The digestion system has a rotor with 10 bombs. Each digestion series must contain two reagent blanks and, if possible, include an appropriate certified reference material. All the samples must be digested in triplicate.

16.3.3.2 Equipment and Material

- Drying oven
- Electronic adjustable pipette
- Analytical balance
- Sub-boiling quartz distillation apparatus
- Microwave digestion system
- High-pressure Teflon digestion vessels
- Polypropylene volumetric flasks (25 mL)
- Polypropylene tubes (13 mL) and tube racks
- Polypropylene beaker (50 mL)
- Polypropylene spatula

16.3.3.3 Reagents

- Nitric acid 65% (e.g., Proanalysis, Merck, Darmstadt, Germany)
- Sub-boiling nitric acid
- Ultrapure deionized water type Milli-Q® (Millipore Corporation, Billerica, Massachusetts) (resistivity 18 MΩ cm)

16.3.3.4 Procedure

Weigh approximately 0.7500 g sample (dry basis) into a decontaminated Teflon decomposition vessel, add 10 mL sub-boiling nitric acid, gently swirl the mixture to homogenize. Let samples sit in acid for several minutes until an aggressive initial decomposition reaction starts, so as to avoid uncontrolled explosions in the interior of the digestion system. Close the vessel and place it in the digestion oven. Using 1000 W power, apply the digestion program, previously optimized. This program consists of the following sequence: 20–90°C for 8 min, 90–110°C for 6 min, 110–150°C for 5 min, 150°C for 3 min, 150–170°C for 3 min, and lastly, 170°C for 3 min. Cool to room temperature in a bath with cold water and open the bomb. Digested samples are diluted to 25 mL in a volumetric flask with ultrapure water and finally transferred to clean polypropylene tubes. Solutions are stored frozen at –20°C until analysis.

16.3.4 Analytical Determination

16.3.4.1 General Principle

The techniques used for minerals and trace elements analysis have been F-AES (Na, K), flame (Ca, Mg, Fe, Zn, Cu, Mn), GF-AAS (Se, Cr, Cd, Pb), and ICP-AES (P, Al).

16.3.4.2 Flame Atomic Emission Spectrometry

16.3.4.2.1 Equipment and Material

- Atomic emission spectrometer (e.g., AAnalyst 800[®], PerkinElmer Inc., Shelton, Connecticut)
- Computer calculation program (AAWinLab[®] instrument control software, PerkinElmer Inc., Shelton, Connecticut)
- Acetylene
- Compressed air
- Polypropylene volumetric flasks (50 mL)
- Polypropylene beaker (50 mL)
- Set of automatic pipettes and tips adjustable in ranges (10–100 μL , 100–1000 μL)

16.3.4.2.2 Reagents

- Ultrapure deionized water type Milli-Q (resistivity 18 M Ω cm).
- Sub-boiling nitric acid distilled from nitric acid 65% (e.g., Proanalysis, Merck).
- Sodium standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Potassium standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Sodium and potassium standard solutions. Calibration standards of sodium (0, 1, 3, 6, and 12 mg/L) and potassium (0, 0.3, 0.9, and 2.0 mg/L) are prepared by diluting the stock solution with enough sub-boiling nitric acid to match the acid concentration found in digestion solutions.

16.3.4.2.3 Sample Dilution

Acid solutions are diluted with ultrapure water (dilution factor 1:250) before carrying out both analyses.

16.3.4.2.4 Operational Parameters and Analytical Determination

Optimized instrumental parameters for sodium and potassium determination are summarized in Table 16.3.

Retrieve the stored program for sodium or potassium and adjust the instrument to find maximum signal according to the manufacturer's instructions. Measure the standard solutions and samples series, including the reagent blank and reference material. An aqueous standard control is run for every 10 sample solutions to provide ongoing quality control.

Table 16.3 Operating Conditions for Atomic Emission Spectrometry

<i>Parameter</i>	<i>Sodium</i>	<i>Potassium</i>
Wavelength (nm)	589.0	766.5
Slit width (nm)	0.2	0.7
Flame gases	Air/acetylene	Air/acetylene
Oxidant flow (L/min)	17.0	17.0
Fuel flow (L/min)	2.0	2.0

16.3.4.2.5 Calculations

$$\text{Na or K (mg)/g dry sample (R)} = \frac{(C \times 0.025 \times F)}{w}$$

$$\text{Na or K (mg)/100 g edible portion} = R \times (100 - M)$$

where

C = concentration in acid solution (mg/L)

F = dilution factor

w = weight of digested sample (g)

M = moisture (%)

16.3.4.3 *Atomic Absorption Spectrometry*

16.3.4.3.1 Equipment and Material

- Atomic absorption spectrometer (e.g., PerkinElmer AAnalyst 800) with flame and graphite furnace atomizers, Zeeman background correction, and furnace autosampler (e.g., AS 800[®], PerkinElmer Inc., Shelton, Connecticut)
- Transversely heated graphite tubes with end caps (e.g., PerkinElmer)
- Single-element hollow cathode lamps
- Computer calculation program (AAWinLab, instrument control software)
- Acetylene
- Compressed air
- Argon
- Polypropylene volumetric flasks (50 mL)
- Polypropylene beaker (50 mL)
- Set of automatic pipettes and tips adjustable in ranges (10–100 μL , 100–1000 μL)

16.3.4.3.2 Reagents

- Ultrapure deionized water type Milli-Q (resistivity 18 $\text{M}\Omega \text{ cm}$).
- Sub-boiling nitric acid distilled from nitric acid 65% (e.g., Proanalysis, Merck).
- Calcium standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Magnesium standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Magnesium standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Iron standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Zinc standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Copper standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Manganese standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Selenium standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Chromium standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Cadmium standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Lead standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Lanthanum(III) chloride solution, 10% (w/v). Suitable volume is added to a final concentration of lanthanum of 1% in working standards and samples to carry out the calcium or magnesium determination.

- Selenium matrix chemical modifier (0.5 g citric acid diluted in 5 mL rhodium nitrate solution [1000 mg/L]).
- Chromium matrix chemical modifier (a solution of 0.15 g magnesium nitrate [Mg(NO₃)₂ 6H₂O] diluted in 100 mL ultrapure water).
- Cadmium and lead matrix chemical modifier (a solution of 0.5 g ammonium monobasic phosphate [NH₄H₂PO₄] and 0.03 g magnesium nitrate [Mg(NO₃)₂ 6H₂O] diluted in 100 mL ultrapure water).
- Calibration standard solutions. Working standards of calcium (0, 1.0, 2.5, and 5.0 mg/L), magnesium (0, 0.5, 1.5, and 3.0 mg/L), iron (0, 1.0, 3.0, and 6.0 mg/L), zinc (0, 0.75, 1.50, and 3.00 mg/L), copper (0, 0.05, 0.15, and 0.30 mg/L), manganese (0, 0.10, 0.30, and 0.60 mg/L), selenium (0, 15.0, 30.0, and 60.0 µg/L), chromium (0, 1.0, 2.0, and 4.0 µg/L), cadmium (0, 0.4, 1.0, and 2.0 µg/L), and lead (0, 2.0, 4.0, and 10.0 mg/L) are prepared by diluting the stock solution with enough sub-boiling nitric acid to match the acid concentration found in digestion solutions.

16.3.4.3.3 Sample Dilution

Acid solutions are diluted with ultrapure water applying a dilution factor of 1:4 and 1:10 for calcium and magnesium analysis, respectively.

16.3.4.3.4 Operational Parameters and Analytical Determination

Optimized instrumental parameters for elemental determination by F-AAS and GF-ASS are summarized in Tables 16.4 and 16.5, respectively. A guide for the temperature-time program to be used with the graphite furnace is shown in Table 16.6.

Retrieve the stored program for the selected element and adjust the instrument to find the maximum signal of absorbance according to the manufacturer's instructions. Measure the standard solutions and samples series, including the reagent blank and reference material. An aqueous standard control is run every 10 sample solutions to provide ongoing quality control.

Table 16.4 Operating Conditions for F-AAS

<i>Parameter</i>	<i>Calcium</i>	<i>Magnesium</i>	<i>Iron</i>	<i>Zinc</i>	<i>Copper</i>	<i>Manganese</i>
Wavelength (nm)	422.7	285.2	248.3	213.9	324.8	279.5
Slit width (nm)	0.7	0.7	0.2	0.2	0.2	0.2
Flame gases	Air/C ₂ H ₂	Air/C ₂ H ₂	Air/C ₂ H ₂	Air/C ₂ H ₂	Air/C ₂ H ₂	Air/C ₂ H ₂
Oxidant flow (L/min)	17.0	17.0	17.0	17.0	17.0	17.0
Fuel flow (L/min)	1.9	2.0	2.0	2.0	2.0	2.0
Wavelength (nm)	10	6	30	15	15	20
Linear range (mg/L)	5.0	0.5	6.0	1.0	5.0	2.0

Table 16.5 Operating Conditions for GF-AAS

Parameter	Selenium	Chromium	Cadmium	Lead
Wavelength (nm)	196.0	357.9	228.8	283.3
Slit width (nm)	2.0	0.7	0.7	0.7
Lamp current (mA)	280	25	4	10
Mode	Area	Area	Area	Area
Background correction	Zeeman	Zeeman	Zeeman	Zeeman

Table 16.6 Graphite Furnace Program for the Determination of Selenium, Chromium, Cadmium, and Lead in Meat Products

Step	Temperature (°C)	Ramp (s)	Hold (s)	Argon Flow (mL/min)	Read On
Drying	130	20/15/15/15	50/40/40/40	250	—
Charring	1400/700/700/1000	10	20	250	—
Atomization	2050/2300/1500/1000	0	5	0	Yes
Cleaning	2500/2500/2450/2450	1	4/5/3/3	250	—
Cooling	20	—	—	250	—

16.3.4.3.5 Calculations

$$\text{Element (mg)/gram of dry sample (R)} = \frac{(C \times 0.025 \times F)}{w}$$

$$\text{Element (mg)/100 g edible portion} = R \times (100 - M)$$

where

C = concentration in acid solution (mg/L)

F = dilution factor

w = weight of digested sample (g)

M = moisture (%)

16.3.4.4 Inductively Coupled Plasma Emission Spectrometry

16.3.4.4.1 Equipment and Material

- Inductively coupled plasma emission spectrometer (e.g., Jobin Yvon JY38 S Plus Sequential[®], Horiba Jobin Yvon S.A.S, Longjumeau, France)
- Pneumatic nebulizer-type Meinhard and cyclonic chamber (e.g., Horiba Jobin Yvon)
- Peristaltic pump
- Argon
- Polypropylene volumetric flasks (50 mL)
- Polypropylene beaker (50 mL)
- Set of automatic pipettes and tips adjustable in ranges (2–200 μ L, 100–1000 μ L)

16.3.4.4.2 Reagents

- Ultrapure deionized water type Milli-Q (resistivity 18 MΩ cm).
- Sub-boiling nitric acid distilled from nitric acid 65% (e.g., Proanalysis, Merck).
- Phosphorous standard 1000 mg/L PO_4^{3-} in water (commercial standard solution, Merck).
- Aluminum standard 1000 mg/L in nitric acid (commercial standard solution, Merck).
- Calibration standard solutions. Working standards of phosphorous (0, 65.2, 130.4, and 293.5 mg/L) and aluminum (0, 40.0, 80.0, and 200.0 mg/L) are prepared by diluting the stock solution with enough sub-boiling nitric acid to match the acid concentration found in digestion solutions.

16.3.4.4.3 Sample Dilution

Acid solutions are diluted with ultrapure water applying a dilution factor of 1:2 and none for aluminum and phosphorous analysis, respectively.

16.3.4.4.4 Operational Parameters and Analytical Determination

Suggested instrumental parameters for elemental determination by ICP-AES are summarized in Table 16.7.

Adjust the instrument according to manufacturer's instructions. Calibration standards may be for single or mixed standard containing both elements. Whether single or mixed standard is used will depend on computer software requirements of the particular ICP instruments in use. After calibration in complete, samples series, including the reagent blank and reference material, are analyzed. Calibration of instruments should be checked after every 10 sample solutions by analyzing a working solution. If reanalysis of this standard indicates a drift (>5% of original signal), instruments should be recalibrated.

Table 16.7 Operating Conditions for ICP-AES

<i>Parameter</i>	<i>Phosphorous</i>	<i>Aluminum</i>
Rf forward power (W)	1000	1000
Rf generator frequency (MHz)	42	42
Outer gas flow rate (L/min)	12	12
Inner gas flow rate (L/min)	0.3	0.3
Nebulizer gas flow rate (L/min)	0.45	0.45
Wavelength (nm)	213.618	396.152
Integration time (s)	0.5	0.5
Internal standard voltage (V)	873	903
Measurement mode	Gaussian	Gaussian
Increment between measurements (nm)	0.0029	0.0018
Number of points	9	13
Window size (nm)	0.0354	0.0214

16.3.4.4.5 Calculations

$$\text{P or Al (mg or } \mu\text{g)/g dry sample (R)} = \frac{(C \times 0.025 \times F)}{w}$$

$$\text{P or Al (mg or } \mu\text{g)/100 g edible portion} = R \times (100 - M)$$

where

C = concentration in acid solution (mg/L)

F = dilution factor

w = weight of digested sample (g)

M = moisture (%)

16.3.5 Reliability of the Method

The primary objective in the elaboration of an analytical method is to provide a procedure that meets established quality criteria and may be widely used in different types of laboratories. Information regarding accuracy, precision (repeatability and intermediate precision), limits of detection and quantification, applicability, and practicability are appropriate for food analytical method characterization.

- *Accuracy.* The accuracy of the analytical method is verified by analyzing the standard reference material SRM 1577b (bovine liver), provided by the National Institute of Standards and Technology. In addition, the validity of the analytical procedure is checked using spiked samples of SRM 1577b and internal standard material. Analytical recoveries of spiked elements (750, 2500, 2500, 37.5, 500, 125, 50, 125, and 7.5 μg of Na, K, P, Ca, Mg, Fe, Zn, Cu, and Mn, respectively; and 500, 118.75, 2500, 250, and 100 μg of Se, Cr, Al, Cd and Pb, respectively, added to the sample in a Teflon vessel before digestion) are evaluated.
- *Precision.* In this method, the evaluation of precision is considered at two levels—repeatability (intraassay) and intermediate precision (interassay). It is investigated using both an internal aqueous standard and an authentic sample of the meat product (mortadella) used as internal standard material. The precision of the analytical procedure is expressed as the coefficient of variation calculated in a series of measurements. Repeatability, also termed intra-assay precision, expresses the precision under the same operating conditions over the same assay session. Intermediate precision expresses variations in the same sample measured in the same laboratory on different days.
- *Limit of detection and quantification.* Blank reagent values are monitored throughout the survey and subtracted from the measured sample concentration to calculate the final result in meat product samples. Limit of detection (LOD) is calculated according to the definition and criteria established by the International Union of Pure and Applied Chemistry ($X_b \pm 3\sigma_b$) as the average of three times the standard deviation of the reagent blank. Analogously, the limit of quantification, considered as the smallest measurement content above which a determination of the analyte is possible with suitable precision and accuracy, is calculated by means of 10 times definition (limit of quantification [LOQ] = $X_b \pm 10\sigma_b$).
- *Quality control.* An internal aqueous quality control (6.0, 2.0, 50.0, 2.5, 1.5, 1.0, 1.5, 0.05, and 0.010 mg/L of Na, K, P, Ca, Mg, Fe, Zn, Cu, and Mn, respectively; and 30.0, 2.0, 20.0, 1.0, and 3.0 $\mu\text{g/L}$ of Se, Cr, Al, Cd, and Pb, respectively) is run concurrently with blank reagent and standard materials throughout the course of the analysis and always measured previously for each batch of samples to satisfy the criteria established in the quality program by lower and upper action limits and to provide ongoing quality control information.

Results of quality control assays for all elements analyzed are summarized in Table 16.8.

Table 16.8 Quality Control of Analytical Determinations

Blank Reagent (mg/L; n = 76)	Concentration		Repeatability RSD (%)				Intermediate Precision				Recovery (%)		Certified Reference Material		LOQ (mg/L)
	Aqueous Internal Standard (mg/L; n = 36)	Internal Standard (mg/100 g; n = 24)	Aqueous		Internal Standard (n = 12)		Aqueous		Internal Standard (n = 24)		SRM 1577b n = 6	Std. Int. n = 6	Certified mg-µg/g	Analyzed mg-µg/g	
			Internal Standard (n = 18)	Internal Standard (n = 12)	Internal Standard (n = 36)	Internal Standard (n = 24)									
Na	<LOD	6.04 ± 0.10	1207 ± 2	0.7	1.8	1.7	2.0	101.6 ± 1.2	102.7 ± 1.3	2.42 ± 0.06	2.40 ± 0.006	0.035	0.042		
K	<LOD	1.98 ± 0.02	248 ± 8	0.4	0.2	0.9	1.1	101.5 ± 0.7	100.7 ± 0.7	9.94 ± 0.02	10.208 ± 0.0323	0.016	0.030		
P	<LOD	50.8 ± 0.8	291 ± 13	1.4	0.6	1.5	0.7	100.4 ± 0.6	—	11.0 ± 0.3	10.57 ± 0.20	2.2	4.3		
Ca	<LOD	2.50 ± 0.02	22.3 ± 1.4	0.4	0.8	0.4	0.9	102.3 ± 1.1	103.2 ± 1.0	116 ± 4	116.4 ± 5.4	0.010	0.018		
Mg	<LOD	1.50 ± 0.01	14.3 ± 0.3	0.7	0.8	0.7	2.8	102.2 ± 0.9	104.3 ± 0.9	601 ± 28	605.2 ± 14.4	0.029	0.036		
Fe	0.08 ± 0.02	1.02 ± 0.02	1.30 ± 0.05	1.4	1.3	1.5	1.4	99.3 ± 0.92	98.3 ± 1.0	184 ± 15	184.4 ± 1.6	0.130	0.251		
Zn	0.006 ± 0.001	1.51 ± 0.01	1.66 ± 0.01	0.6	0.4	0.7	0.5	97.3 ± 1.1	98.7 ± 1.3	127 ± 16	127.2 ± 2.2	0.009	0.016		
Cu	0.031 ± 0.003	0.051 ± 0.002	0.076 ± 0.003	3.0	2.0	3.3	2.5	99.0 ± 1.4	100.3 ± 0.6	160 ± 8	164.9 ± 1.1	0.039	0.057		
Mn	<LOD	0.103 ± 0.006	0.124 ± 0.009	3.7	2.7	5.8	4.5	101.3 ± 1.7	99.4 ± 0.4	10.5 ± 1.7	10.67 ± 24	0.015	0.075		
Se	<LOD	30.2 ± 0.6 ^a	9.1 ± 0.3 ^a	1.9	2.0	2.1	2.4	99.4 ± 1.3	99.1 ± 1.3	0.73 ± 0.06	0.73 ± 0.03	0.04	0.05		
Cr	<LOD	2.00 ± 0.03 ^a	5.0 ± 0.8 ^a	1.3	0.6	1.7	1.5	102.9 ± 0.8	103.6 ± 1.2	—	0.022 ± 0.002	0.66	1.82		
Al	15.0 ± 0.4	19.8 ± 0.2 ^a	125 ± 7 ^a	0.6	1.2	0.9	1.2	99.2 ± 0.3	100.2 ± 0.4	(3)	1.99 ± 0.76	16.2	18.9		
Cd	<LOD	1.04 ± 0.03 ^a	0.35 ± 0.02 ^a	2.2	2.8	2.5	3.4	102.3 ± 0.9	104.9 ± 1.0	0.50 ± 0.03	0.50 ± 0.01	0.07	0.21		
Pb	<LOD	3.00 ± 0.05 ^a	3.5 ± 0.1 ^a	1.3	1.3	1.6	1.7	99.4 ± 0.7	101.6 ± 1.0	0.129 ± 0.004	0.131 ± 0.003	0.12	0.37		

^a µg/L.

Note: RSD, relative standard deviation.

Source: Adapted from Barbarin, C., Análisis de macrominerales y oligoelementos en derivados cárnicos. Evaluación del aporte a la ingesta dietética, Ph.D. thesis, University of Navarra, Pamplona, 2004.

Table 16.9 Mineral Composition (Mean ± Standard Deviation) in Meat Product (Number of Each Type of Sample = 32, Analyzed in Triplicate) Provided by 100 g of Edible Portion

Meat Product	Na (mg)	K (mg)	P (mg)	Ca (mg)	Mg (mg)	Fe (mg)	Zn (mg)	Cu (μg)	Mn (μg)	Se (μg)	Cr (μg)	Al (μg)	Cd (μg)	Pb (μg)
Bulk pâté	865 ± 85	184 ± 36	202 ± 25	23.0 ± 7.0	10.1 ± 1.8	6.23 ± 2.00	2.45 ± 0.48	625 ± 277	294 ± 113	12.0 ± 2.1	5.4 ± 1.9	171 ± 64	1.35 ± 0.29	3.0 ± 1.0
Canned pâté	720 ± 112	131 ± 27	180 ± 73	14.6 ± 3.8	8.9 ± 1.8	4.94 ± 2.42	1.98 ± 0.62	694 ± 504	239 ± 73	15.6 ± 4.9	4.6 ± 1.6	656 ± 1548	1.28 ± 0.60	2.6 ± 1.7
Blood sausage	445 ± 118	69 ± 19	49 ± 15	23.2 ± 10.0	11.3 ± 6.1	8.81 ± 2.57	0.46 ± 0.25	110 ± 36	344 ± 143	5.3 ± 1.6	6.1 ± 2.3	290 ± 275	0.27 ± 0.13	2.8 ± 1.7
Bacon	1655 ± 428	412 ± 57	250 ± 39	14.8 ± 3.9	21.9 ± 3.7	1.15 ± 0.28	2.99 ± 0.51	92 ± 25	51 ± 25	7.0 ± 2.8	2.7 ± 1.3	153 ± 119	0.37 ± 0.09	3.4 ± 0.9
Bacon (vacuum packed)	1303 ± 397	329 ± 73	250 ± 79	9.6 ± 3.6	15.9 ± 3.8	1.14 ± 0.84	2.41 ± 0.58	80 ± 26	9 ± 15	7.6 ± 2.6	2.8 ± 3.1	112 ± 111	0.28 ± 0.07	2.9 ± 1.9
Mortadella	1115 ± 104	239 ± 28	289 ± 51	22.5 ± 9.7	13.9 ± 1.8	1.09 ± 0.23	1.58 ± 0.38	88 ± 14	118 ± 37	9.2 ± 1.2	5.5 ± 6.3	142 ± 79	0.35 ± 0.06	2.8 ± 0.6
Canned sausage	649 ± 152	63 ± 23	102 ± 30	23.8 ± 18.3	7.9 ± 1.4	1.48 ± 0.33	1.19 ± 0.46	96 ± 26	75 ± 27	6.8 ± 0.9	4.5 ± 1.5	110 ± 50	0.24 ± 0.07	1.7 ± 0.2
Frankfurt-type sausage	1088 ± 184	177 ± 22	230 ± 23	56.7 ± 22.8	12.5 ± 1.3	1.81 ± 0.35	1.17 ± 0.22	124 ± 52	125 ± 46	8.6 ± 2.5	10.7 ± 5.7	175 ± 55	0.32 ± 0.11	1.6 ± 0.9
Fresh sausage	803 ± 198	297 ± 70	179 ± 33	12.3 ± 3.3	16.8 ± 2.9	0.94 ± 0.25	2.07 ± 0.60	77 ± 19	56 ± 30	8.7 ± 2.7	7.7 ± 14.2	103 ± 54	0.17 ± 0.05	2.8 ± 1.7
Chistorra (spicy sausage)	879 ± 203	403 ± 71	225 ± 54	22.1 ± 5.3	24.1 ± 4.5	2.36 ± 0.60	2.59 ± 0.75	133 ± 26	141 ± 67	4.4 ± 1.5	6.5 ± 4.2	641 ± 300	0.65 ± 0.14	5.3 ± 2.3
Chorizo	1600 ± 283	444 ± 86	245 ± 65	39.4 ± 10.9	23.1 ± 6.0	2.54 ± 0.59	2.40 ± 0.43	137 ± 44	310 ± 195	5.1 ± 1.6	8.1 ± 2.5	624 ± 163	0.49 ± 0.24	3.9 ± 1.0
Chorizo de Pamplona	2091 ± 296	690 ± 69	425 ± 71	35.7 ± 13.0	37.4 ± 4.0	3.20 ± 0.65	3.82 ± 0.77	209 ± 41	578 ± 343	12.4 ± 2.9	10.0 ± 4.7	672 ± 417	0.47 ± 0.11	3.7 ± 1.8
Salami	1996 ± 245	429 ± 79	353 ± 89	57.4 ± 24.1	23.1 ± 6.1	1.63 ± 0.35	2.94 ± 0.64	130 ± 35	262 ± 259	13.3 ± 2.3	4.9 ± 4.1	328 ± 120	0.55 ± 0.16	4.9 ± 1.1
Cured ham	2099 ± 361	540 ± 64	281 ± 43	17.3 ± 3.7	29.4 ± 22.0	1.60 ± 0.28	3.15 ± 0.52	132 ± 18	50 ± 23	13.2 ± 4.2	2.0 ± 1.2	103 ± 34	0.26 ± 0.07	3.5 ± 0.8
Cured ham (vacuum-packed)	1996 ± 296	502 ± 67	262 ± 49	16.7 ± 4.0	27.7 ± 3.6	1.42 ± 0.35	3.33 ± 0.65	123 ± 21	15 ± 13	8.7 ± 2.7	2.2 ± 1.1	86 ± 32	0.21 ± 0.06	2.3 ± 0.7
Cooked ham	949 ± 133	334 ± 68	319 ± 55	12.0 ± 10.0	18.7 ± 2.2	0.89 ± 0.16	1.81 ± 0.35	68 ± 13	17 ± 20	3.8 ± 1.2	3.6 ± 3.3	145 ± 101	0.35 ± 0.13	2.1 ± 1.3
Cooked ham (vacuum-packed)	967 ± 114	346 ± 38	285 ± 32	10.7 ± 6.7	21.6 ± 2.5	0.80 ± 0.11	1.80 ± 0.26	57 ± 6	24 ± 26	3.9 ± 1.1	2.5 ± 1.0	90 ± 47	0.33 ± 0.18	2.7 ± 4.0
Cured pork loin	1687 ± 394	618 ± 110	378 ± 41	13.5 ± 4.6	42.1 ± 7.4	1.20 ± 0.49	2.97 ± 0.93	111 ± 29	75 ± 28	5.9 ± 3.0	3.3 ± 1.8	127 ± 58	0.34 ± 0.11	3.4 ± 1.0
Mean ± s.d.	1274 ± 577	353 ± 183	255 ± 102	23.8 ± 18.1	18.8 ± 10.1	2.39 ± 2.33	2.31 ± 0.99	170 ± 221	157 ± 195	8.4 ± 4.4	5.2 ± 5.3	269 ± 458	0.45 ± 0.37	3.1 ± 1.8
Median	1102	332	250	19.7	20.1	1.54	2.33	118	96	8.1	4.7	149	0.34	2.8
Range	445–2100	63–690	49–425	9.6–57.3	7.9–42.1	0.80–8.81	0.46–3.82	57–696	4–578	3.8–15.6	2.1–11.0	86–672	0.15–1.35	1.1–5.3

Source: Adapted from Barbarin, C., Análisis de macrominerales y oligoelementos en derivados cárnicos. Evaluación del aporte a la ingesta dietética, Ph.D. thesis, University of Navarra, Pamplona, 2004.

16.4 Reference Values of Selected Minerals and Trace Elements in Meat Products

In the literature, several studies were found on the mineral content in animal muscles and tissues [37,57–65]. Unfortunately, there is little information on the content in meat derivatives, although these products involve an important proportion of the total meat consumption in developed countries.

Table 16.9 shows the macromineral and trace element content found in a wide variety of meat-derived products [34]. These findings and those of diverse research works [66–92] suggest that meat derivatives might be an important mineral source, providing a significant nutritional contribution to dietary intake.

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SENSORY QUALITY



Chapter 17

Color Characteristics of Meat and Poultry Processing

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17.1 General Aspects of Color

Color can be defined as a subjective sensation resulting from a complex series of physiological, physical, chemical, and psychological responses to electromagnetic radiation at a wavelength between 400 and 700 nm [1].

17.1.1 Color Attributes

Many color spaces (e.g., Munsell, Hunter, or CIELAB), color order systems, and color appearance models, such as the Munsell color system [2], are used for color specification. CIELAB [3], which is used for formulating color differences, and CIECAM02 [4], which is used for the specification of color appearance, use lightness, hue, and chroma to specify color attributes.

Hue, lightness, and chroma are the perceptive aspects of colors defined in color science [5], whereas color memory, color meaning, and color harmony are cognitive aspects of color [6]. Hue is the dominant factor in color emotions, whereas the meaning of a color comes mainly from its lightness and chroma and to a lesser extent from its hue.

CIELAB color space can be represented by CIELCH space (lightness: L^* , chroma: C^* , and hue: H^*). The L^*C^* plane is divided by three emotion indices into six regions based on the dependence of “soft–hard,” “warm–cool,” and “light–dark” on L^* and C^* . Soft and warm colors are clearly lighter and correspond to colors with high lightness and high chroma. Colors in the low chroma and low lightness region are cool, hard, and dark. Colors with high lightness and low chroma feel cool and soft, while colors with high chroma and low lightness feel hard and warm. The responses vary from dark to light with the increase of lightness or chroma [7], but an inconvenience of both the CIELAB and CIELCH planes is their nonuniformity.

17.1.1.1 Appearance

The perception of color is a complex process in the human visual system. The majority of researchers consider that color preference is cultural and even completely individual, yet they also agree that the conformity between different people about the general cognitive characteristic of colors is considerable [8]. To describe the appearances of color, it is generally agreed that five perceptual dimensions, or attributes, are necessary: brightness, lightness, colorfulness, chroma, and hue [9–12]. For color reproduction, hue and the relative color attributes, chroma and lightness, are typically used for color specification.

17.2 Practical Aspects of Meat Color Measurement

From an objective point of view, meat color is the result of an interaction of four factors: the light falling on the meat or meat product; the object (meat or meat product) that reflects or absorbs the light; the observer, and the surroundings [13].

17.2.1 Color Physics

Important tools in the study of color are the reflection spectra that represent the spatial distribution of radiation bands and that are obtained by separating the monochromatic components, thus permitting the composition of the whole band to be understood.

Reflectance spectroscopy is a major remote-sensing technique used to study the chemical composition and microstructure of various light-scattering media [14]. The reflected light spectrum is measured and used to decode the relevant information with respect to the inherent properties of a food layer in the framework of the radioactive transfer theory [15].

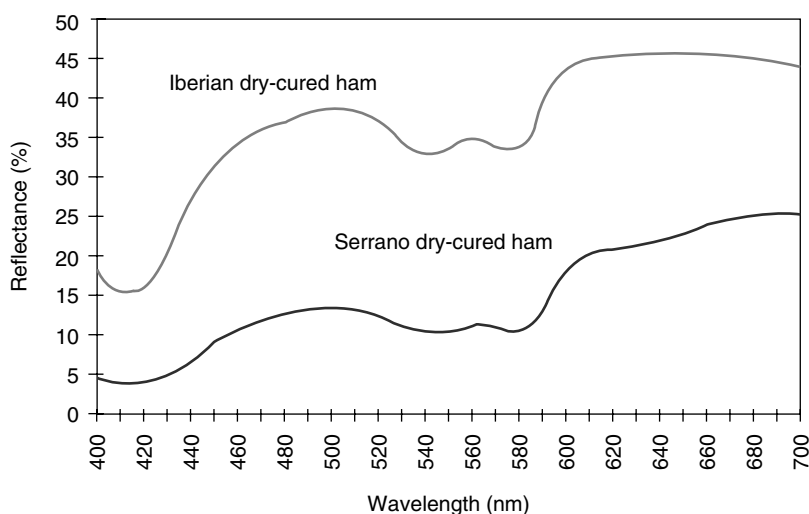


Figure 17.1 Reflectance spectra (400–700 nm) of Serrano and Iberian dry-cured ham.

In many cases, the scattering measured in foods depends on the optical thickness, and so on the food geometry, creating a problem if one is interested in the bidirectional reflectance determined by illumination and viewing angles. Optical phenomenon occurring at the air–food interface are of great importance in the field of food optics. Surface reflection can be used for color matching [15].

Color can be determined objectively by reflectance spectrophotometry both of the primary materials used in the industry [16–21], as can be seen in Figure 17.1, and of meat products derived [22–26]. Each of the primary materials has its own particular absorption and reflection spectrum [1,27]. Moreover, each of the states of myoglobin (Mb) can be quantified spectrophotometrically; for example, the maximum absorption of Mb and metmyoglobin (MetMb) are at 555 and 505 nm, respectively. The use of this technique permits measurements to be made of the product's surface without destroying the sample, and permits such measurements to be made as time elapses [28].

For the same reasons, this technique can be used to control the quality of the materials used in the elaboration of meat products.

Color measurement is dependent on the size of the port used to observe the samples, also known as the aperture size [29]. The same author [29] also found that the 10-mm aperture gives lower L^* , a^* (redness), and b^* (yellowness) values (darker, less red, and less yellow) than the 50-mm aperture size. When the smaller aperture size is used, the reflectance values from the longer, red wavelengths in the visual range are reduced by a greater percentage than the shorter wavelengths (blue).

Sánchez-Rodríguez [30] reported that in Iberian dry-cured ham from pig fed with acorn L^* , a^* , b^* and chroma showed metamerism by illuminant (C and D_{65}) both in the CIELAB and in HunterLab space for *Biceps femoris* (BF), *semitendinosus* (ST), and *semimembranosus* (SM) muscles. The same author found that observer metamerism occurred for lightness and yellowness for the same color spaces and illuminants.

For Karamucki et al. [31], L^* , b^* , and C^* values in porcine *Longissimus lumborum* muscle were closely connected with meat quality. Meanwhile, redness and hue values showed medium and low

correlations with meat quality traits. The application of illuminant D_{65} and an observer angle of 10° for measurements of meat color proved to be more suitable in the case of a^* and C^* parameters, whereas the use of illuminant C and observer an observer angle of 2° was more suitable in the case of the hue angle (h°) parameter. For chicken and beef color, Zhu and Brewer [32] reported that a^* , h° , and reflectances differences (R630–R580) could be used to predict visual redness under halogen light and cool white fluorescent light.

17.2.2 Meat Color

Meat color, primarily due to the presence of Mb, is an important characteristic in the consumer acceptance of meat products. However, color may vary greatly in fresh and processed products, and is strongly influenced by handling during storage and display [33]. Color development depends on methods used in manufacture as well as on the nitrosation of meat pigments [34].

Several color measurements are applied to meat; for example, Sahoo et al. used high Lovibond tintometer red color units and chroma, together with the MetMb content [35], but meat color researchers generally use CIELAB, and in some countries, such as Spain, this color space is used as official standard [1].

Some coordinates have been used as a tool for quality control. Schivazappa and coworkers correlated the color assessed by sensory evaluation with the a^* value and (K/S572)/(K/S525) ratio, whereas Santamaria et al. [34] used spectrophotometric measurements of the nitrosation index (at R560/R500 nm) and the red color stability index (RI; at R570/R650 nm).

17.2.3 Color during Meat Processing

During meat and meat product processing, color may be very difficult to define in a single way, because several factors (chemical, physical, biochemical, and microbiological, among others) and complex reactions are interrelated [19].

The techniques of mincing, mixing, cooking, and drying, together with the incorporation of additives and spices, all have an effect on color. Such processes are common in the production of meat products and play a fundamental role in the development of the essential characteristics of each product. Although these are very common operations, they are, from a theoretical point of view, quite complex and no complete description exists regarding their influence on color.

17.2.3.1 Slaughter

In fresh meat, such factors as the interaction of the slaughter process and animal stress or exhaustion can result in massive variation in meat appearance. This is usually a direct consequence of differences in the rate and extent of the pH fall that occurs after slaughter coupled with the speed of carcass chilling and the length of aging. Colors that would normally be an attractive bright red-pink may appear dark jelly-like translucent purple or pale opaque pink due to the light scattering properties of the meat. This greater than twofold range in the scattering power of the product leads to unacceptable quality variations both in color and texture [36].

The energy status of the chicken at slaughter has a large impact on the development of post-mortem pH and hence color. Nisen and Young [37] found that creatine monohydrate and glucose

supplementation to slow- and fast-growing chickens had no effect on these parameters. In ostriches, Fasone et al. [38] reported that meat lightness was strongly affected by stress, the color of meat from the “stress group” being darker than the meat from the “non-stressed group” (L^* : 34.30 and 38.10, respectively). The other color parameters were not affected by stress.

Bianchi et al. [39] reported that holding time and temperature exerted the most important effect on broiler breast meat color. However, other factors, such as genotype, live weight, and transportation, may influence breast meat color. These authors also reported that market live weight influenced color parameters; for example, the heavier birds (>3.3 kg) produced a darker breast meat ($L^* = 51.67$) than did the lighter birds (<3.0 – 3.3 kg; $L^* = 52.63$). When the birds were transported for distances of <40 km, the breast meat color exhibited higher redness (a^* : 3.59), whereas for longer distances a^* diminished.

Linares et al. [40], in their study of how stunning systems affect the color of lamb meat, found that the animals slaughtered after CO_2 stunning showed lower redness and yellowness values than control groups (without previous stunning).

After slaughter, food inspectors and veterinarians use the color of livers to tell whether this offal is suitable for human consumption. It is also possible to approximately determine whether liver has a high or low amount of hepatic lipids. Thus, lighter liver colors in full-fed broilers were associated with higher hepatic lipid concentrations; in contrast, darker livers from fasted broilers had lower levels of lipids [41].

For processing purposes, chicken color can be classified on the basis of lightness values as dark ($L^* < 50$), normal ($50 \leq L^* \leq 56$), or pale ($L^* > 56$) according to Petracci et al. [42]. These authors also reported that paler ($L^* > 56$) chicken breast meat is associated with lower ultimate pH and lower water-holding capacity (WHC), whereas darker ($L^* < 50$) chicken breast meat is associated with higher pH and cooking yield. In beef, meat color values decrease with increasing pH, and increase with increasing backfat thickness [43]. Dvorak et al. [44] reported that a^* (redness) was the most important aspect of color for objective pork quality evaluation on a production line in a large slaughterhouse, although this coordinate showed a very low correlation coefficient for pH and drip loss.

17.2.3.2 Aging

Aging is a common practice in meat production and plays an important role in several quality characteristics, such as texture and color. With regards to color, aging improves blooming, and Lindahl et al. reported that aging increased the oxymyoglobin (OMb) content of pork *Longissimus dorsi* muscle and the decreased content of deoxyMb, resulting in increased lightness, redness, and yellowness. Also, aging has a smaller effect on color stability, with slightly lower MetMb being observed in aged meat [45]. In other studies, the aging of pork loins increased lightness and slightly increased yellowness; tenderness was also improved [46]. In beef, aging has a marked influence on all color parameters [47]. The maturity of the carcass increases the redness and lightness of meat and the yellowness of fat [48]. In *Longissimus dorsi* muscle, the chilling rate affects lightness, yellowness, and h° differences, whereas fat cover thickness affects the influence that aging has on lightness and yellowness [47]. In Rubia Gallega (RG) breed aging, meat color increased redness, yellowness, hue, and chroma [49], but Oliete et al. [50,51] reported that pigment concentration decreased with aging time. In the RG breed, color variables were the most determinant characteristic of meat quality.

17.2.3.3 *Fresh Meat Products*

Several studies have pointed to the importance of the different states of Mb in determining the color changes that take place during processing [19]. In fresh pork lean meat, color parameters (L^* , a^* , b^* , h° , C^* , and a^*/b^* ratio), reflectance spectrum (400–700 nm), and reflectance ratios (R560/R500, R650/R570, R630/R580, and R630-R580) varied according to the state of the Mb.

Biochemical aspects, such as lactate dehydrogenase (LDH) activity, was negatively correlated with the Mb content of muscle tissue. LDH activity (%) in the sarcoplasmic fraction is higher in porcine than in bovine muscle, and this can affect the color of meat products manufactured with these meats [52].

During the storage of fresh sausages, products change to a brown color, a change related to MetMb formation. Schivazappa et al. [53] described that the brightest red color is only stable for up to 7 days when the sausages are packaged under atmospheres containing O_2 , although this period can be increased if the product is packaged under an O_2 -free atmosphere. The bright red color (OMb) in pork sausages is related to the presence of oxygen in the pack atmosphere; but when it decreases (after 7 days), the pigment changes mainly to MetMb. These authors found a correlation between Mb oxidation and lipid oxidation.

Fresh meat and meat products are normally exposed in a light box or in a supermarket display case. The sample is illuminated from both above and from the sides. In fresh meat, such factors as the interaction of the slaughter process and animal stress or exhaustion can result in massive variation in meat appearance [54].

The addition of potassium lactate (KL) improved the microbial counts of patties, with no effect on color or lipid oxidation. But when this additive was added to chunks, color stability increased and microbial counts decreased [55]. KL stabilizes postmortem muscle color via the interaction of KL with lactic dehydrogenase to regenerate reducing equivalents (nicotinamide adenine dinucleotide), which subsequently increases MetMb-reducing activity [56,57]. Most pigment oxidation occurs when meat is exposed to light (decreasing a^* , b^* , and C^*), but KL can help protect against the negative influence of light, via the mechanism described by Seyfert et al. [58].

17.2.3.4 *Dry-Cured Meat Products*

Dry-cured meat products can be described from a technological point of view as products that, during their elaboration, are treated at controlled temperature and humidity for variable times both to favor microbiota development and for dehydration. During this process, a complex series of chemical, enzymatic, and ultrastructural reactions takes place, together with different physical processes, which modify the properties of the product [1].

17.2.3.4.1 *Dry-Cured Sausages*

The manufacturing process of these dry-cured meat products consists of four basic stages: mincing, mixing, fermentation, and dry-maturation [54].

In the first step, the raw materials are reduced in size and mixed with additives and spices to make a batter, which is generally left to rest for 12–24 h (depending on the type of product). The meat batter is then stuffed into a casing, before being allowed to ferment and mature.

Color formation in dry-cured meat products takes place during the different stages of elaboration in response to biochemical mechanisms related to the characteristics of the materials used, the technological operations applied, and the additives or spices incorporated.

During the manufacture of dry-cured sausages the pH falls as a consequence of the metabolic activity of the microorganisms present [1]. The extent to which light rays penetrate or are reflected depends on the pH, so that dispersion increases as pH falls [59].

In studies investigating such changes in dry-cured pork products, variations in the color coordinates have been observed to take place during the resting stage [60].

In the case of dry-cured sausages, and from a strictly color point of view, this will depend on such diverse factors as the composition of the sausage, the fat–lean ratio, salt content [61], curing salts used, the presence of organic acids, the proportion and type of spices, other additives (polyphosphates, sugars, etc.), the technological treatments applied (e.g., the degree of mincing, mixing time, or fermentation time), dry-maturation, type of animal meat, the composition (content and distribution of fat and muscle fraction), technological treatment applied, the different phenomena that occur during the elaboration process, and storage (since this type of product undergoes different physical, chemical, biochemical, and ultrastructural transformations), all of which modify the color of the final product. Reactions such as the formation of nitrosomyoglobin (NOMB), a characteristic pigment of this type of product, the availability of water on the product surface, degree of integrity of the muscle tissue, and the state of the ultrastructure, are phenomena to be taken into account when evaluating the color of this type of product [62].

Chasco et al. [63] reported that nitrites react with Mb to form NOMB and MetMb during the fermentation stage; this MetMb is subsequently reduced to NOMB during the drying process. But the main color changes of Spanish dry-cured sausages took place during the fermentation stage [25,60,64]. Pérez-Alvarez [1] established that the color evolution (CIELAB) in dry-cured meat products (low fermentation temperature, fermented sausages, with and without paprika addition, and whole dry-cured meat cuts such as dry-cured loin and dry-cured ham) was lightness, yellowness, hue, and chroma decreasing, and redness and a^*/b^* ratio increasing. This behavior can be observed in Figure 17.2. For Sucuk, Turkish sausage color parameters changed during the dry-curing processing; thus, lightness, yellowness, and h° decreased during the ripening period. For this meat product, Bozkurt and Bayram [65] found that the main color changes take place between the fifth and ninth days of the ripening period. These authors also found a positive relationship between redness and sensory color scores.

17.2.3.4.1.1 Paprika The addition of paprika to meat products has been observed to modify color coordinates in several ways. For example, its addition reduced L^* [54,62], whereas a^* and b^* values of meat increased. The meat color itself was masked by paprika [66]. Paprika also affects reflectance spectra, regardless of the meat product or processing conditions. The color modifications observed in dry-cured meat products containing paprika are due principally to changes that occur in the paprika, as opposed to changes in meat color itself, as can be seen in Figure 17.2.

17.2.3.4.1.2 Starter Cultures Several studies dating from the 1950s have shown that the use of starter cultures improves the color of meat products. However, recent studies have described a new type of bacteria used in meat products, and Khankhalayaeva et al. [67], for example, showed that propionic acid bacteria increase the formation of nitrous pigments and stabilize the color of meat products. They also mentioned that the use of sodium nitrite can be reduced (30%) without reducing the final color of the same products. When *Staphylococcus carnosus* and *S. xylosus* were added to Chinese-style sausage, they improved the color stability of this sausage [68]. At the same time, it is important to mention that these starter cultures in this sausage showed higher concentrations of

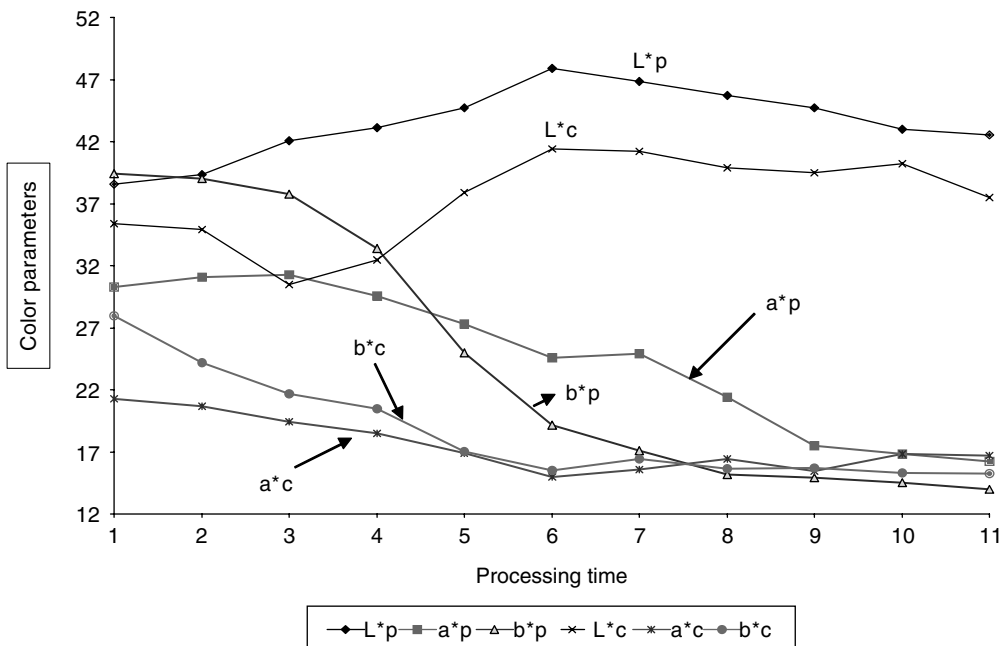


Figure 17.2 CIELAB color coordinates evolution during paprika oxidation under ultraviolet light exposure and dry-cured sausage added with paprika (chorizo) elaboration process. L*, lightness; a*, redness; b*, yellowness; p, paprika, and c, chorizo.

nitrosyl-pigments and lower MetMb contents. Inoculated sausages showed higher redness values than control samples, whereas L* and b* decreased during processing.

According to Moller et al. [69] *Lactobacillus fermentum* strains (JCM1173 and IFO3956) and *Pediococcus pentosaceus* PC-1 and *S. carnosus* XII can generate nitrosylated derivatives from Mb. All of these bacteria can convert brown MetMb into red OMb, but only the lactobacilli can specifically produce nitrosylmyoglobin, especially in smoked fermented sausages.

Vural [70] reported that *P. acidilactici* can be used as starter culture for a Turkish semidry-fermented sausage. This culture increases the conversion of heme pigments to cured meat pigments and improves the development of the typical Turkish sausage color.

17.2.3.4.2 Dry-Cured Ham

In this type of meat product, salt diffusion plays an important role in the development of color. Thus, when salt diffuses through meat, the color coordinates change. This is related to changes in the WHC and interconversion of Mb states. But when the WHC does not change during maturation, such color changes are related to ultrastructural disorganization and modification of the Mb structure. There is a gradient of color coordinates in this type of product, and each muscle has different color parameters, according to its anatomical distribution. Indeed, in the same muscle, it is possible to obtain a color gradient due to salt diffusion and ultrastructural disorganization of the muscular structure [71]. At the end of the aging step (“afinado”) of Serrano dry-cured ham, the most important muscles, such as SM, ST, and BF, showed different color values: SM showed

the lowest values of the color parameters, except for the a^*/b^* ratio; BF had the highest values, except for the a^*/b^* ratio. BF and ST showed similar values for redness, yellowness, and hue. This author also obtained a correlation between L^* and the moisture content and water activities [26]. From a sensorial point of view, Sánchez-Rodríguez [30] reported that “veteado” (intramuscular fat) of Guijuelo acorn-fed Iberian dry-cured ham reduces the red meat color and contributes to the “brilliance” of the cut surface.

17.2.3.4.2.1 Parma and Iberian Dry-Cured Ham Pigments The stable, bright red color of some dry-cured hams, such as Parma ham, is achieved without the need for added nitrate or nitrite [72]. In dry-cured hams, Moller et al. [73] observed the presence of chemically identical red chromophores with properties similar to a complex of transition metals and protoporphyrin IX (Zn–porphyrin complex). Similar findings in Parma ham were obtained by Wakamatsu et al. [74].

The substitution of iron by zinc in Mb structure during the maturation of Parma ham is concomitant with Mb modifications [75]. These changes begin during the resting period following salting and seem to precede the formation of zinc porphyrin. Electron spin resonance spectra showed that the pigment in dry-cured Parma ham is at no stage a nitrosyl complex of ferrous Mb, as found in brine-cured ham and Spanish Serrano hams [76]. Protein denaturation/degradation facilitates the substitution of iron by zinc, but pigment polymerization may also be the result of noncovalent protein association with zinc porphyrin in denatured or partly degraded Mb [75].

Parma ham increases its lipophilic character during processing, suggesting that a combination of drying and maturing yields a stable red color [77].

At the end of processing, pigments become less extractable with water. The heme moiety of Parma ham pigment is gradually transformed from a Mb derivative into a nonprotein heme complex, thermally stable in an acetone/water solution [76].

The Zn–porphyrin complex has better color stability than those pigments from dry-cured ham with added nitrites. This Zn–porphyrin complex showed faster initial discoloration when exposed to light, under retail conditions [78]. Thus, Adamsen et al. [79] found that apomyoglobin did not show antioxidative capacity, although the heme moieties had antioxidative properties. These authors also found that lipid oxidation was inhibited by the addition of Parma ham pigment (0.12–0.24 ppm), and that this Zn–porphyrin complex can protect alpha-tocopherol degradation.

Spectral patterns from Parma ham differed from those of the Mb derivatives [80]. Among other hypotheses proposed, some authors have suggested that the reddening of Parma ham is probably caused by the action of bacteria; the *Staphylococci* isolated from Parma ham also generate the red Mb derivative from MetMb.

In other dry-cured meat products, the use of nitrite inhibits the reduction of heme pigments in meat products in the presence of NaCl [81].

17.2.3.5 Cooked Meat Products

Fernández-Ginés et al. [82] established the pattern of color formation during the whole cooking process, lightness, and yellowness decreasing but redness increasing during heat treatment. These authors reported that color changes are related to changes into internal temperature and the state of the sarcoplasmic pigments. Also, the color characteristics of the batter were closely related to the degree of pigment nitrosation, and the redness of the batter may be used as an index for assessing

the mincing process. It must also be taken into account that the color of freshly minced batters is closely related to the color of the final products [83].

In a model system, Sakata and Nagata found that low molecular weight of the sarcoplasmic fraction of *Longissimus thoracis* muscle, in combination with preheating Mb, enhanced cured color formation during the subsequent reaction with nitrite. Studies with pork sausage indicated that denaturation and the nitrosation of heme pigments followed a similar pattern. Sakata and Nagata suggested that this behavior might be due to the acceleration of the heat denaturation of heme pigments, resulting in their rapid nitrosation [82,84].

The addition of polyunsaturated fatty acids to animal diets, for example, the use of rich linoleic acid feeds, may produce several problems during the cooking process of this meat. Bergamaschi et al. [85] found that a high level of linoleic acid favored an increase in rancidity and color oxidation in muscles liable to oxidation, such as *masseter*, but had no significant effect on glycolytic-type muscles (shoulder). But when mortadella bologna was elaborated with these muscles, an unacceptable color was sometimes observed after cooking, accompanied by an increase in rancidity.

When diphosphate is added to sausages, the development of NOMb is not affected; the color of the sausages was only slightly less red than control sausages when this additive was used with ascorbate. It is very interesting to note that the addition of diphosphate increased the cured color of sausages [86], whereas the residual nitrite concentration decreased.

Several attempts have been made to obtain natural colorants from blood, and to apply this to meat products, especially cooked meat products. For example, Ito et al. [87] used the electrolytic reduction of heme protein (MetMb and methemoglobin) solutions, and found a stable nitrosyl derivative by means of limited electrolysis in the presence of sodium ascorbate and nitrite, at neutral pH values, especially in the presence of 1 M sucrose. When these electrolytically obtained pigments were used in meat products (sausages), their redness increased.

Blood derivatives are used as ingredients in cooked meat products [88]. The red cell fraction of slaughtered cattle blood is treated to obtain nitrosated hemoglobin for use as a colorant in cooked meat products. This nitrosohemoglobin improved the color of sausages [88]; the color remained stable for 2 weeks at 2°C and no differences were detected, but redness retention was better than for control samples. When this colorant was applied to pork sausages (0.5 or 1%), redness increased [89]. Krysztofiak [90] described how the use of plasma proteins negatively affected the color of the cooked meat products (sausages) in which they were incorporated. In general, discoloration (brown color) in meat occurs when L^* and b^* values increase, and a^* values decrease. Other new techniques have been used to improve the healthy appearance of meat products; for example, Kwon [91] used supercritical carbon dioxide to produce meat with a low fat concentration, and found that this technique produced meat with a lighter color. If this meat is used for meat products, the product is also lighter than control products.

If exposed to light, the redness of cooked meat products diminishes during storage. This phenomenon, in pork bologna, is related more with photochemical than thermal processes, and the action of temperature on color was attributed by Carballo et al. [92] to microbial growth, which in turn affects oxygen availability. Turkey bologna color is modified by light during retailing, the extent of its color fading depending on increases in yellowness, rather than decreases in redness [93].

Deep fat frying of beef meat balls affects the kinetics of surface color [94]; thus, surface colors (L^* , a^* , and b^*) decrease exponentially with frying time, whereas total color change increases. During this process, color evolution follows first-order reaction kinetics.

Redness in cooked ham is the most sensitive parameter in color measurement, characterizing red color and color stability [95]. In this product, consumers prefer a lighter color and less redness

(higher value of L^* and a lower a^*), a deeper red color suggesting the addition of artificial coloring. Válková et al. [96] reported that the color characteristics for cooked ham ranged from 61.57 to 68.97 L^* value (lightness), 8.14–13.95 a^* value (redness), and 6.60–9.70 b^* value (yellowness).

17.2.3.6 *Marinated Meat Products*

As other technologies, marination can affect color. In this technique the additives used in the bath modify color co-ordinates. For example, the use of sodium tripolyphosphate dicalcium hydrogen phosphate solution modified lightness, but when citric acid was used, yellowness increased [97]. Pérez-Alvarez and Fernández-López [13] suggest that most of the phosphates used in meat processing decrease lightness because of their influence on WHC and acid ingredients.

The use of phosphates in marinated cooked broiler leg muscles did not affect color parameters, except that yellowness increased [98].

17.3 Functional Foods

Since the 1980s, the scientific–technological advances made in the food industry relating food with health have given rise to a new sector in the world of food technology known as “functional foods” [99]. According to Fernández-Ginés et al. [100], the meat sector in recent years has been one of the most dynamic in the agro-food field, because it has rapidly incorporated functional foods into its repertoire, using a wide variety of new ingredients with the aim of making healthier meat products.

From a technological and scientific point of view, many food ingredients have been shown to play a beneficial role in health [101], improving the physiological functions of the human organism and permitting the design and optimization of foods that prevent or diminish the risk of certain chronic diseases [102]. However, from the consumer’s point of view, it is very important for functional meat products to possess almost the same sensory characteristics as similar or traditional meat products [100], in which color plays an important role. However, one of the inconveniences associated with the incorporation of new ingredients in any food, and meat products are not the exception, is the effect that this might have on the corresponding technological, nutritional, and sensory properties. Some ingredients or bioactive compounds may produce undesirable colors. This is particularly problematic in the case of dry-cured sausages [103,104], to which botanical and herbal ingredients (depending on the region or country) are frequently added [105].

Along with botanical or herbal additives, increased protein levels and vitamin and mineral fortification can lead to unacceptable flavors and marked alterations in the product’s color [1]. It must also be taken into account that the concentration of added ingredients can disrupt the normal evolution (chemical, biochemical, enzymatic, etc.) of these types of meat product, especially dry-cured products [106]. Ingredients such as polyphenols can act as antioxidants (in low doses) [107] or prooxidants (in high doses) [108], and, as is known, the color stability of dry-cured meat products is related to lipid oxidation:

Plum fiber. In meats, dried plum purée has moved beyond functioning as a fat replacer. It has been used in frozen, precooked meats (such as precooked hamburgers). Plum purée is used in meat between 3 and 5%. In pale-colored applications, the 3% level improves the end result, taking some of the pinkish color out of poultry [109,110].

Apple fiber. It is used in the elaboration of reduced-fat cooked meat sausages (mortadella). Color differences observed between sausages were mainly due to reduction because the addition of fiber slightly modified the color of the sausages, making them more yellow than the control sausages [111].

Citrus fiber. Citrus albedo (raw and cooked) added to cooked and dry-cured sausages produced significantly lower residual nitrite levels (higher with raw than cooked albedo) [103,104,112,113]. Yellowness was the only color parameter not affected by albedo type and concentration in both types of sausages. In bolognas, lightness increased when albedo was added, and this increase was higher when raw albedo was added rather than cooked albedo. These results mean that the albedo addition gave a lighter-colored product. On the contrary, when albedo was added to dry-cured sausages, lightness values decreased, especially in the case of cooked albedo. Albedo affected redness in a different way, depending on the type of sausage, increasing in dry-cured sausages and decreasing in cooked sausages.

When lemon albedo was added to the drying step of dry-cured sausage processing, color evolution was not affected by the presence of albedo. However, CIELAB color parameters L^* and b^* decreased, whereas a^* increased during drying. According to Fernández-Ginés et al. [114], citrus fiber extract up to 25 g/kg affects color properties, mainly a^* and b^* , which increase independently of citrus fiber addition. These authors also found that this type of fiber can act as nitrite scavenger, which was also observed by Aleson-Carbonell et al. [103,104] in several meat products. Garcia et al. [115] manufactured conventional and low-fat cooked sausages (mortadella type) with fruit fiber (15 and 30 g/kg) and reported that the fiber only increased yellowness.

Pea fiber and soy protein. These effectively protected the product against surface oxidation. The addition of pea fiber improved color stability of ground beef, although soy protein gave the best protection against the auto-oxidation of red OMB to brown MetMb. The addition of soy protein concentrate mixed with κ -carrageenan (0–3%) [116] favorably affected the WHC and thermal stability of the processed sausages regardless of the fat content, but did not improve the textural parameters, and no significant influence on color parameters was observed.

Rice bran. This has been used as an ingredient in frozen sausages [117], resulting in products showing a higher tendency toward red.

Rye bran. This has been used as a fat substitute in the production of meatballs. Its incorporation produces a lighter and yellower product than the control [118].

Olive oil. This has been used as a fat replacer in fermented sausages, the resulting products being lighter in color and more yellow [119].

Interesterified vegetable oils. Interesterified vegetable oils (60–100%) in meat products led to a significant increase in the oleic and linoleic acid content of meat products and a higher polyunsaturated fatty acids (PUFA)/saturated fatty acids (SFA) ratio, without any change in appearance, including color [120].

Conjugated linoleic acid. Conjugated linoleic acid as fat substitute improved the color stability, possibly by inhibition of lipid oxidation and OMB oxidation [121].

Lycopene. In meat products such as minced meat, the addition of lycopene from natural sources produces a meat product with better color and a well-documented health benefit [122].

Some of the new functional ingredients have color in themselves, which, in many cases can act as colorants, changing reflectance spectra and the color characteristics of a dry-cured sausage and

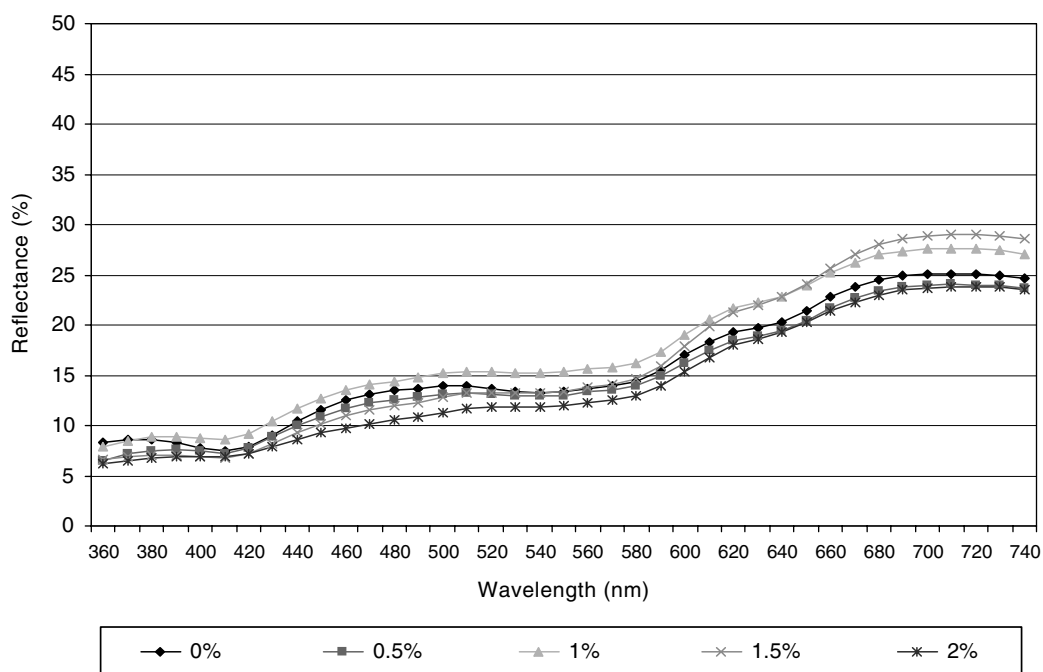


Figure 17.3 Reflectance spectra (360–740 nm) of a dry-cured sausage added with different concentrations of OFEs at time 28 days.

of other meat products. Ruíz-Cano et al. [123] reported that orange fiber extracts (OFE) as a “functional ingredient” did not modify the sausage meat batter reflectance spectrum (Figure 17.3), and found the highest reflectance values in the orange and red wavelengths. This was correlated with higher redness values in the meat products.

17.3.1 Additives

Salt replacers in meat products can affect the color of the product. For example, a mixture of NaCl, KCl, MgCl₂, and CaCl₂ (1.00, 0.55, 0.23, and 0.46%, respectively) was used in dry fermented sausage to replace NaCl alone [124]. Gimeno et al. [124] reported that these salt replacers decreased the micrococcaeae and nitrosoheme pigments. Ibanez et al. [125] reported that the replacement of NaCl by KCl in dry-cured sausages, using *L. plantarum* and *S. carnosus* as starter culture, led to a faster and more intensive nitrosation process than when NaCl was used alone. The chemical conversion of heme pigment percentages (nitrosopigments/total pigments × 100) were higher in the sausages made with NaCl + KCl during the whole ripening process. Also, the RI was higher, indicating that less oxidation of heme pigments occurred when this salt mixture was used.

To improve the health benefits of restructured meat products, new ingredients are now incorporated. Thus, Cofrades et al. [126] added walnuts, which provoked changes in the color coordinates (lightness decreased, whereas redness and yellowness increased). Koo et al. [127] added enoki mushroom; the redness and yellowness of a fish meat paste increased and the product was darker. When palm fats (palm oil and palm olein) were added to chicken frankfurters, the color significantly changed in all the coordinates [128].

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Chapter 18

Texture Analysis

Shai Barbut

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18.1 Background

Studying and evaluating the textural parameters of meat products is an essential part of being able to offer consumers high quality and consistent products. A whole muscle product, such as a beefsteak, is unacceptable if it is too tough to chew due to high levels of connective tissue. On the other hand, if the meat does not have enough connective tissue (e.g., turkey breast from heavy toms), it would be too mushy and could fall apart. Such textural parameters can be the result of numerous factors (e.g., breed and age of the animal, stress prior to slaughter, chilling, and cooking methods). Therefore, studying and understanding factors contributing to the texture of meat products is of great interest to breeders, farmers, the meat industry, and obviously the consumer. Overall, meat textural parameters can be measured by different tests, which usually are classified as large and small deformation tests. The large deformation tests, which are more

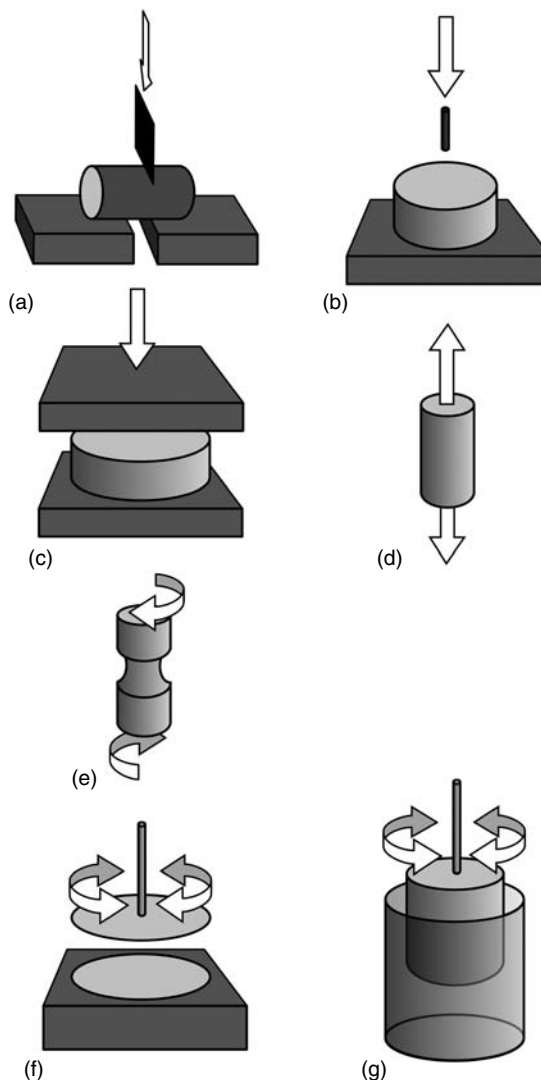


Figure 18.1 Different texture analysis tests: (a) shear; (b) penetration; (c) compression, which can be single or double for the texture-profile-analysis test; (d) tension; (e) torsion; (f) small oscillatory deformation between parallel plates; (g) similar to “f” but a bob and cup configuration. See text for further explanation. (Illustrated by O. Barbut, School of Engineering, University of Toronto, Canada.)

common, usually include shear, penetration, compression, tension, and torsion (Figure 18.1). The small deformation tests (i.e., nondestructive testing) are used more for research purposes and mainly include dynamic scanning rigidity monitoring, using a low-strain or a low-stress rheometer. The small deformation tests are mostly used for monitoring gelation processes (e.g., heating of meat proteins and cooling of gelatin) and interactions among different meat and nonmeat gelling components.

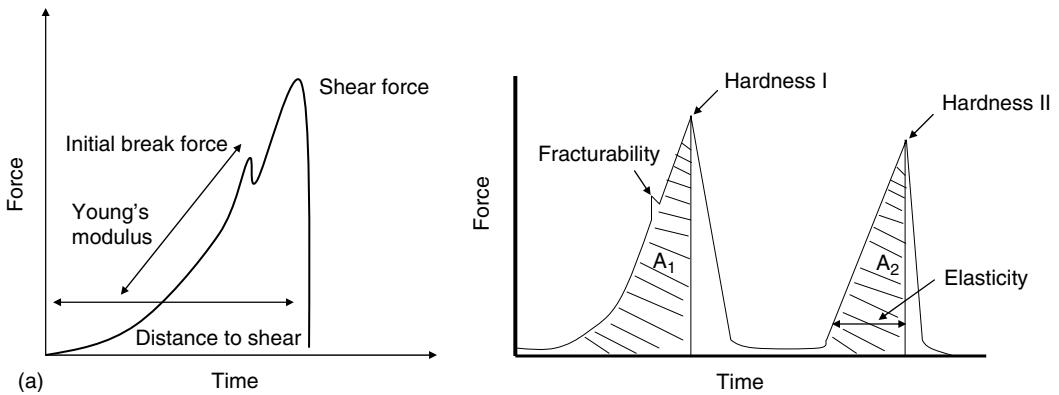


Figure 18.2 Generalized force deformation curve for a single compression test (a) and for a two cycle compression test also known as texture profile analysis (b). For texture profile analysis: cohesiveness = A_2/A_1 ; gumminess = hardness I \times cohesiveness; chewiness = gumminess \times elasticity. (Based on Bourne, M. C., *Food Technol.*, 32(7), 62, 1978.)

18.2 Shear Test

The shear test was one of the first developed to determine the toughness of raw/cooked whole muscle and/or meat products [1]. The most popular test is the Warner Bratzler Shear (WBS), named after its inventor [2]. Bratzler explained that, “It is of paramount importance that some method of measuring variation in tenderness be devised in order that the factors that cause this variation may be studied.” Overall, the procedure employs a single blade used to cut a core meat sample (Figure 18.1a). The result provides a measure of the force required to shear the sample (Figure 18.2a). Instrumental determination of texture, or tenderness, is usually evaluated on intact pieces or core samples, large enough to ensure representative sampling so that the treatment effect can be accurately measured. Various researchers have noted that factors such as sample size, location within the muscle, and orientation of the fiber relative to the shearing blade are critical to ensure reproducible results. Evaluating the same sample but positioning the muscle fiber perpendicular or parallel to the blade will result in different outcomes since it takes more force to shear the fibers positioned perpendicular to the blade; the standard WBS test calls for perpendicular positioning. Using a modified WBS shear apparatus (texture analyzer fitted with a flat Volodkewick shear blade), Hansen et al. [3] reported a significant transverse muscle variation in pork *longissimus dorsi* muscle, that is, increased shear force from dorsal to lateral sampling position. Sensory evaluation revealed a similar nonsignificant trend from dorsal to medial sampling position. Both WBS and sensory analysis showed that muscles from left and right sides of the carcass differed significantly. This was postulated to be caused by a greater amount of work performed by right muscles compared to left muscles. As the authors indicated, “in general, sensory and instrumental analyses were found largely to be predictive indexes of each other.” They also stressed that the significant variation between longitudinal locations should be taken into account when designing an experiment.

Another device used to evaluate food texture is the Allo-Kramer Shear (AKS), introduced in the 1950s. It has been adapted to meat texture and is routinely used by researchers and quality control personnel. The same considerations of size, location, and fiber orientation have been noted for the AKS as for the WBS. The AKS employs a cell consisting of 10–13 blades guided into a square

Table 18.1 Warner-Bratzler and Allo-Kramer Shear Values of Intact and Diced Cooked (80°C) Samples of Broiler Breasts Deboned at Three Postmortem (PM) Times as Well as Sensory Evaluations (Category Scale—Untrained Panel) of Cooked Diced Chicken

	Texture		Sensory		
	Warner-Bratzler ^a (intact) (kg)	Allo-Kramer ^b (20-g diced; kg/g)	Juiciness ^c	Tenderness ^c	Acceptability ^d
Deboning time (h PM)					
2	9.5 ± 3.9 ^e	5.2 ± 1.0 ^e	3.5 ± 1.3	2.5 ± 1.3 ^g	2.0 ± 0.9 ^g
6	4.7 ± 1.6 ^f	3.4 ± 0.8 ^f	3.4 ± 1.2	3.8 ± 1.2 ^f	2.6 ± 0.9 ^f
24	3.2 ± 0.9 ^g	2.2 ± 0.2 ^g	3.5 ± 1.3	5.1 ± 0.8 ^e	3.0 ± 0.9 ^e
Correlations (R values)					
With Warner-Bratzler			0.06	-0.90	-0.92
With Allo-Kramer			0.00	-0.99	-0.93

^a Bench-top Warner-Bratzler device was used to shear a 1.9-cm-wide intact strip.

^b Multiple bladed Allo-Kramer attached to an Instron was used to shear 20 g of diced sample.

^c Category scales: 1 = very dry, tough to 6 = very juicy, tender.

^d Category scales: 1 = poor to 5 = excellent.

^{e-g} Values ($\bar{x} \pm SE$) within a column with no common superscript differ significantly ($p \leq 0.05$). For texture, mean values are averages of 66 observations (22 birds \times 3 replications) for each deboning time. For sensory, 22 panelists \times 3 replications.

Source: Adapted from Lyon, B. G., Lyon C. E., *Poultry Sci.*, 75, 812, 1996.

box to shear the sample. Lyon and Lyon [4] compared the effect of broiler breast meat deboning time by using both the WBS and AKS methods; they correlated the results with a sensory panel. For the WBS, intact 1.9-cm-wide strips of cooked meat were evaluated. For the AKS, 20-g strip portions of 1 cm² (top size) were evaluated. Sensory characteristics were evaluated by category scales—untrained panel (results reported in Table 18.1) and descriptive analysis—trained panel (results not presented here).

The shear values of the intact (WBS) and diced (AKS) samples indicated significant differences due to deboning times (Table 18.1). Both shearing procedures were sensitive enough to discriminate differences among each of the three deboning times. As indicated by the authors, reduction in shear values as postmortem deboning time increases is well documented in the literature. The sensory panel reported the same trend, and results for tenderness were highly correlated with both instrumental methods (Table 18.1). Juiciness was not found to be affected by deboning time. The overall texture acceptability was significantly affected by deboning time and also showed high correlations with both instrumental methods.

A typical force deformation curve for the shear test is shown in Figure 18.2a. Besides the peak shear force, other parameters such as work required to shear (area under the curve), initial fracture force, and the Young's modulus (slope of the curve) can be obtained. It should be pointed out that although the WBS is the most frequently cited texture analysis test used by meat researchers, variations in test procedure (sample preparation, dimensions, end point cooking temperature, cross head speed, blade dimension) are fairly common among research groups [1]. Some call their procedure a modified WBS, while others give it a more specific name such as the slice-shear force [5]; the latter has been used fairly extensively by the group cited as well as by others. Overall, it

appears that developing a universal procedure can benefit us all and make it easier (or at least possible) to compare results among different research groups. However, as concluded by Janz and Aalhus [1], “at the moment there is no reason to discount the use of specialized procedure, provided methodology is reported in full” (i.e., they also recommended moving toward a standardized test).

18.3 Penetration

In this test, a probe descends into the product at a constant speed (Figure 18.1b), with the force required to rupture the sample being recorded. A variety of different probes have been used, including flat and rounded tips with different diameters. The test can be used to determine the resistance to puncture in products such as cooked whole muscle, ground and comminuted meat products, or fine gels (e.g., Jello). The results are commonly used to compare relative toughness/hardness (see also the puff test discussed in Section 18.8). This is an easy test to perform, and some companies use it on a routine basis as a rapid quality control test. The gelatin industry, for example, employs the test to standardize gelatin strength, also known as “bloom.” Another area where this test can be useful is in monitoring changes in a raw meat batter during cooking. The test is applicable for this purpose because the researcher starts with a paste-like, raw meat batter (which cannot be subjected to a shear test); later, this stiffens during the cooking process. An example of results obtained for a poultry meat batter prepared with 2.5% salt, showed penetration force (using a 9 mm diameter flat tip probe attached to a texture analyzer) rising from 30 to 43, 60, 190, 355, and 475 N as temperature was increased from 20 to 40, 50, 55, 60, and 70°C, respectively [6]. The transition from a viscous to a more rigid sample was clearly seen at the point where myofibrillar protein started to gel, at about 50–55°C. The amount of extractable protein was also used to evaluate the quantity of soluble proteins going into structure building (i.e., gelling). As temperature was raised, the amount decreased from 1.6 to 1.5, 1.4, 1.2, 1.0, and 0.4 mg/mL at 20, 40, 50, 55, 60, and 70°C, respectively. The changes could also be followed under a microscope as the protein strands became thicker and the number of connections among them increased as temperature was raised.

18.4 Single Compression and Texture Profile Analyses

In this configuration, a sample is compressed axially between two flat plates (Figure 18.1c). The test can be performed as a single compression or double compression. The single-compression test can be done to failure, meaning that the sample is compressed until it totally breaks. Alternately, the test can be done to a predetermined point where the deformation is measured. Two extreme food examples would be a hard candy and a marshmallow. In the first case, when force is slowly applied, the sample will hardly deform; however, at a certain point, it will shatter. In the second case, a relatively low force will quickly deform the sample, but the sample can easily recover (highly elastic sample). Meat samples fall in between these two extremes and show moderate elasticity. Voise et al. [7] reported that in a single compression test, the force required to produce failure in a wiener-type product they studied was strongly correlated ($R = 0.89$) with sensory chewiness.

A two-cycle compression test, known in the food industry as texture profile analysis (TPA), was developed by a group of scientists at General Foods in the early 1960s. A cylindrical sample is compressed to a certain predetermined deformation during the first cycle, pressure is released,

and then the sample compressed a second time [8]. The General Foods group has established some very useful parameters that correlate well with sensory data (Figure 18.2b). The test is commonly used by food scientists for various products. However, over the years, different test parameters have been used by various researchers, which has made it difficult, if not impossible in some cases, to compare results from different laboratories. Mittal et al. [9] reviewed test parameters reported in the literature to evaluate meat samples, and indicated that meat specimen length or height (L) varied from 10 to 20 mm, diameter (D) from 13 to 73 mm, and D/L ratio from 1 to 4. The compression ratio varied from 50 to 85%, and compression speed from 5 to 200 mm/min. The effects of varying D/L , speed, and compression rate are shown in Table 18.2. The results are for a commercial-type frankfurter made of beef meat (55.9% water, 28.5% fat, 12.6% protein, and 2.9% ash). The authors have also tested a whole muscle product (corned beef) and a ground meat (salami) product; results are not included here. The data show that a decrease in D/L resulted in a lower hardness I and II, cohesiveness, and gumminess, while springiness and chewiness increased. Increasing the compression ratio resulted in decreased springiness, cohesiveness, gumminess, and chewiness. According to Peleg [10], at the same deformation rate, a shorter specimen is actually deformed at a higher strain rate, and therefore should exhibit higher stress than those of a longer specimen and the same strain. Thus, TPA

Table 18.2 Duncan's Test Results for Different Texture Profile Analysis Parameters of Frankfurters

	$H1$ (N/cm ²)	$H2$ (N/cm ²)	E (m/cm ²)	COH	GUM (N/cm ²)	$CHEW$ (J/cm ⁴)
Mean Values						
D/L	30.11 ^a	23.47 ^a	0.024 ^c	0.405 ^a	11.76 ^a	0.33 ^c
2.0	27.39 ^b	20.40 ^b	0.047 ^b	0.388 ^a	10.25 ^b	0.56 ^b
1.5	24.14 ^c	14.52 ^c	0.084 ^a	0.338 ^b	7.69 ^c	0.77 ^a
1.0						
Speed (cm/min)						
2.0	29.51 ^a	20.64 ^a	0.053 ^a	0.369 ^a	10.41 ^a	0.58 ^a
1.0	26.81 ^b	20.16 ^a	0.052 ^a	0.369 ^a	10.27 ^a	0.57 ^a
0.5	25.32 ^c	17.59 ^b	0.051 ^a	0.366 ^a	9.02 ^b	0.50 ^a
Compression (%)						
25	22.72 ^c	20.52 ^a	0.070 ^a	0.686 ^a	15.58 ^a	1.05 ^a
50	34.41 ^a	18.51 ^b	0.055 ^b	0.299 ^b	10.54 ^b	0.50 ^b
75	24.50 ^b	19.36 ^{ab}	0.031 ^c	0.147 ^c	3.59 ^c	0.11 ^c
Correlation Coefficients						
D/L	0.38 ^d	0.63 ^d	-0.78 ^d	0.11	0.29 ^d	-0.37 ^d
Speed	0.27	0.19	0.02	-0.01	0.09	0.06
Compression	0.11	-0.08	-0.52 ^d	-0.92 ^d	-0.85 ^d	-0.78 ^d

^{a-c} Data with the same superscript letter in a column, within a category, are not significantly different at $p > 0.05$ level.

^d $p \leq 0.0001$.

Note: $H1$ = hardness-1; $H2$ = hardness-2; E = springiness; COH = cohesiveness; GUM = gumminess; $CHEW$ = chewiness; D/L = diameter to length ration.

Source: Adapted from Mittal, G. S., Nadulski, R., Barbut, S., Negi, S. C., *Food Res. Intern.*, 25, 411, 1992.

parameters are comparable when the tests are performed by a standard procedure. From the results reported in Table 18.2 and results obtained for the salami and corned beef, Mittal et al. [9] recommended the use of the following test parameters: $D/L = 1.5$, compression ratio = 75%, and rate of compression of 1–2 cm/min. As with the WBS test previously discussed, employing such standard conditions should be beneficial in allowing for a meaningful comparison of results among different laboratories.

Several researchers have compared the use of TPA to other methods such as the WBS test. Caine et al. [11], for example, have indicated that TPA explained more of the variation in subjective sensory tenderness of beef rib steaks they evaluated compared to the WBS test. However, this is not necessarily a universal finding.

18.5 Tension

The test is done by pulling apart a meat sample (Figure 18.1d), and is done to determine the strength of products such as sliced cooked meats. If a whole muscle product is tested, the orientation of the muscle fibers is, again, very important because muscle fibers pulled at 90° to their longitudinal axis will require lower force than fibers pulled along their longitudinal axis.

Results obtained for commercial whole muscle turkey breast meat products showing good slice integrity, with an average value of 2.5 N, while those from a product that showed poor sliceability had an average value of 1.1 N (sample size: 150 mm [long] \times 20 [wide] \times 3 [thick], pulled apart at 50 mm/min). The samples were evaluated after a meat processor got a product complaint indicating a problem obtaining thin slicing (also called shaving). Under the microscope, the poor integrity areas revealed poor connective tissue binding among the individual muscle fibers.

18.6 Torsion

The torsion test is usually performed by twisting a sample that has been carved into a dumbbell shape (Figure 18.1e). The controlled rotation can be done with a viscometer after the sample ends have been glued to plastic disks. The force required to break the sample is measured and is used to calculate true shear strain and shear stress values. The advantage of using this test is that volume changes are minimized and the squeezing out of water prior to the breakpoint (which typically occurs in a compression test) is avoided. The test has been shown to reveal differences in muscle protein gelation and functionality (Table 18.3). The table indicates that shear strain is a measure of protein functionality (i.e., the ability of the salt-soluble proteins to form a heat-induced

Table 18.3 Degree of Influence of Different Factors on Failure Stress and Strain of Processed Muscle Foods

<i>Factor</i>	<i>Shear Stress</i>	<i>Shear Strain</i>
Protein functionality	Strong	Strong
Protein concentration	Strong	Weak
Filler ingredients	Strong	Weak
Thermal process	Strong–moderate	Weak

Source: Data from Hamann, D. D., *Food Technol.*, 42(6), 66, 1988.

cohesive gel). Shear stress is also dependent on protein functionality, but it is strongly influenced by other factors as well. Overall, shear stress relates to sensory hardness and shear strain to sensory cohesiveness. Hamann [12] described the relationships between shear strain and shear stress in terms familiar to a customer evaluating a food sample: A product with low shear strain and shear stress = mushy; high shear strain and low shear stress = rubbery; high shear stress and low shear strain = brittle; high shear strain and stress = tough. An important implication of the data presented in Table 18.3 is that it is more difficult to modify sensory cohesiveness by adding different ingredients than it is to modify sensory hardness of meat gels. Montejano et al. [13] compared torsion and TPA results with sensory notes (springiness, hardness, cohesiveness, denseness, chewiness, gel persistence). Overall, these two instrumental parameters correlated strongly ($R = 0.83$), as did the six sensory notes. Stress and TPA hardness correlated strongly ($R = 0.94$) but did not correlate as strongly with the sensory notes as did the parameters based on deformation to failure.

18.7 Small Deformation

This test is used to monitor a process such as meat protein progressive gelation during heating; it involves a scanning rigidity evaluation, applying low strain or stress to a sample (Figures 18.1f and 18.1g). Several studies have shown that the information is valuable in monitoring physical changes in meat proteins and possible interactions with other nonmeat ingredients (e.g., soy proteins and hydrocolloids gums) during the gelation process. These interactions can be related to molecular changes [12]. It should be pointed out that several studies have indicated that results are well correlated with sensory texture and the penetration test, while others have indicated that results are not necessarily related to the shear test and TPA. The reason for the latter is that changes in rigidity provide circumstantial evidence of changes taking place during gelation (structure-building phase). These changes are associated with protein unfolding, bonding of molecules, and interactions with other nonmeat components such as soy proteins and gums.

One of the first laboratory devices employed a microscope coverslip glass submerged in a meat protein solution. The coverslip was moved up and down, at a very slow rate and over a short distance, while the sample was heated. The resistance to movement was recorded and plotted against temperature to study the effect of actin to myosin ratio on the gel formed during heating [12]. The data provided valuable information on gelation temperatures, effects of the myosin:actin ratio, and the magnitude of protein–protein interactions during the actual gel forming stages. Today, various commercial computer-controlled stress/strain rheometers are available in the market that provide very precise motion and temperature control, as well as easy calculations. An example of results obtained with a commercial controlled stress rheometer is shown in Figure 18.3. Kerry et al. [14] used a rheometer in the oscillatory mode to show that adding modified starch or pectin significantly increased storage modulus (G') compared to a control. The results demonstrate a synergistic effect during the gelation of the meat protein system at $\geq 55^\circ\text{C}$. Sodium alginate, on the other hand, caused a disruption in the meat protein matrix formation. Upon cooling, further texture building by the starch and pectin was observed as the two polysaccharides formed more hydrogen bonds.

18.8 Other Noncontact Methods

Over the past few years there has been a drive to develop rapid methods for fast online evaluations. The area of greatest interest is tenderness; several methods, mainly based on light reflectance and response to applied pressure, have been or are under development for commercial use. For an

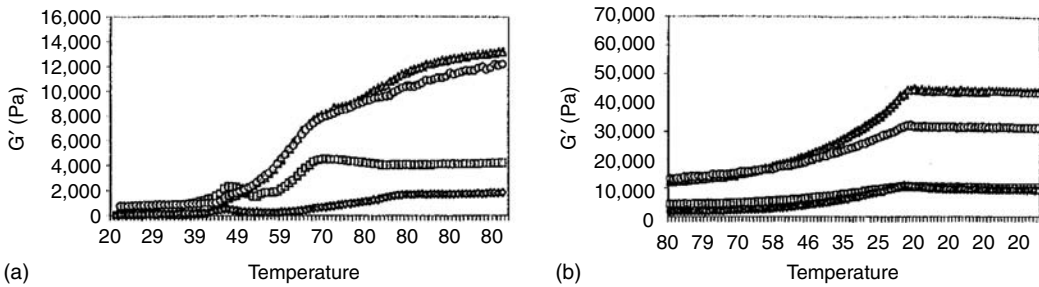


Figure 18.3 Plots of storage modulus G' (Pa) values as a function of increasing (a) and then decreasing (b) temperature (1°C per min) of meat exudates prepared with whey protein (control), and treatments with 2% modified potato starch (Δ), 2% low-methoxy pectin (\circ), and 0.5% sodium alginate (\diamond). (From Kerry, J., Morrissey, P., Buckley, D., *J. Sci. Food Agri.*, 79, 1260–1266, 1999. With permission.)

overall reference explaining the basic principles of most such methods, the reader is referred to Swatland [15]. The goal of such methods is to directly or indirectly predict the texture of meat and other food products. Two examples are an optical sensor and an applied stress method. Shackelford et al. [16] reported an online application of visible and near infrared spectroscopy to U.S. Select beef carcasses during grading, using shear force and trained sensory panel tenderness ratings as their basic reference methods. They indicated that “the technology might be useful for identifying U.S. Select carcasses that excel in *longissimus* tenderness.” Using an applied stress in the so-called puff test is based on a controlled puff of air (applied from a nozzle) that impinges on a surface. The deformation at the center of this small indentation is measured with a laser displacement sensor [17]. Fresh meat and meat products are some of the items being tested, but currently there is no commercial puff equipment available on the market. This will probably change as the demand for fast, online individual carcass grading/evaluation is growing.

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Chapter 19

Flavor of Meat Products

Mónica Flores

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19.1 Introduction

Meat products constitute a wide variety of products in terms of sensory characteristics. Their diversity of colors, flavors, and textures is due to different traditional practices. There are many different meat products, but in general, meat products are cured by the addition of sodium nitrite and salt along with other additives, such as sugars, certain reducing agents, and appropriate seasonings that impart specific properties to the final product. These cured meat products can be grouped in two major groups—dry and wet curing [1]. During dry curing, the meat is mixed or rubbed on the surface with a dry cure containing salt plus nitrate or nitrite and without any added water, followed by a drying stage. The most representative products of the dry curing process are dry-cured

ham and dry-fermented sausages. In the wet curing process, a pickle injection or brine solution is used as the vehicle for cure penetration into the meat, and afterwards the meat is cooked to obtain the final sensory characteristics [1].

Some of the most well-known dry-cured meat products are those manufactured using pork meat such as dry-cured hams—Parma and San Daniele hams (Italy), Serrano and Iberian hams (Spain), Bayonne ham (France), and Jinhua ham (China). Other dry-cured meat products are processed from beef meat, as in the case of jerked beef (Brazil) and pastirma (Turkey). However, many different dry-fermented products are manufactured around the world; some of them are known as salami (Italy), saussicon (France), salchichon, and chorizo (Spain). The most representative products obtained from wet curing processes are bacon, roast beef, frankfurters, and cooked ham.

Flavor is one of the most important factors contributing to the quality of meat products. Raw meat possesses little odor and only a mild serum-like taste that has been described as salty, metallic, and bloody with a sweet aroma [2]. The flavor of meat products depends on composition and processing conditions; it can also be modified by the use of spices and condiments. Moreover, thousands of volatile compounds have been identified in meat products, but the exact contribution of each compound to flavor is not yet known.

19.1.1 Flavor Precursors

The constituents of raw pork meat act as important taste compounds, flavor enhancers, and aroma precursors. These constituents are essentially proteins, amino acids, nucleotides, sugars, lipids, vitamins, and other compounds. During meat processing, these constituents undergo degradation reactions, forming hundreds of volatile compounds with the characteristic pork flavor [2]. Generally, pork flavor has not been attributed to a single chemical compound, although it is frequently accepted that meaty notes are produced by sulfur-containing compounds generated from water-soluble precursors in lean meat. However, fat and fat-soluble substances contribute to flavor differences among species [3].

The main reactions involved in the development of flavor in meat products are lipid degradation, thiamine degradation, Strecker degradation, degradation of ribonucleotides, microbial amino acid degradation, carbohydrate degradation (heating or fermentation), and Maillard reactions [4].

19.1.2 Flavor Analysis

In the study of the flavor of meat products it is necessary to obtain a sufficient amount of volatile compounds. The sample used should be a good representative of the product with a good aroma and without off-flavors or artifacts.

Sample preparation will depend on the technique used for volatile extraction due to the number of factors that affect it. This is the case for the concentration levels of aroma compounds that generally are low and for the matrix effect, because the complex matrices contain lipids, proteins, and carbohydrates that complicate the isolation process. Other factors affecting the extraction are the complexity of food aroma composed of a large number of different chemical compounds with different volatility and, finally, the instability of aroma components [5].

The analysis of volatile compounds is generally accomplished by an extraction step, followed by concentration, chromatographic separation, and subsequent detection. Thermally labile compounds may decompose in the heated zones of the instruments, producing a chromatographic profile that it is not truly representative of the sample.

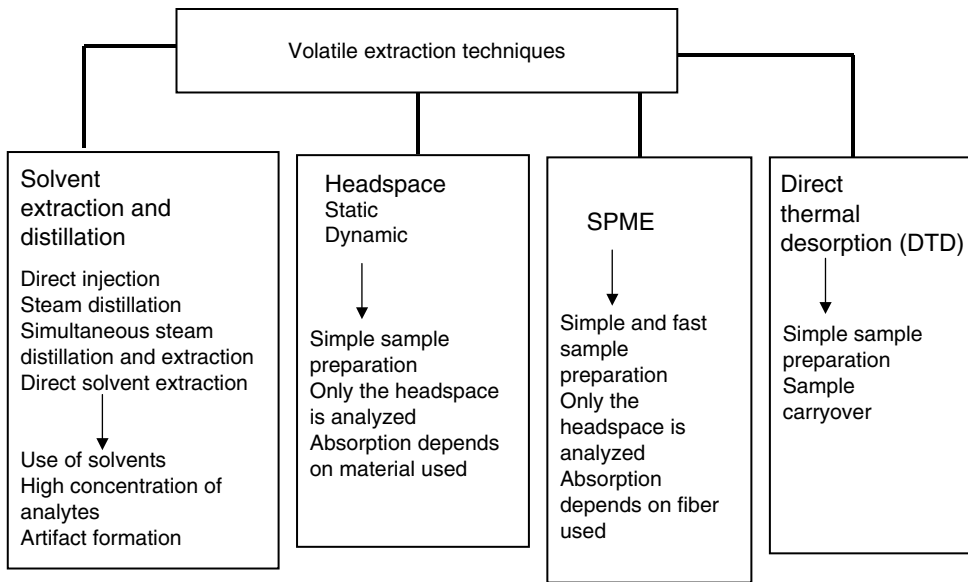


Figure 19.1 Extraction techniques of volatile compounds.

The techniques used for analyzing food aroma have been widely described by Marsili [6] and Cadwallader and MacLeod [7]. Figure 19.1 summarizes the extraction methods, including the main advantages and disadvantages. Generally, the most common techniques are solvent extraction and headspace analysis. Distillation and solvent extraction techniques are critical to assess the organoleptic quality of the isolate. This evaluation is made to ensure that decomposition and loss of desirable components have not occurred.

The headspace techniques do not use solvents to remove the analytes from the sample matrix, avoiding the presence of the solvent peak in the chromatographic elution. This fact could be of special interest when the important analytes are of low boiling points [8].

Currently, solid-phase micro extraction (SPME) is becoming a suitable technique to analyze food aroma. In this method, the analytes are adsorbed onto a coating and an exhaustive extraction is not necessary; ultimately, an equilibrium is reached between the matrix and the stationary phase coating the fiber [9].

Direct thermal desorption consists of sparging the volatiles from the sample matrix and transferring them directly onto the head of the chromatographic column. This technique allows the qualitative analysis of volatile compounds without sample preparation [10].

In past years, flavor research seems to have been directed not only to the identification of compounds but also to characterizing their components based on their organoleptic importance; one of the major problems in aroma research is to select those compounds that significantly contribute to aroma. Different techniques have been used to distinguish the most potent odorants. In the beginning, the techniques used required the identification and quantification of a great number of volatile compounds and the determination of their threshold values, calculating what was known as “aroma value” [11]. To select the aroma-active components from a complex mixture, several techniques using gas chromatography with olfactometry (GCO) detection have been described (Figure 19.2).

In GCO analyses, the effluent of a GC column is analyzed by human subjects describing the eluted compounds. However, the description process is not an easy task, because several

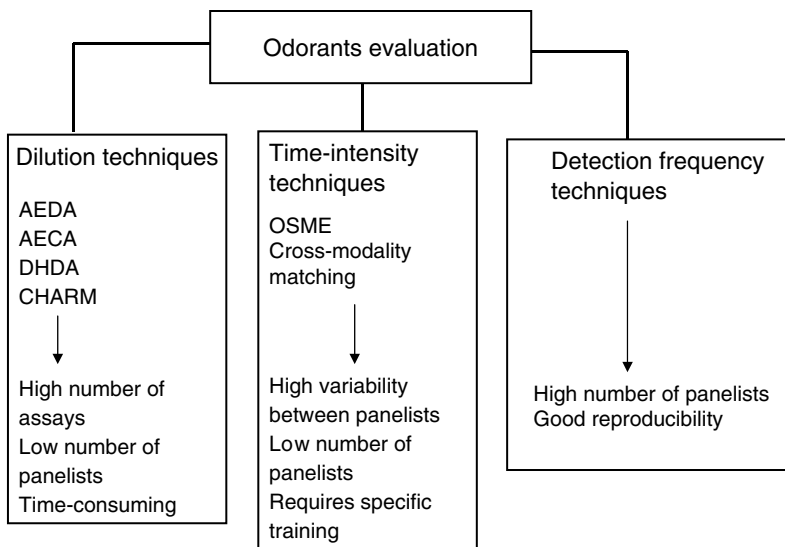


Figure 19.2 Techniques to evaluate odorants. AEDA, aroma extract dilution analysis; AECA, aroma extract concentration analysis; DHDA, dynamic headspace dilution analysis; CHARM, combined hedonic aroma response; *osme*, Greek word meaning smell.

compounds exhibit different odor qualities at different concentrations. In addition, panelists analyzing the effluent can use different terms to describe the same odor and can suffer fatigue, sensory saturation, and adaptation.

The different olfactometry techniques developed (Figure 19.2) are dilution, time intensity, and frequency techniques. In dilution techniques, the key odorants are ranked in order of potency by sniffing a food extract and its successive dilutions. The dilution factor (DF) value is the last dilution at which an odor compound is detected; this technique is known as aroma extract dilution analysis (AEDA) [12,13]. Several modifications of this technique have been proposed [14–17].

In time-intensity techniques, the panelist directly records the intensity of the compound perceived, using a computerized scale. The main problem with this technique is that it requires a well-trained panelist [18,19]. On the other hand, the detection frequency method uses a number of panelists detecting an odor in the effluent as a measure of the intensity of a compound [20,21].

19.2 Flavor of Cured Meat Products

The study of the flavor of meat products has been done by the identification of volatile compounds and their different chemical structures. Many studies have been done on the flavor of pork and beef meat [22]. In addition, the flavor of cured meat products has also been studied [23], but more attention needs to be paid to the different processing techniques, which affect the flavor formation on cured meat products. In general, there is no clear path that indicates the compounds responsible for the cured meat flavor notes [23]. However, the concentration of carbonyl compounds was higher in uncured pork than in cured meat [24]. Therefore, in this chapter the studies of flavor in each product are presented individually to determine the effects of the different processing technologies.

19.2.1 Bacon

Bacon is manufactured using pork bellies pumped with a curing pickle containing salt, sugar, sodium nitrite, and sodium erythorbate. The pumped bellies are vacuum tumbled, smoked, and then heat processed. Afterwards, the bellies are chilled, sliced, and packaged.

The role of nitrite in cured meat flavor has been widely reviewed [25] because it was questioned due to its involvement in the formation of *N*-nitroso compounds in cured meats. Moreover, bacon is a widely consumed meat product and its acceptability is highly dependent on its flavor. Generally, the flavor volatile compounds have been extracted by distillation [26,27] or by dynamic headspace analysis [28,29]. Hundreds of volatile compounds have been identified in bacon, although the number of compounds extracted depends on the cooking process. Thus, a higher number of compounds (around 150) have been extracted from fried bacon [26,30], 80 from cooked bacon [28,29], and around 40 from bacon without a previous cooking [27]. The chemical classes isolated are indicated in Table 19.1.

Mottram [28] reported qualitative differences between cured and uncured bacon, such as the presence of several unsaturated and aromatic aldehydes in the nitrite free sample and the presence of benzonitrile, phenylacetone, and alkyl nitrates in the cured bacon samples. Later, Mottram et al. [29] reported that the possible origin of these nitrogen compounds was the reaction between fatty acids in the lipids and sodium nitrite. In addition, the aroma of alkyl nitrates and alkyl nitriles was similar to their corresponding aliphatic aldehydes, and their odor thresholds were relatively high; therefore, they concluded that their contribution to the aroma of cured meats was unimportant.

Ho et al. [26] indicated the impact of each chemical class on bacon flavor; hydrocarbons, alcohols, and carbonyl compounds are probably not primary contributors to the flavor of bacon. Also, a high number of pyrazines were identified, contributing to a roasted nut sensory perception, whereas furans contributed to a sweet, nutty, and caramel-like odor impression. The thiazols

Table 19.1 Chemical Compounds Isolated in Bacon

Chemical Compound	Number of Identified Compounds				
	Ho et al. [26]	Timón et al. [30]	Ai-Nog and Bao-Guo [27]	Mottram et al. [29]	Ho et al. [26] (from smoke)
Hydrocarbons	18	60	9	25	2
Alcohols	16	19	1	12	1
Ketones	11	14	1	9	5
Aldehydes	8	24	1	15	3
Ethers	3				
Esters	7		7		
Acids	4		5		4
Phenols	10		12		8
Pyrazines	22	5		4	4
Furans	12	7		3	6
Thiazoles	3	5		2	
Oxazoles	3				
Pyrroles	6	1			1
Pyridines	4	4		2	
Alkyl nitrates		4		6	

Table 19.2 Main Aroma Compounds Detected in Bacon by GCO

<i>Chemical Group</i>	<i>Chemical Compound</i>	<i>Aroma by GCO</i>
Aldehydes	Pentanal	Rancid, grassy
	2-Pentenal	Rancid
	Hexanal	Rancid, grassy, oily
	Heptanal	Fruity
	2-Heptenal	Oily
	Octanal	Lemon
	2-Octenal	Rancid, biscuit
	Nonanal	Fruity, fatty
	2-Nonenal	Cooked meat, grassy
	Decanal	Chemical, sweet
	2,4-Nonadienal	Meaty
	2-Decenal	Oily, meaty, rotten
Pyridines	Pyridine	Burnt, rubber
Pyrazine	Methylpyrazine	Meaty
Furan	2-Methyl-(3-methylthio)furan	Meaty
Alcohols	3-Hexen-1-ol	Sweet
	1-Octen-3-ol	Mushroom
Ketones	2-Heptanona	Chemical, bitter

Source: Adapted from Timon, M.L., Carrapiso, A.I., Jurado, A., van de Lagemaat, J., *J. Sci. Food Agr.*, 84, 825–831, 2004.

and oxazoles detected in bacon produced a green, nutty, and vegetable-like aroma. However, a high number of phenols were identified, and it is clear that these compounds are derived from the smoke flavor, as indicated in Table 19.1; many of these have been reported in wood smoke vapor.

However, until now there has been only one olfactometry study available on bacon [30]; the main odorants identified are reported in Table 19.2. This study used only one assessor, who described the odors and repeated the analyses several times. In this case, the aldehydes contributed their typical odor notes such as oily, grassy, and rancid, although the meaty characteristic was imparted by pyrazine and furan compounds.

Recent studies on bacon flavor have been focused on those factors that affect the sensory characteristics. This is the case with the study of the effect of pork belly thickness on bacon [31] and the addition of *n*-3 polyunsaturated fatty acids on bacon quality [32].

19.2.2 Frankfurter

This comminuted product is a complex food system formed by an oil-in-water emulsion. The standard process consists of the preparation of a batter that mostly contains meat (beef, pork, others) and fat, with water, salt, sodium nitrite, sodium ascorbate, polyphosphates, thickeners, and a spice mix. All the ingredients are mixed in a vacuum cutter in different steps to obtain the maximum yield. Once fully mixed, the batter is stuffed, cooked, and, in some cases, smoked.

Many volatile compounds have been identified in frankfurters; for the most part, they have been extracted using headspace techniques, such as purge and trap using Tenax as the adsorbent [33–35] and SPME [36]. The volatile compounds isolated were derived mainly from meat ingredients such as aldehydes, alcohols, ketones, alkanes, and aromatic hydrocarbon, whereas many other compounds were derived from smoke and spices, as the case of phenolic compounds, terpenes,

and several sulfur compounds. Few reports have studied the odor activity of the isolated volatile compounds [33]. Odor assessment by olfactometry was done by four assessors who described the odor eluted from the column. They indicated that the compounds derived from the smoke, such as phenols, produced an important contribution to the aroma, whereas the meaty, roasted, and grilled notes were produced by sulfur-containing compounds. Finally, they found the various sulfur compounds can be generated during the cooking process [33].

19.2.3 Cooked Ham

Cooked ham is produced using the pork leg cleaned of skin, bones, connective and adipose tissues, and zones not attractive to consumers. The hams are injected with a brine containing salt, sugar, nitrite or nitrate, ascorbate or erythorbate, aromas, and in some cases polyphosphates and other authorized additives. Once injected, the hams are tumbled, vacuum packaged, and cooked to stabilize the product and to develop the sensory characteristics, coagulated texture, and flavor.

Several reports have studied the volatile composition of cooked ham by simultaneous steam distillation-extraction [37,38] and vacuum distillation [39]; however, only Guillard et al. [39] determined the odor active compounds in cooked ham. Many of the volatile compounds isolated from this product are derived from smoke and seasonings, whereas others come from reactions occurring in the meat. In general, the identified compounds were aldehydes, alcohols, ketones, furans, sulfur compounds, esters, and phenols derived from the smoke, as indicated earlier for bacon. However, the main odorants identified in cooked ham by olfactometry are sulfur compounds such as methional, dimethyl disulfide, and allyl isothiocyanate; acids such as 3-methylbutanoic acid; and compounds derived from spices, such as terpenes. These compounds were identified by three assessors who repeated the analysis twice. In summary, the technique used in olfactometry analysis was only the source for description of the aroma and the number of times it was detected; in general, this corresponds to the technique called detection frequency [20].

19.2.4 Roast Beef

Only a reduced number of beef cuts are manufactured into meat products in comparison to pork or chicken because of the high cost of beef [40]. The roast beef process consists of injecting or immersing the roast cuts in a brine containing salt, sugar, polyphosphates, and occasionally other additives such as sodium nitrite, garlic, and others. After injection the roast cuts can be tumbled, vacuum packaged, and cooked. The flavor characteristics are developed during the cooking process, but the final flavor also depends on the additives added in the brine. There are few reports about the flavor of cured beef; only Ramarathnam et al. [23] studied the difference in volatile compound composition of cured and uncured beef, reporting the presence of 31 hydrocarbons, 26 carbonyls, 3 alcohols, and 2 acids. They reported a lower carbonyl compound concentration in the cured beef, as was also detected in cured pork meat.

19.3 Flavor of Dry-Cured Meat Products

19.3.1 Dry-Cured Ham

The dry-cured ham process is generally very long and significantly affects the final quality of the product. The process consists of different stages: salting, in which the salt containing nitrite

and nitrate is rubbed onto the surface and hams are maintained at refrigeration temperatures; postsalting, in which the hams are cleaned of the salt and kept at refrigeration temperatures for salt equalization or distribution; and finally, drying/ripening, in which the temperature of the chambers is progressively increased, while the relative humidity is decreased to dry the hams and develop the flavor.

Approximately 240 volatile compounds have been identified in dry-cured hams [41]; however, the use of different extraction techniques has shown differences among the studies. Several authors have employed dynamic headspace analysis for the extraction of volatile compounds [42–48], whereas others have used vacuum distillation [49,50]. A higher number of carboxylic acids, lactones, and aliphatic hydrocarbons were extracted when vacuum distillation was used than in extraction by dynamic headspace. Most recently, the use of the SPME technique has allowed the extraction of a high number of volatile compounds from dry-cured ham [51–53].

Only a few studies have determined the odor impact of the volatile compounds detected in dry-cured ham [41,47,48,54]. The number of volatile compounds from each chemical class identified in dry-cured ham is reported in Table 19.3. Table 19.3 also includes the volatile compounds that showed an odor property. It is observed that although a high number of hydrocarbons were identified in dry-cured ham, they do not contribute to any odor character, whereas the contribution of aldehydes, alcohols, ketones, and so on was important. The most important group that produces meaty notes are the sulfur compounds; however, there is no specific compound that possesses the characteristic cured aroma.

The olfactometry techniques used for identification of aroma compounds are the description of the aroma by several assessors [41] and the detection frequency method [47,48,54], in which the intensity of the aroma is defined as the number of times that an odor is detected by the assessors. In these analyses, several sulfur compounds (methanethiol and 2-methyl-3-furanthiol) showed the highest detection frequency values together with several aldehydes (3-methyl-butanal and hexanal). Also, many other volatile compounds contributed to the odor of the dry-cured ham, as shown in Table 19.3.

19.3.2 Dry-Fermented Sausages

Dry-fermented sausages are manufactured by mixing minced meat and additives (salt, sugar, nitrite and nitrate, ascorbate, spices, and others) and then stuffing them into casings. Then the sausages are allowed to ferment and dry to develop their organoleptic characteristics.

Many volatile compounds have been identified in dry-fermented sausages using different extraction techniques. Dynamic headspace analysis has been used many times, yielding a large number of volatile compounds [55–64]. In addition, distillation techniques have been used [65–68], giving similar results, but this technique allows the extraction of a higher number of high-boiling and sulfur compounds than do headspace techniques. In recent years, the use of SPME has been applied and many volatile compounds were extracted; however, the nature of the volatile compounds extracted depended on the fiber used [69–72].

The contribution of volatile compounds to the aroma of dry-fermented sausages has been studied using olfactometry techniques. The first studies on dry-fermented aroma used the description of the aroma eluted from the gas chromatograph [57,64]. Then other studies used the AEDA technique to select the flavor DF of each odor compound [65–68]. Authors reported that the greatest contribution to aroma was due to sulfur compounds derived from spices such as garlic, but also to acids, 3-methyl-butanoic and acetic acids, methional, and 2-acetylpyrroline.

Table 19.3 Chemical Compounds Isolated from Dry-Cured Ham and the Main Odorants Reported by GCO

<i>Group of Compounds</i>	<i>Number of Compounds^b</i>	<i>Compounds</i>	<i>Odor^a</i>
Aldehydes	48	2-methyl-propanal	Fruity, pungent
		3-methyl-butanal	Fruity, almond like
		Hexanal	Green
		(E)-2-hexenal	Fruity, green
		(Z)-3-hexenal	Fruity
		(E)-2-nonenal	Fatty, leather-like
		Alcohols	41
		1-Pente-3-ol	Onion, toasted
Hydrocarbons	48	—	—
Sulfur compounds	7	2-Methyl-3-furanthiol	Cured-ham like
		Methanethiol	Toasted
		Hydrogen sulfide	Rotten eggs, meat
		Methional	Boiled eggs, sewage
		Dimethyl disulfide	Dirty socks
Ketones	24	2-Heptanone	Nutty
		1-Penten-3-one	Rotten, fruity
		2,3-Butanedione	Buttery
		3-Hydroxy-2-butanone	Fruit red jello
		2-Hexanone	Floral, apple
Esters	38	Ethyl-2-methylbutanoate	Fruity, apple like
		Methyl butanoato	Sweet caramel
Pyrazines	4	Methylpyrazine	Nutty
		2,6-Dimethyl-pyrazine	Toasted nuts
Lactones	7		
Acids	18	Acetic acid	Vinegar
Furans	5	2,5-Dimethyl furan	Sulfury, fishy
		2-Pentyl furan	Ham-like
Pyrrol	2	2-Acetyl-1-pyrroline	Roasty, popcorn
		Pyrrol	Meaty

^a Odor detected by assessors in Refs 46–48, 54.

^b Number of compounds identified in Refs 46–48, 54.

Recent studies on the aroma of dry-fermented sausages showed the contribution of a large number of different chemical compounds (Table 19.4), alcohols, esters, acids, aldehydes, etc. [73]. The contribution of each volatile compound to the aroma of the dry-fermented sausage was determined by the detection frequency method (Figure 19.3). In this study the authors reported that the high concentration of compounds such as 1-octen-3-ol, hexanal, and heptanal would be important contributors to the aroma because their concentrations were above their detection thresholds.

In summary, the content of volatile compounds in dry-fermented sausages depends on the process due to the considerable differences among northern and southern European cured products [4]. Therefore, the contribution of each volatile compound to the aroma of the food product should be carefully evaluated.

Table 19.4 Odor-Active Compounds Identified in the Headspace of Fermented Sausages

Number ^a	GCO		N ^a	GCO	
	Descriptor	Compounds		Descriptor	Compounds
1	Acetone, alcohol	Acetone	29	Meat broth, rancid, savory snack	Methional (3-methylthio-propanal)
2	Rotten eggs, cauliflower	Methanethiol	30	Rotten, strawberry, sweet	Unknown (2)
3	Bread dough, yeast	Ethanol	31	Onions, savory, rancid	2-Pentylfuran
4	Sweet, snacks	Butanal	32	Rancid, dirty	2-Heptenal (E)
5	Cheese, snacks	Diacetyl (2,3-butanedione)	33	Mushroom	1-Octen-3-ol
6	Fruity, toffees	Ethyl acetate	34	Sweet, fruity, cherry	Ethyl hexanoate
7	Rancid, dry-cured ham	3-Methylbutanal	35	Geranium, herbal, floral	Octanal
8	Vinegar	Acetic acid	36	Citrus, orange	Limonene
9	Toasted, garlic	2-Ethylfuran	37	Roasted, butter, soap	2,4-Heptadienal
10	Roasted, sweet	2-Pentanone	38	Cooked meat, nutty	2,4-Heptadienal (E,E)
11	Fresh cut grass, rancid	Pentanal	39	Roses	Phenylacetaldehyde
12	Butter, cheese	2,3-Pentanedione	40	Dry-cured ham, dry-cured sausage	2-Octenal (E)
13	Strawberry	Ethyl 2-methylpropanoate	41	Rancid nuts, woody	Unknown (3)
14	Cheese	Propanoic acid	42	Mushroom	1-Octanol
15	Roasted, roasted meat	1-Pentanol	43	Roasted, burnt	2-Nonanone
16	Fresh-cut grass, rancid	Hexanal	44	Plastic, soap	Nonanal
17	Strawberry	Ethyl butanoate	45	Waxy, smoke	Unknown (4)
18	Fatty, savory snacks	2-Methylpropanoic acid	46	Green, fresh	Unknown (5)
19	Strawberry	Ethyl 2-methylbutanoate	47	Rotten cheese, rotten orange	Unknown (6)
20	Cheese	Butanoic acid	48	Rancid, dry-cured ham	Heptanoic acid
21	Salty meat, dry-cured ham	2-Hexenal (E)	49	Roasted nuts, fried snacks	Unknown (7)
22	Green grass, plastic	1-Hexanol	50	Burnt plastic, stable	Unknown (8)
23	Strawberry	Ethyl pentanoate	51	Winery	Phenylethyl alcohol
24	Cheese, feet, dirty socks	3-Methylbutanoic acid	52	Plastic, salty, rancid	Unknown (9)

Table 19.4 (Continued)

Number ^a	GCO		N ^a	GCO	
	Descriptor	Compounds		Descriptor	Compounds
25	Medicinal, fruity	2-Heptanone	53	Herbal, crushed leaves	Unknown (10)
26	Citrus, soap, rancid cured ham	Heptanal	54	Cucumbers, herbal, woody	2-Nonenal (E)
27	Plastic, pork scratchings	2-Heptanol	55	Rancid, woody	Octanoic acid
28	Roasted nuts, fried snacks	Unknown (1)			

^a Number of the aroma in order of chromatographic elution and in accordance with Figure 19.3. Source: Adapted from Marco, A., Navarro, J.L., Flores, M., *J Agr. Food Chem.*, 55, 3058–3065, 2007.

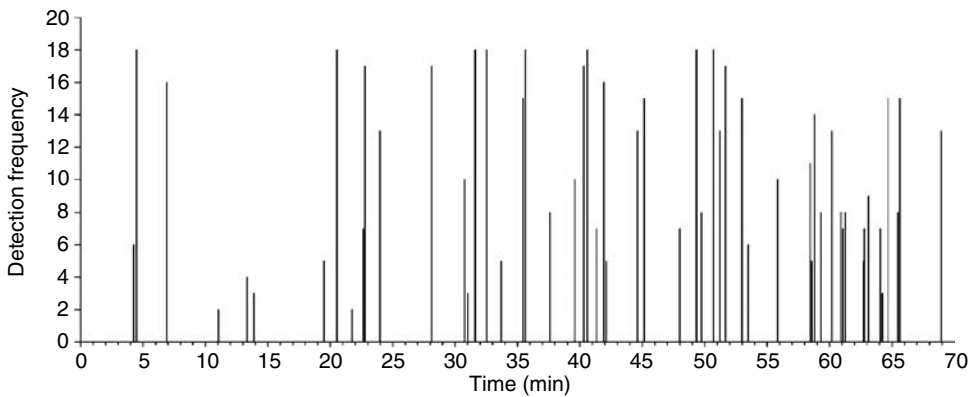


Figure 19.3 Detection frequency signal of a dry-fermented sausage obtained from the olfactometry detector using six assessors.

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Chapter 20

Sensory Descriptors for Cooked Meat Products

Jenny E. Hayes

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20.1 Introduction

The flavor and texture of a food product are determined by its components (character notes) and the arrangement (order of appearance) and the intensity of these components. Sensory analysis is the analysis of product attributes perceived by the human senses of touch, smell, sight, taste, and hearing. Descriptive analysis is a sensory method that provides quantitative and qualitative descriptions¹ of products, based on their perceptions by a group of qualified assessors. Trained descriptive panels should not be asked to evaluate flavor or any other attribute in terms of like/dislike or acceptability; these responses should be obtained only from a consumer panel.² The qualitative aspects of a product include all aroma, appearance, flavor, texture, mouth feel (aftertaste), and sound properties of the product which distinguish it from others. The sensory assessor then quantifies these product attributes to facilitate description and intensity of the perceived product attributes.

Descriptive analysis of any food requires a descriptive technique and a lexicon or language to describe the sensory properties. A group or family of attributes (lexicon) describes specific sensory attributes in products that can be used to evaluate development or change in these attributes. The results from descriptive analysis tests provide complete sensory descriptions of the products, provide the basis for mapping product differences and similarities, and provide a basis for determining those sensory attributes that are most important for acceptance. In many cases descriptive analysis can be used as a method to provide information that cannot be obtained by other analytical means. For example, the analysis of lipid oxidation does not indicate how rancid a meat product tastes or the level or intensity of off-flavors present. It is also very difficult to monitor subtle changes in shelf life or package stability using analytical instrumentation. The only useful way to monitor complex changes in lipid oxidation, rancidity or flavor intensity, as well as the inclusion of new descriptors that may often occur during the storage of meat products, is by using descriptive analysis methods. This chapter will outline the various descriptive sensory analysis methods, panel selection, and approaches to descriptor development related specifically to cooked meat products and possible examples of where different descriptors have been used in meat product research.

20.2 Methods of Descriptive Analysis

20.2.1 *Flavor Profile*

The Flavor Profile Method (FPM) was introduced by Arthur D. Little Inc., in 1949 and is the only formal qualitative descriptive procedure.³⁻⁵ It is based on the idea that flavor consists of identifiable taste, odor, and chemical feeling factors plus an underlying complex of sensory impressions not separately identifiable. The FMP utilizes a panel of four to six panelists who are screened for sensory acuity, that is, normal abilities to smell and taste, including odor perception, odor recognition, and taste sensitivity.³ During training the panel is instructed to precisely define the flavors of the product category. The panel is exposed to a wide range of samples in the product category, and during training panelists evaluate and refine the flavor vocabulary. A good prospective flavor panelist should be able to discriminate between taste and aroma sensations.⁶ The panel leader is a member of the panel, is a key member of the group, and works with them to generate the language and the method for sample preparation and evaluation. Panel members consider aspects of overall flavor and the detectable flavor components of foods. The scale used for each descriptor is a 4-point scale (0 = not present, 1 = slight, 2 = moderate, 3 = strong). This scale is further refined into half units to show narrower ranges. The key components of the flavor profile are amplitude, identification of perceptible aroma and flavor notes, the intensity and order of appearance of each character note,

and any aftertaste sensations. A disadvantage of FPM is that due to the small number of highly trained experts, the absence of one panel member can have a severe impact on the sensory program. However, the amount of work in running FPM is usually less due to this small number, and the panel is very cohesive in comparison to a Quantitative Descriptive Analysis (QDA) panel.⁷

20.2.2 Texture Profile

In 1963, the Textural Profile Method (TPM) was developed at the General Foods Research Center.^{8–10} Brant et al.⁸ defined the Textural Profile as “the sensory analysis of the texture complex of a food in terms of its mechanical, geometrical, fat and moisture characteristics, the degree of each present and the order in which they appear from the first bite through to complete mastication.” The development of the texture profile method was based on the adaptation of rheological principles to sensory evaluation and on the overall concept of the FMP. Attributes are defined to describe texture from initial bite to residual sensations remaining after swallowing. Terminology and references were developed to illustrate various classifications of textural characteristics. Following classification, the various texture attributes (or parameters) can be grouped into three main categories: mechanical characteristics, geometrical characteristics, and fat and moisture content (Table 20.1).

Mechanical characteristics deal with the resistance to breakdown and have primary parameters. Geometrical characteristics deal with the (a) particle size and shape of particles and (b) particle shape and orientation. Fat and moisture content and the rate and manner of their release are related to mouth feel. Screening procedures are conducted to eliminate candidates with dentures and those who are unable to discriminate between and describe texture differences.¹¹ The original TPM used expanded 13-point scales, however,¹⁰ TPM panels have recently been trained using

Table 20.1 Relationship between Textural Properties and Popular Nomenclature

<i>Primary Parameters</i>	<i>Secondary Parameters</i>	<i>Popular Terms</i>
Mechanical Characteristics		
Hardness		Soft, firm, hard
Cohesiveness	Brittleness	Crumbly, crunchy, brittle
	Chewiness	Tender, chew, tough
	Gumminess	Short, mealy, pasty, gummy
Viscosity		Thin, viscous
Elasticity		Plastic, elastic
Adhesiveness		Sticky, tacky, goeoy
Geometrical Characteristics		
<i>Class</i>		<i>Examples</i>
Particle shape and size		Gritty, grainy, coarse, etc.
Particle shape and orientation		Fibrous, cellular, crystalline, etc.
Other Characteristics		
Moisture content		Dry, moist, wet, watery
Fat content	Oiliness	Oily
	Greasiness	Greasy

Source: Reproduced from Szczesniak, A.S., *J. Food Sci.*, 28, 385–389, 1963.

category, line, and magnitude estimation scales.⁴ Jeremiah et al.¹² stated that the dimensions of the profile method (i.e., flavor and texture) include: (a) character notes of perceptible aroma, flavor, and texture factors; (b) degree of intensity of each factor; (c) the order in which these factors are perceived; (d) aftertastes or after feelings; and (e) amplitude or overall impression.

20.2.3 The Spectrum™ Descriptive Analysis Method

The Spectrum Descriptive Analysis Method, a universal intensity method, provides a complete, detailed, and accurate descriptive characterization of a product's sensory attributes.^{1,13} The Spectrum examines the complete "spectrum" of product attributes. This method emphasizes both qualitative and quantitative aspects of descriptive measurement; that is, the Spectrum panel describes the sensory language of the product in detail (characteristics of flavor and texture) and order of appearance. It is a descriptive attribute method that uses a 0–15 point universal scale, where 0 = none and 15 = extremely intense, to evaluate the sensory attributes of foods. The specific attributes of a product or attributes of interest are identified through ballot development sessions or using a standard lexicon. By examining a wide range of products within the category, panelists develop their list of attributes. The method provides an array, or lexicon, of standard attribute names, each with a set of standards that define the scale of intensity. The scales used in the Spectrum method are based on wide use of reference points along their range that correspond to food reference samples. It is reported that the use of these points may reduce panelist variability, which allows for better correlations with other data—for example, instrumental data.¹⁴ Results are usually graphically represented using histograms. Johnsen and Civille¹⁵ successfully applied the Spectrum method, developing a standard lexicon of meat warmed-over flavor (WOF) descriptors. Several other published studies have been carried out on pork and chicken, and many other studies use the principles applied by this method in descriptive sensory analysis.^{16,17}

20.2.4 The Quantitative Descriptive Analysis Method

The method of QDA is based on the principle of a panelist's ability to verbalize perceptions of a product.^{18–20} The method represents a formal screening and training of panelists, development and use of sensory terminology, and scoring of products to obtain a complete quantitative description. The panel leader is a sensory professional who acts as a facilitator but does not participate as a panelist, coordinating the training and screening of panelists. The order of appearance of the attributes and the order of assessment of the sensory descriptors is also determined by the panel. Definitions of the sensory descriptors are generated, which can further clarify the descriptors. The method uses a continuous 15-cm line scale anchored at each end to quantitate each sensory attribute. The data can be analyzed statistically and is reported graphically in the form of a web plot where a branch from the center point in the web represents the intensity of that attribute. QDA has been carried out in numerous studies with a variety of meat products, including beef,^{21,22} cooked ham and dry-cured ham,²³ dry-cured sausages,²⁴ and pork.²⁵

20.2.5 Quantitative Flavor Profiling

The Quantitative Flavor Profiling (QFP) method was developed by Givaudan-Roure, Switzerland^{26–28} and is a modification of the QDA technique.¹⁸ QDA profiles all sensory attributes of a product, whereas QFP concentrates solely on the flavor attributes of the product. The language used in QFP is technical; a supposed advantage of this method is that no invalid terms will be included, as the

flavorists have a wide technical knowledge. QFP is characterized by the development of a standardized flavor language (flavor descriptors and physical references) and the quantification of selected flavor characteristics. QFP depends largely on the set of reference standards and a common standardized flavor language to demonstrate concepts and intensity. The use of reference standards plays an important role in the development of appropriate flavor terminology and also reduces the amount of time required to train sensory panelists, while providing documentation for terminology. Stamparoni²⁶ focuses on the quantification of flavor characteristics for ham and bacon using QFP.

20.2.6 *Generic Descriptive Analysis*

Many organizations require the use of generic descriptive analysis, which allows the most suitable philosophies of various methods to be used and combined according to the project requirements (e.g., Lawless and Heymann,⁵ Jahan et al.,²⁹ Veberg et al.³⁰).

20.2.7 *Free Choice Profiling*

Free Choice Profiling (FCP), developed by Williams and Arnold,³¹ involves no screening or training but allows the assessors to develop their own descriptive terms; yet panelists must be able to detect differences between samples, verbally describe the perceived attributes, and quantitate them.³² Assessors should be guided in their description with advice to provide descriptors under the headings “appearance,” “texture,” “odor,” and “flavor.” The data is analyzed by General Procrustes Analysis (GPA), a multivariate technique which adjusts for the use of different parts of the scale by different panelists and then manipulates the data to combine descriptors that appear to measure the same parameter.³³ As a result, GPA allows for individual variation in descriptors of the panelists to be examined by correlating them with principal axes of the centroid of the assessor sample spaces.³⁴ When developing free-choice profiling for muscle foods, it is desirable to provide a range of the product type—for example, different textural properties or variety of off-flavors—and to use a sufficient number of samples to allow significant correlations to be obtained. The advantage of FCP is the avoidance of panel training, as panelists only need to be able to use the scale and to be considered consumers of the product under evaluation.³⁵ Cristovam et al.³⁶ stated that while FCP can show large differences between the samples, it does not show the more discriminatory differences that would be revealed by conventional sensory profiling. In previous studies FCP has been applied to beef,^{37,38} turkey,³⁹ ostrich,⁴⁰ chicken,²⁹ and pork.⁴¹ Recently, Sieffermann⁴² suggested combining free choice profiling with a comparative evaluation of the product set in a technique named Flash profile. Rason et al.⁴³ used the Flash profile method when evaluating sensory characteristics of traditional dry sausages.

A more structured approach to FCP has been developed incorporating the repertory grid method (RGM) as a prior step. This method is mainly suited to vocabulary development and can therefore solve the difficulties in generating sufficient and suitable descriptors, a problem which usually arises when working with consumers.⁴⁴ The RGM was applied to chicken in conjunction with FCP.²⁹

20.3 Panel Selection

Many researchers have discussed the selection of panelists for descriptive analysis, indicating the screening tests to be performed and also how the panelists can be monitored.^{4,5,45–47} All descriptive analysis methods require a panel with some degree of training. In most cases panelists are also required to have a reasonable level of sensory acuity, with the exception of FCP. Previous papers have

discussed the selection of sensory panelist and screening tests to be performed. Panelist selection is a critical aspect of descriptive analysis.^{4,5} A prospective flavor panelist should be able to describe sensations and should be able to discriminate between taste and aroma sensations.⁶ Panelists must be able to detect and describe the perceived sensory attributes of the product. The qualitative aspects of a product combine to define the product; they include all the appearance, aroma (odor), flavor, texture, or sound characteristics of the product that differentiate it from other products. Panelists must also learn to differentiate and rate quantitatively the intensity of the product and to define the level of each characteristic present in the sample. Two meat products might contain the same qualitative descriptors but their intensity may differ significantly between the two samples, thus resulting in different and quite distinctive sensory profiles of the product. The two samples below have the same qualitative descriptors but they differ considerably in the intensity of the characteristic (quantitative). The level or intensity of the characteristic is recorded on a 15-point scale (0 = none and 15 = extreme).

Attribute	287	131
Browned	4.5	3.1
Meaty	8.0	1.4
Rancid	1.1	8.7

The two samples of beef have the same flavor descriptors; however, they differ significantly in the intensity of each flavor note. Sample 287 has a distinct browned, meaty flavor, with a small underlying rancid note. Sample 131 also has a browned flavor note with a slight meaty flavor and a predominant rancid flavor.

A good descriptive analysis panel requires extensive training by an experienced panel leader. This sensory professional will be trained and experienced in the analytical method being applied. Once trained, a descriptive panel operates as an instrument, meaning that data must be replicated, as with any instrument.⁵ This training begins once the panelist screening and selection stages are complete. The overall goal of the training is to familiarize the panelist with the test procedures, to improve their ability to recognize the sensory attributes, and also to improve the panelist's sensitivity to and memory for test attributes so that sensory judgment will be precise and consistent. Reaching agreement among assessors is one of the main objectives of training a descriptive panel.⁴⁸

These basic guidelines should be followed as part of the sensory analysis of meat products: (1) As part of the training for a panel on meat products, panelists should be instructed on the general biology of muscle foods, that is, to understand what muscle fiber structure is and what connective tissue is, etc. (2) Panelists should also be instructed on how to chew the meat sample, how to swallow or regurgitate, and to rinse the palate between samples. This is important in texture evaluation as experimental treatment effects may be concealed or not clearly differentiated if biting and mastication are not uniform. (3) All samples must be prepared as uniformly as possible to ensure sample-to-sample variation is due to experimental treatments rather than during preparation. (4) Samples should be served as quickly as possible after cooking to avoid changes that may occur (e.g., development of off-flavors or drying of sample).

20.4 Descriptor Development

The general background to descriptive analysis aims at agreement by the panel on the use of a common list of descriptors. The selected descriptors should therefore account for and refer to the human perception of the sensory attributes of the product (with the exception of FCP). Inherent

product variations, processing effects, and storage effects can be effectively compared from a sensory perception. Sensory terminology is very important in descriptive analysis, and perceptions are greatly influenced by the language. The final descriptive language should be accurately defined and should contain enough terms to include all attributes likely to be encountered; however, it should not be so large as to be unmanageable.⁴⁹ The sensory descriptors should be descriptive, with clear and precise meaning for consistent panel performance and for a platform from which results can be repeated and compared in the future.

Generally, a new panel will develop their own sensory language with the input from an experienced leader. An existing language may also be used; however, if it is developed in another country, difficulties in understanding the interpretation may arise. In this situation, definitions are essential for translation purposes as word-for-word translations of descriptors are known to be difficult to achieve or even confusing.⁵⁰ Panelists often prefer to link descriptors together to assist in the panel's interpretation of particular attributes (e.g., rancid/painty and burnt/caramel/browned). One of the main applications of a defined sensory language is to improve the understanding of a product's flavor and texture. Using definitions is an attempt to ensure that panelists refer to the same sensory concept⁵¹ and may also reduce any ambivalence in meaning that may arise in the panel. However, many sensory attributes are not easily defined and often fail to provide a frame of reference for all panelists to describe the concept. The use of full definitions and references/standards maximizes clarity and minimizes confusion. Ishii and O'Mahony⁵² recommend multiple references, as panelists identify better with certain references. For example, the flavor descriptor oxidized/rancid/free fatty acid is defined as the flavor associated with rancid fat or as the aromatics associated with short-chain fatty acids. Panelists may be unfamiliar with the words or concepts; hence, the definition does not necessarily provide a frame of reference. Many authors have therefore recommended the use of reference standards to achieve concept alignment in sensory panels.^{6,7,53,54} For example, when a panelist is provided with butyric acid as a chemical reference or feta cheese as a food reference, a concept and common point of reference becomes readily grasped by all panelists. Descriptor references can be qualitative, quantitative, or both.⁵⁵ For lexicons, qualitative or intensity references are not generally provided for each attribute. Qualitative references allow panelists to associate the concept of the term and reduce the amount of time required to train the sensory panel⁵⁴ and also to calibrate the panel in the use of the intensity scale. Quantitative references are used as part of the training of panelists to allow the panel to concentrate on the identified language and are a mandatory part of most descriptive panel training.⁶

20.5 Meat Texture Descriptors

Descriptive texture analysis aims to allow the description of texture from first bite through to complete mastication and also accounts for temporal aspects of attributes. It occurs in several logical stages which may vary slightly between studies. The stages are evaluation of the surface properties, partial compression properties, first bite properties, mastication, and residual properties. The descriptors for muscle foods do not tend to vary from one species to another. Table 20.2 shows the descriptors used for a variety of muscle foods (pork, beef, restructured beef, frankfurters, chicken, and lamb). The first evaluation stage involves the surface properties of the meat product such as smoothness, surface moisture, fat type, and roughness. The amount of particles and the debris on the surface of the product can also be evaluated. The partial compression stage analyses the degree to which the meat sample returns to its original form, that is, the springiness, elasticity, or rubberiness of the product.

During the first bite stage, the texture properties which are analyzed are springiness, hardness/firmness, cohesiveness/disintegration, moisture release/juiciness. The terms denseness, coarseness,

Table 20.2 Texture Descriptors for Various for Cooked Meats

<i>Character Note</i>	<i>Pork⁵⁶⁻⁵⁸</i>	<i>Chicken^{56,59}</i>	<i>Lamb⁶⁰</i>	<i>Beef⁵⁵</i>	<i>Restructured Beef^{61,62}</i>	<i>Frankfurters⁶³</i>
Surface properties	Smoothness Surface moisture Fat type	Oiliness/wetness Roughness	Smoothness Surface moisture Fat type	Oiliness/wetness Roughness	None	Surface moisture Type of moisture Surface smoothness
Partial compression properties	Greasy (%) Greasy and oily (%) Fat amount Amount of particles Elasticity	Springiness	Elasticity	Springiness	Springiness	Elasticity
First bite properties	Springiness Hardness Compressibility	Firmness/hardness Cohesiveness Juiciness/moisture release	Compressibility (cohesiveness) Moisture release Amount of fat	Firmness/hardness Cohesiveness Juiciness or moisture release	Hardness Cohesiveness Moisture release	Hardness Cohesiveness Uniformity
Mastication properties	Moisture release Fat amount Fat type Greasy (%) Greasy and oily (%) Cohesiveness Cohesiveness of mass Hardness of mass	Cohesiveness of mass Uniformity of mass	Type of fat Cohesiveness (disintegration) Chewiness Number of chews	Uniformity of bite Cohesiveness of mass Uniformity of mass	Uniformity Sample breakdown after two chews Juiciness	Moisture release Denseness Coarseness Graininess Chewiness Moisture release

Fibrousness (between teeth) Chewiness	Juiciness Fibrousness	Stringiness (fibrousness) Moisture release	Juiciness Gristle	Size of chewed pieces Gristle	Oiliness Moisture absorption Cohesiveness of mass Lumpy Grainy
Number of chews	Saliva produced Particle size and shape Chewiness	Moisture absorption Cohesiveness of mass Fat type	Connective tissue	Cohesiveness of mass Uniformity of mass Webbed connective tissue	
Stringiness					
Moisture release					
Moisture absorption Cohesiveness	Chew count Bolus size	Fat amount Rate of breakdown Uniformity		Number of chews Overall gristle	Skin Description of breakdown
Fat type	Bolus wetness			Overall webbed connective tissue	
Greasy (%) Greasy and oily (%)		Density Connective tissue Connective tissue amount			
Fat amount					
Rate of breakdown Uniformity Density Connective tissue type Webbed fibers (%) Webbed fibers and gristle (%) Connective tissue amount					

(Continued)

Table 20.2 (Continued)

<i>Character Note</i>	<i>Pork⁵⁶⁻⁵⁸</i>	<i>Chicken^{56,59}</i>	<i>Lamb⁶⁰</i>	<i>Beef⁵⁵</i>	<i>Restructured Beef^{61,62}</i>	<i>Frankfurters⁶³</i>
Residual properties						
Oily/greasy film		Ease of swallowing				Ease of swallowing
Ease of swallowing		Tooth pack		Tooth pack	Tooth pack	Mouth coating
Mouth coating type		Residual particles		Number of particles	Mouth coating	Oiliness
Particles and grease (%)		Mouth coating		Oiliness/greasiness		Particles
Particles (%)		Oiliness/greasiness				
Particles and fibers (%)						
Mouth coating amount						
Loose particles						
Particle type						
Particle amount						
Tooth packing						

and graininess were used in a texture lexicon to describe frankfurters.⁶³ The amount of fat and type of fat (greasy % and greasy and oily %) were also evaluated in pork and lamb.^{56,60}

During the mastication/chewdown stage, cohesiveness of mass, fibrousness/stringiness, chewiness/number of chews, description/rate of breakdown, uniformity of mass and density were important factors which were evaluated in the majority of meat products in Table 23.2. Other factors which were assessed included moisture release and absorption, bolus size and wetness, fat type and amount, connective tissue type, and gristle. Civille and Liska⁶³ also used the terms lumpy, grainy, and skin under mastication properties of frankfurters.

Residual characteristics (after swallowing) included the ease of swallowing, presence of mouth coating and type, tooth pack, oiliness or greasiness, particle type and amount, residual particles type, and amount.

Several studies have reported the use of a texture profile panel for measuring the effects of processing procedure on the texture of restructured meats.^{62,64,65} In a study by Guerrero et al.⁶⁶ four texture descriptors were evaluated (hardness, pastiness, crumbliness, and adhesivity) using a nonstructured quantitative scale ranging from 0 (absence) to 10 (intense). To illustrate the maximum intensity for each of these descriptors in dry-cured ham, reference products were used. The texture descriptors' hardness, adhesiveness, crumbliness, pastiness, fibrousness, and fat melting were evaluated on dry-cured ham samples.²³ Examples of texture terms and their definition are shown in Table 20.3.

Table 20.3 Examples of Texture Terms Used in Sensory Texture Profiling of Meat Products

<i>Term</i>	<i>Definition</i>
Chewiness	Number of chews required to masticate the meat sample to a consistency suitable for swallowing
Cohesiveness of mass	Degree to which the bolus holds together after product mastication
Cohesiveness	The amount the sample deforms before it ruptures or comes apart when biting between the molars
Ease of swallowing	Degree to which bolus can be readily swallowed
Hardness	Force required to compress the sample with molars or incisors
Fibrousness	The amount of grinding of fibers required to chew through sample
Graininess	Amount of small particles present in sample
Gristle	Amount of rubbery particles present
Juiciness	Amount of juice released after 10 chews
Loose particles	The amount of particles left in and on the surface of the mouth after swallowing
Moisture absorption	Amount of saliva absorbed by product
Moisture release	The amount of wetness/moistness felt in the mouth after one bite or chew
Mouth coating	Type and amount of oily residue left on surface of mouth
Number of chews	The amount of chews required to prepare sample for swallowing
Smoothness	Absence of particles, small bumps/lumps or grains in the product
Springiness/ Elasticity	Degree to which a product returns to its original shape after partial compression (without failure) between the tongue and palate or teeth
Tooth pack	Amount of sample remaining in, around, and between teeth
Surface moisture	Degree to which sample feels wet/oily on the surface
Uniformity of bite	Evenness of force during mastication
Uniformity of mass	Degree to which sample is uniform
Webbed tissue	Amount of webbedlike connective tissue present

20.6 Meat Flavor Descriptors

Historically, flavor has been considered an important quality attribute of meat and comprises mainly of the two sensations taste and aroma or smell. Although both of these factors affect the overall acceptability of foods, the aroma or flavor volatiles are of utmost importance because they influence the judgment of the panelist, even before the food is consumed. In their raw state, muscle foods have little flavor of their own; however upon heat processing their specific meaty aroma develops.^{67,68}

Scientists describe five basic tastes: bitter, salty, sour, sweet, and umami. The basic tastes of sweet, sour, salty, and bitter can be found at low to moderate intensities in the various muscle foods. Sweet and salty naturally have low intensities in fresh meat but can be more intense in cured meats. Taste compounds are nonvolatile or water-soluble compounds with taste or tactile properties, including inorganic salts and sodium salts of certain acids (salty), hypoxanthine, peptides and some amino acids (bitter), sugars and some amino acids (sweet), and acids (sour).⁶⁹ Umami is a Japanese word meaning “savory,” “meaty,” or “delicious flavor” and thus applies to the sensation of savoriness. It is represented by glutamates and 5'-nucleotides, which are especially common in meats and other protein-heavy foods. The action of umami receptors explains why foods treated with monosodium glutamate (MSG) often taste *fuller*. Several umami substances are used as flavor enhancers.⁷⁰

The recent identification by Laugerette et al.⁷¹ of CD36 (fatty acid transporter [FAT]) as a taste receptor for fatty acids provides insight into the molecular basis of our preference for fat. As we gain more information regarding the function of this receptor, we may be able to devise better strategies to address the addictive potential of dietary fat which could serve as a possible target for treatment of obesity. Hence, if there is a connection between CD36 and our preference for fat, this would allow fat to join the five previously identified tastes that govern the experience of food.⁷¹

Substances in raw meat of particular importance for flavor forming reactions include free amino acids, peptides, sugars, and also phospholipids and their fatty acids, while various vitamins and minerals change the rate and extent of these reactions.^{72,73} The amounts and proportions of these compounds will determine the progress of these flavor forming reactions and therefore the ultimate flavor of the cooked meat. Amino acids, peptides, and carbohydrates constitute the basis of meat flavor precursors. It has been suggested that the basic meaty aroma of beef, pork, and lamb is the same and is derived from the water-soluble fraction of the muscle which is a reservoir of low-molecular weight compounds.^{74,75}

Meat flavor develops during cooking by complex reactions between natural components present in the raw meat. The cooking of meat generates many hundreds of volatile compounds, but relatively few make a key contribution to the odor and flavor of cooked meat.⁷⁶ The role of lipids in meat flavor generation has been studied extensively. An example of the link between lipid composition and flavor is that of concentrate-fed versus grass-fed beef. It has been suggested that the flavor of the concentrate-fed beef is preferred with the flavor of the grass-fed beef described as having “milky,” “grassy” flavor notes.⁷⁷ The reactions between amino compounds and reducing carbohydrates are another important route to formation of flavor compounds in cooked foods.⁷⁸ Whitfield⁷⁹ showed that temperatures above 110°C promote Maillard reactions in meat. The Maillard reaction occurs when the denatured proteins on the surface of the meat recombine with the sugars present. This combination creates the “meaty” flavor and changes the color of the meat. The water content in meat is high, and the formation of flavor compounds by the Maillard is, therefore, generally located in the areas of the meat where the heat source has dehydrated the meat.⁸⁰

Apart from microbial spoilage, lipid oxidation is one of the major causes of quality deterioration in muscle foods and processed meats⁸¹ resulting in rancidity, off-flavors and off-odors as well as color and texture deterioration.^{82–85} The interaction of lipid oxidation products in the thermally-induced reaction between amino acids and sugars is very important for the development of desirable meaty flavors in cooked meats.^{86,87} The relationship between rancidity and flavor is unclear. As rancid flavors develop there is a loss of desirable flavor notes.⁸⁸ A study was conducted by Campo et al.⁸⁸ to determine the relationship between human perceptions, as determined by a trained panel, to a chemical measurement of lipid oxidation. Oxidation provokes deterioration of beef flavor throughout display and it was this that could be closely related to thiobarbituric acid reactive substances (TBARS).⁸⁸ The study concluded that TBARS value of 2 could be the limiting point from where rancid flavor overpowers beef flavor, and therefore, was considered as the maximum level for the positive sensory perception of beef.

20.6.1 Mouthfeel Characteristics in Meat Products

There are two mouthfeel factors commonly found in all muscle foods, astringent and metallic.^{89,90} Astringency is not a taste or odor sensation but it must be included in flavor evaluation because it is common to many foods. Metallic mouthfeel in meat products is attributed to high myoglobin and hemoglobin contents since these proteins release iron during cooking. Increased hemoglobin may also be caused by improper blood removal during processing. This off-flavor may be reduced by cooking beef to a lower degree of doneness. Ruiz Pérez-Cacho et al.⁹¹ also included two trigeminal sensation descriptors, “irritant” (describes a product causing a sensation of heat/burning in the buccal cavity as produced by pepper) and “piquancy” (describes a product causing sharp sensations of nasal mucous membrane as produced by pepper) in a detailed lexicon for dry-cured sausages.

20.6.2 Lipid Oxidation and Warmed-Over Flavor in Meat Products

The term warmed-over flavor was first introduced by Tims and Watts⁹² to describe the development of off-flavors in cooked meats within the first 48 h of refrigeration. In meats this distinctive off-flavor can become readily apparent within a few hours of thermal processing and is most evident in refrigerated cooked meat products when they are reheated. This rapid development was the most significant factor that differentiated WOF from the ordinary rancidity flavors that develop during long term storage. This is in contrast to the slowly developing rancidity that becomes evident only after long periods of storage.⁹³ Stale or off-flavor notes such as “ice box,” “rancid,” and “freezer burn” have been used to describe this occurrence. Meat products develop stale off-flavors which are undesirable to consumers and at the same time the desirable meaty flavors notes are lost. After cooking, lipid oxidation is often considered synonymous with WOF, characterized by the loss of fresh cooked meat aroma and a simultaneous increase in undesirable off-flavors commonly described as “warmed-over,” “stale,” “rancid,” “wet cardboard,” “painty,” or “grassy.”^{88,94,95}

The high susceptibility of phospholipids to oxidation is attributed to the high concentration of polyunsaturated fatty acids. Phospholipids are generally accepted as the main substrate for the formation of oxidation products associated with WOF.^{94,96,97} Meat flavor deterioration (MFD) is also currently used to describe this undesirable flavor.⁹⁸ However, autoxidation, which is a continuous free radical chain reaction,⁹³ is still hypothesized as the major reaction responsible for WOF of precooked roast beef. The normal resistance of meat to the development of rancidity depends on the balance between the presence of antioxidants in the animal tissues, the level of unsaturation

and the concentration of fatty acids present.⁹⁹ Poultry meat is composed of relatively high levels of unsaturated fatty acids and low levels of natural tocopherols and thus poultry products are very susceptible to the development of off-flavors. In chicken, lack of α -tocopherol is the main reason for MFD and the formation of undesirable WOF products. However, cooked turkey meat, despite a higher content of unsaturated lipids, may not readily develop WOF because it contains endogenous α -tocopherol.¹⁰⁰ Researchers have shown that lipid oxidation in muscle foods takes place in the order, fish > poultry (turkey and chicken) > pork > beef > lamb.^{81,101} This ordering is associated with the increasing levels of more susceptible fatty acids in each of the tissue phospholipids of these species and to the level of natural antioxidant present.⁷⁷

Johnsen and Civille¹⁵ developed a standardized lexicon of meat descriptors and reported that WOF was identifiable in meat from various species (beef, pork, turkey, and chicken) or various treatments (grilling, steaming, baking) within species, while samples varied in intensity. Sensory terminology to describe WOF has also been described by researchers in pork,^{102–104} chicken,^{105–107} lamb,¹⁰⁸ and beef.⁹⁴ The development of WOF descriptors and references by sensory analysis has much potential in the further elucidation of WOF and lipid oxidation in muscle foods. Several sensory descriptive vocabularies for WOF in meats of different species have been prepared. These WOF vocabularies can be used by panelists to describe perceived sensory characteristics in a sample set. The resultant profile is a perceptual map of the variations in the sample type that can be applied alone or in combination with chemical or instrumental data to help explain or elucidate underlying sensory and chemical relationships.

St Angelo et al.¹⁰⁸ used the following lexicon to describe WOF in lamb: meaty (the flavor associated with cooked muscle meat, such as beef), gamey/muttony (the flavor associated with muscle meat from wild game or older lambs), musty/herby (associated with wet soil/mulch and dried herbs such as rosemary or thyme), browned/caramel (associated with the outside of grilled or broiled lamb, seared but not burnt), grainy/cowry (associated with cow meat and/or meat in which grain fed character was detectable), bloody/serum (associated with raw lean meat), livery (associated with organ meats such as liver), fatty (associated with cooked lamb fat), painty (similar to linseed oil and associated with rancid fat or oil), and cardboardy (similar to wet cardboard and associated with refrigerated cooked meat). The basic tastes of sweet, sour, bitter, and salty along with the aftertaste astringent were also used as descriptors. Byrne et al.^{103,106} described sensory terms for WOF. In chicken sensory profiling with WOF and oven cooking temperature variation, WOF was described by increased “rancid” and “sulfur/rubber” sensory descriptors associated with a simultaneous decrease in chicken “meaty” flavor. Cooking temperature was described by increased “roasted,” “toasted,” and “bitter” sensory descriptors. A sweet, fresh pork, or chicken meatlike to linseed oillike, rancidlike flavor note, indicating a loss of freshly cooked “meatiness” as oxidation proceeds and WOF develops, was apparent in the three vocabularies.^{103,104,106}

20.6.3 Boar/Sex Taint

Boar/sex taint is a well-known off-flavor in meat from pigs, mostly male pigs, that is, boars. Boar taint/sex taint is an unpleasant urinelike odor that is released during cooking from some pork and products made from the meat and fat of noncastrated male pigs; however, only a proportion of boars produce this odor and not all consumers are sensitive to it. It is primarily due to high levels of androstenone and/or skatole in pig carcasses. Font-I-Furnois⁴¹ described the sensory characteristics of boar taint using a modified FCP technique and the odor and flavor descriptors “urine,” “sweat,” “chemical,” and “rancid,” and flavor descriptors “turpentine,” “viscera,” “pig/animal,” “naphthalene,”

and “piquant.” Other descriptive analysis studies on boar taint in pork have also used the descriptors “boar flavor,” “pig,” “manure/stable,” “abnormal,” “sweet,” “naphthalene/mothballs.”^{109,110}

20.6.4 Flavor Lexicons

A flavor lexicon is a set of words to describe the flavor of a product. The lexicon is then applied using descriptive sensory techniques as described previously. A key characteristic of a good flavor lexicon is that it be discriminating and descriptive.⁶ Characteristics of flavor lexicons have been discussed in the past by Lawless and Heymann⁵ and Civille and Lawless.¹¹¹ A lexicon is a source list to describe a category of products (beef, pork, lamb) or finished products (meat patties, frankfurters). Table 20.4 shows the flavor languages used in the sensory analysis of meat flavor.

The flavor attributes mustiness (flavor that evokes the smell of a damp poorly ventilated cellar), sweetness, piquantness (pungent stimulating to the palate), bitterness, aged flavor (a pleasant incipient rancid flavor typical of dry-cured meat products aged for a long period), cured flavor (complex flavor developed during the curing and aging process and typical of dry-cured meat products) were evaluated using a flavor lexicon for dry-cured pork shoulders by Sárraga et al.²³ Fishy (cod liver oil or old fish flavor), saltiness, and metallic flavors were evaluated in dry-cured pork shoulders and also cured hams and cooked ham flavor (typical cooked ham flavor) in cooked hams only.²³

A specific descriptive lexicon was developed by Flores et al.¹¹² to evaluate the flavor of Spanish dry-cured ham. The following aromatic descriptors “fat complex,” “boar taint,” “barnyard,” “haylike/musty,” “brown spice,” “pickling spice,” “smoky,” “pork,” “serum,” “pungent,” with basic taste descriptors “sour,” “salty,” “bitter” and feeling factors “astringent,” “metallic,” and “mountfilling” were evaluated. The flavor lexicon is used by researchers to study the flavor development during the curing process. Previous sensory attributes to describe dry-cured ham flavor were not well defined using terms such as dry-cured flavor, aged taste, aroma typical of dry-ham that are very subjective and will differ depending on the origin of the dry-cured ham.

A lexicon for describing the sensory attributes of a Spanish dry-cured sausage (salchichón) was developed.⁹¹ A highly trained, descriptive sensory panel generated, defined, selected, and referenced the main sensory characteristic of commercial salchichón elaborated from meat of white pig. The language was not only descriptive but also discriminative. Panelists initially produced a vocabulary of 108 terms that were later modified to 15 attributes: four for appearance (luminance, presence of crust, fat/lean connection, and exudate); four for odor (black pepper, lactic acid, mold, and other spices); two for texture (hardness and initial juiciness); and five for flavor (black pepper aroma, mold aroma, other spices aroma, acid taste, and salty taste).

20.7 Odor in Meat Products

The sensation of odor is produced by volatile substances which stimulate receptors in the nasal epithelium. Odor plays a major part in defining the characteristic flavor of a food and is a crucial sensory attribute that may determine whether consumers will accept a food product. While odor is generally caused by low-molecular-weight volatile compounds, taste substances are usually much larger and water soluble. More than 1000 volatile compounds have been identified in cooked meat aroma.⁷⁸ However, it is believed that only a small number of compounds actually play an important role in the overall aroma of cooked meat. A further class of nonvolatile

Table 20.4 Flavor Languages Developed and Used for Sensory Analysis of Meat Flavor

<i>Meat Product</i>	<i>Descriptive Vocabularies</i>
Ground beef ^{f113,114}	Odor: putrid, sweaty, rancid, animal, blood, fatty, oily, meaty, raw meat, fishy, painty, herbal, milky-oily, cooked beef fat, sour
Beef ^{f15,38,88,115,116}	Flavor: milky-oily, cooked beef fat flavor, sour, fishy, liver, metallic, off-flavor Flavor: beefy, brothy/meaty, cooked beef fat, serum/bloody, browned, livery/organ meat, grainy/cow, herbal, acidic, chemical, beef fat, browned, dry, salty, rich, fruity, toasty, burnt, nutty, milky, turpentine, juicy, oatmeal, oily, caramel, cooked vegetable, fresh, creamy, kerosene, bland, beef, broth, popcorn, tangy, musty, citrus, perfumelike, earthy Aromatics: cooked beer/brothy, grainy/cow, serum/bloody, cardboard, painty, fishy, livery, abnormal, rancid, greasy, metallic, acidic, vegetable, grassy, dairy Basic tastes: sour, bitter, salty, sweet Aftertastes: metallic, astringent
Pork ^{56,57,109,117–119}	Odor: roasted, caramel, burnt caramel, fresh cooked pork, piggy, bouillon, linseed oil/paint, oxidized, egg/sulfur/rubber, fried meat, boiled meat, sour, sweet Tastes: sweet, sour, salt, bitter, MSG/umami Flavor: porky, piggy, sex taint/boar odor, fatty, bloody, heart/liver, browned, metallic, fresh cooked chicken, fresh cooked pork, rancid, cardboard, lactic acid/fresh sour, vegetable oil, briny, bread, fried meat, burnt caramel, boiled meat, umami Aftertastes: lactic/fresh sour, metallic, oxidized, fat, astringent
Poultry ^{104,120–122} (turkey and chicken)	Aromatic: chickeny, poultry flavor, meaty, brothy, liver/organy, browned, burned, cardboard/musty, warmed-over, rancid/painty Flavor: chickeny/poultry, meaty, brothy, browned, liver/organy, peanut/hazelnut, Basic tastes: sweet, bitter Aftertastes: metallic
Lamb ^{108,123–125}	Odor: blood, lamb, meaty, sheep meat, boiled meat, bouillon, liver, poultry, animal, rancid, fat, oil, butter Flavor: gamey/muttony, bloody/serum, browned/caramel, livery/organ meat, lamb, meaty, sheepmeat, cabbage, roast, liver, poultry, animal, rancid, fat/fatty, oil, butter, rubber, milk, oxidized, fish, abnormal
Pork sausages ²⁵	Odor: fermenting, sausagey, vinegary, musty, sour, winey, rancid, sweet, yeasty
Pork patties ¹²⁶	Odor: boiled meat, linseed oil, rancid, sweet, sour Flavor: boiled meat, linseed oil, rancid, bouillon Basic tastes: sweet, sour, bitter, salt
Ham ²³	Odor: metallic, cooked ham, fishy Flavor: metallic, cooked ham, fishy, salty
Dry-cured pork ²³	Odor: metallic, cooked ham, fishy Flavor: mustiness, sweetness, piquantness, bitterness, aged flavor, cured flavor, fishy, salty, metallic
Bacon ⁵⁷	Flavor: smoked, sweet, salt, cured fat, cured lean, cured meaty/fatty Brown sugar Molasses, burnt Aftertastes: sweet, salty, smoked, meaty

components, known as flavor enhancers, do not necessarily possess a taste or aroma themselves but enhance the flavor of other compounds.⁷³ In cooked meats many of the volatile compounds are formed by chemical reactions caused by heating and these reactions are the main source of odor compounds in cooked meat.⁷²

Roller et al.²⁵ used QDA to obtain nine odor attributes (fermenting, musty, rancid, sausagey, sour, sweet, vinegary, winery, yeasty) of pork sausages. The panelist were trained to recognize standardized odor attributes and to assess their intensity on a 0–4 scale (nondetectable—very strong) and similarly the overall acceptability in terms of odor and appearance on a 1–7 scale (extremely acceptable—extremely unacceptable). Campo et al.¹²⁷ investigated the contribution of muscle components in the development of cooked meat odor in an aqueous model system using trained sensory panelists. Following training and assessment, the following odor descriptors completed the odor profile of cooking mixtures of fatty acids, sugar and amino acids, corned beef, meaty, fatty, cooking oil, oily, wax, fish oil, linseed oil, grassy, creosote/tarmac, rubbery, gassy/eggy, sharp, pungent, and sweet.

Instruments that separate compounds and indicate their concentration include gas chromatography (GC), high pressure liquid chromatography, and sensing devices referred to as “electronic noses.”¹²⁸ Investigations of odor/aroma of meat products can be aided with gas chromatographic separation and olfactory analysis in which a human subject qualitatively evaluates the aromas of individual compounds separated by GC. The analysis of characteristic food odors has been commonly carried out by human assessment and headspace/direct gas chromatography-mass spectrometry (GC/MS).¹²⁹

20.8 Conclusions

Descriptive analysis is the most comprehensive, flexible, and useful sensory method, providing an in-depth description on all of a product’s sensory properties. In meat research, sensory descriptors are an essential tool for accurately documenting the description of cooked meat products. Sensory scientists should adhere to those recommended practices required to guarantee sensory panel performance: thorough screening and selection of panelists, extensive training that includes descriptor development, definitive references, continuous monitoring of sensory panel, and experimental controls for sample preparation and evaluation.

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Chapter 21

Sensory Descriptors for Dry-Cured Meat Products

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21.1 Introduction

The wide variety of processed meat products depends on the ingredients and additives used in their formulation as well as the type of processing [1]. The sensory characteristics developed in cured meat products treated with salt and sodium nitrite are completely different depending on the process applied—cooking, smoking, or drying [2]. In dry curing, the curing additives are rubbed onto the surface of a ham, which is then left for ripening and drying, or, in the case of

dry-fermented sausages, the ingredients are mixed with the minced meat and fat, stuffed in casings, and left to ferment and dry. Frequently, depending on traditions, the products can be smoked [3].

The acceptability of dry-cured meat products is dependent upon its sensory characteristics, including all aroma, appearance, flavor, texture, aftertaste, and sound properties. Differences among these aspects allow one to distinguish the various products from each other. These aspects are quantified by sensory judges to describe the perceived product attributes [4].

Descriptive sensory analyses are distinguished from other sensory methods in that they give a profile of a product on all its perceived sensory characteristics; therefore, its purpose is to identify and measure the presence or intensity of a particular characteristic [5].

Descriptive sensory analyses are used to relate descriptive sensory data with instrumental or consumer preference measurements. They are also used for quality control, for comparison of product prototypes, and for sensory mapping and product matching [4], as well as for many other uses, including investigating the effects of different ingredients or product changes over storage time. Dijksterhuis and Byrne [6] concluded that a trained set of assessors could be a reliable and valid measure instrument, although this depends on the quality of the sensory training carried out before the profiling. Also, it is difficult to generalize profiling results to a consumer population because sensory profiling is focused on nonaffective parts of food perception.

There are different methods of descriptive analysis, including flavor profile method, texture profile, quantitative descriptive analysis (QDA[®]), quantitative flavor profile, spectrum method, generic descriptive analysis, and free choice profiling [4,6]. The main differences among these methods are shown in Table 21.1. These methods reflect different philosophies and approaches.

The flavor profile method is a consensus technique in which vocabulary development and rating sessions are carried out during group discussions [4]. The advantage of this method is that the panelists are highly trained and, therefore, are sensitive even to small product differences. The texture profile method allows the description of texture from the first bite through complete mastication and also accounts for the temporal aspects of attributes [4]. The panel is highly trained and attributes are rated on scales anchored with specific food products; some of these have become unavailable or difficult to find outside the United States. The vocabulary used in QDA is a nontechnical everyday language to avoid bias response resulting from using a provided language. Reference standards are only used when a problem exists with a particular term. One limitation is that it is difficult to compare results from this technique between panels or laboratories, and from one time to another [4].

The quantitative flavor profile method focuses on the description of flavor only, using a technical standardized flavor language and reference standards to demonstrate concepts; estimated intensities are highly suitable for cross-cultural and cross-laboratory projects [4]. In the spectrum method there is an extensive use of reference lists, specialized panel training, and scaling procedures; however, as in the texture profile method, the reference products for anchoring attribute intensities are not available outside the United States [4]. Generic descriptive analysis combines the most suitable philosophies and techniques of the various methods, depending on the needs of the project. Finally, free choice profiling uses consumers with their own attribute definitions to define and quantify products.

In general, these methods use a reduced number of trained panelists; the main differences are in the use of vocabulary, attribute references, and the intensity scale. An attribute reference is defined as the background information and reference points that assessors mentally use when choosing words and intensities to describe and quantify perceptions [7]. Without training, assessors use their own, usually different, points of reference to evaluate products, and therefore responses vary widely. All traditional descriptive methods follow a training regimen to establish a common frame of reference. There are various attribute-specific scaling philosophies—universal, product-, or

Table 21.1 Methods of Sensory Profiling

Method	No. of Panelists	Vocabulary Development	Qualitative Attribute References	Training	Scale
Flavor profile method	4–6	Consensus vocabulary obtained through discussions	Technical language Definitions and references only during training	2–3 weeks	Product scale 5-point scale
Texture profile method	10	Only texture	Scale points anchored by specific food products	6–7 months (130 h)	Product scale 13 points
QDA®	10–12	Nontechnical vocabulary	None used, only when there is a problem	10–15 h	Unstructured 15-cm line Relative differences among products
Quantitative flavor profile technique	6–8	Only flavor technical language	Reference standards	2–3 weeks	
Spectrum method®	6–8	Panelists develop their list of attributes with the use of a reference list	References for anchoring attribute intensities different from the product	50–95 h	Universal reference scale unstructured 15 cm line
Generic descriptive analysis		Combine different methods according to their needs			
Free choice profiling		The panelist are consumers, not trained, and they use their own attributes	Able to use a scale and be consumers of the product	No	Unstructured 15-cm line

Source: Adapted from Murray et al., *Food. Res. Int.*, 34, 461–471, 2001; Dijksterhuis, G. B. and Byrne, V., *Crit. Rev. Food Sci.*, 45, 527–534, 2005.

attribute-specific—that are based on how the highest intensity of the frame of reference is chosen. In universal scaling, the attribute intensities are rated on an absolute and universal basis. Intensities are established considering all products and intensities to define the highest intensity point on the scale. In product-specific scaling, the attribute intensities are rated only within the product category being studied. Finally, in attribute-specific scaling, each attribute is rated independently from the others within a product, and each attribute has its own scale and intensity references [7].

The development of sensory descriptors or sensory descriptive attributes for dry-cured meat products depends largely on the processing conditions and geographical location, as will be shown. In this chapter are presented the different methodologies applied to dry-cured meat products and the main descriptors that have been defined and used.

21.2 Dry-Fermented Sausages

21.2.1 Sensory Methods Applied to Dry-Fermented Sausages

Dry-fermented sausages vary depending on the raw materials and processing conditions, as well as on geographic locations. Briefly, the minced meat, fat, and additives are mixed and stuffed into casings. Then the sausages are fermented in curing chambers for 1–2 days, then subjected to a ripened process for about 7–90 days depending on the type of product, diameter, and desired flavor. The most common spices and condiments added to dry-fermented sausages are pepper, paprika, mustard, oregano, rosemary, garlic, and onion [8], producing a major contribution to the aroma profile of the product.

The principal sensory profiling methods applied to dry-fermented sausages are generic descriptive analyses, followed by QDA, free choice profiling, and flavor profile (see Table 21.2). In generic descriptive analyses, the number of trained or semitrained panelists has been between 5 and 12, and in most of the studies, the training process was briefly described. The sensory descriptors evaluated were mainly appearance, flavor, and texture descriptors. The scale used varied depending on the work but generally was a 5–10 point intensity scale.

The QDA method was applied exclusively to Spanish dry-fermented sausages using between 10 and 15 trained panelists, and the training sessions were briefly described. Free choice profiling and flavor profiling are also applied to dry-fermented sausages but less frequently (Table 21.2).

However, attribute references were only used by a small number of authors (Table 21.3). A product-specific scale, based on different Italian salamis, was used to evaluate specific flavor attributes, corresponding to the upper extreme of the scale [9]. In the evaluation of French and Spanish dry-fermented sausages, various attribute specific references were used [10,11]. However, these attribute references were included only during the training process to help the panelists to describe the perceptions.

Other sensory analyses have been applied to dry-fermented sausages, mainly acceptability and preference tests (Table 21.4). In both tests, the principal attributes evaluated have been appearance, flavor, and texture, using a large number of consumers.

21.2.1.1 Flavor Descriptors

The flavor descriptors developed for dry-fermented sausages are quite varied, as reflected in Figure 21.1. Depending on their origin, the flavor descriptors can be classified as process- and meat-related flavors and other flavors. Process-related flavors include terms like “hot,” “spice,”

Table 21.2 Methods of Sensory Profiling Applied to Dry-Fermented Sausages

No. of Panelists	Attributes	Dry-Fermented Sausage	Qualitative Attribute References	Training	Scale	Reference
Generic Descriptive Analysis						
10 trained 5 trained	Appearance, flavor, texture Flavor, taste	American salami Italian	No references Product-specific scaling (attributes are based on the own-product scale) No references	3 sessions	5 and 7 point 7 points	12 9
13 semitrained	Appearance, taste, flavor, texture	Italian	No references	2 sessions	1–7 points	13
10 semitrained	Appearance, taste, flavor, texture	Italian (Sicilian salami)	No references	1 session	6-point intensity	14
10 untrained	Appearance, flavor, texture, sensations	Italian	No references		10-point intensity	15
12 trained 11 trained	Appearance, flavor, taste Flavor	Italian Sausage	No references No references A reference sample presented at the beginning of the session	12 sessions	10 point 15-cm unstructured line	16 17
12 trained	Aroma, taste, sensation	French	No references	9 sessions (1.5 h/session)	20-cm unstructured	18
11 trained	Appearance, taste, flavor, texture	Spanish (chorizo)	No references		7-point intensity	19
20 semitrained	Appearance, flavor, texture, sensations	Spanish (salchichón)	No references		9-point intensity	20
5 trained	Texture, taste, flavor	Spanish (salchichón)	References Attribute-specific scaling (each attribute has its own reference and scale)	3 sessions	Unstructured 10-point intensity	21
5 trained	Appearance, taste, flavor, texture, sensations	Spanish (salchichón)	Attribute-specific scaling (each attribute has its own reference and scale)	18 h	5-point intensity	11

(Continued)

Table 21.2 (Continued)

No. of Panelists	Attributes	Dry-Fermented Sausage	Qualitative Attribute References	Training	Scale	Reference
QDA						
10 trained	Texture	Spanish (chorizo)	No references		5-point intensity	22
10 trained	Appearance, flavor, texture	Spanish (chorizo)	No references		5-point intensity	23
12 trained	Appearance, taste	Spanish (chorizo)	No references	3 sessions	9-point intensity	24
			Control sample used as reference	(1 h/session)		
12 trained	Flavor, taste, sensations	Spanish (chorizo)	No references	3 sessions	9-point intensity	25
			Control sample used as reference	(1 h/session)		
15 trained	Texture, flavor, aroma, sensations	Spanish (salchichón)	No references	120 h	Unstructured 10-cm line	26
Free Choice Profiling						
12 untrained	Appearance, aroma, texture, flavor	Australian salami	No references	7 sessions	Unstructured line (0–100) with end points defined by consensus	27
16 untrained	Appearance, taste, flavor, texture	Spanish (ostrich)	No references		Unstructured 10-cm line scale anchored with weak and strong	28
Flavor Profile						
10 trained	Flavor	French	References for each attribute different from the products	7 sessions	Unstructured	10

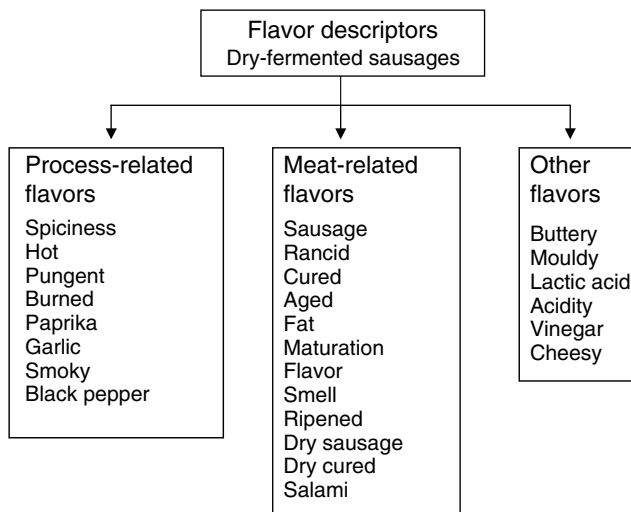
Table 21.3 Attribute References Used in the Sensory Evaluation of Dry-Fermented Sausages

<i>Virgili et al. [9]</i>		<i>Rousset-Akrim et al. [10]</i>		<i>Ruiz Pérez-Cacho et al. [11]</i>	
Attribute	Product-Specific References (Italian Salami) ^a	Attribute	Attribute-Specific (French)	Attribute	Attribute-Specific (Spanish)
Aged	10-month-old salami (pH: 5–5.5, salt: 5–5.5%)	Dry-cured sausage flavor	Dry-cured sausage		
Pungent	Salami (salt: 3–3.5%), proteolysis (20–22%), acetic acid > 0.1%, moisture/protein > 2				
Rancid	Dry-cured ham, external fat				
Buttery	250 mg/kg diacetyl in water	Butter	2,3-Butanedione		
Sweetish	Salami, fat/protein > 2				
Salty	Salami, salt: 7–7.5%	Vinegar	Acetic acid	Salty	—
Acid	Salami, pH < 4.5	Nutty	Dried fruit (hazelnut)	Acid	Vinegar, old cottage cheese
		Milk	Fresh cow milk		
		Pâté	Pâté de campagne		
		Fat	Fresh pork fat		
				Black pepper aroma	Black pepper ground
				Lactic acid odor	Yogurt
				Mould aroma	1-Octen-3-ol (0.4 mg/L)
				Spices aroma	Mixture of ground nutmeg, anise, cloves, cinnamon, and cumin
				Hardness	Raw frankfurter–American yellow cheese–toasted peanuts
				Initial juiciness	Egg white–cooked ham

^a Corresponds to the upper extreme of the scale.

Table 21.4 Other Sensory Analyses Applied to Dry-Fermented Sausages

<i>Method</i>	<i>No. of Panelists</i>	<i>Attributes</i>	<i>Dry-Fermented Sausage</i>	<i>Scale</i>	<i>Reference</i>
Acceptability					
Acceptability test	116	Appearance, flavor, tenderness, juiciness, color, overall satisfaction	Salami (USA)	Hedonic	12
Acceptability test	25	Odor, color, texture, taste, acceptability	Spanish	Hedonic	29
Acceptability test	18–20	Color, texture, odor, flavor	Spanish	Hedonic	30,31,32–34
Acceptability test	98		Spanish	Hedonic	35
Preference					
Preference test	279		Spanish		40
Paired preference test	60		Spanish		36
Paired preference test	50–100	Color, aroma, taste, overall quality	Spanish		37–40
Paired comparison test	27	Aroma, color, and taste	Spanish		41,42
Preference ranking test	35	Appearance, taste, texture	Spanish		43

**Figure 21.1 Main flavor descriptors used in the sensory analysis of dry-fermented sausages.**

“paprika,” “smoky,” etc., which result from the addition of spices and condiments or a smoking process during the manufacture of the dry-fermented sausage. These terms were used in regard to sausages from various origins—French [10,18], Spanish [19,25,28], Italian [9,16], and Australian [27]. They varied depending on the condiments added during the processing due to local traditions.

Meat-related flavors are those related to meat constituents, such as lean meat and fat; therefore, the flavor descriptors involved are mainly related to the terms “cured” [26], “aged” [9], “maturation” [13], “rancid” [9,13–17,19,26,28], and “fat” [10,17,18]. Other flavor descriptors also described in Danish and French dry-cured sausages were “vinegar” and “cheese” [10,17,18], although other terms like “mould,” “acidity,” and “buttery” were also defined in Spanish [11] and Italian [16] sausages.

The principal taste descriptors used in all types of dry-fermented sausages were “bitter,” “acid,” and “salty” [11,13,14,18,19]. The salty taste comes from the sodium salt added with the other condiments, and the sour or acid taste is due to carbohydrate fermentation, which generates significant amounts of acetic and lactic acids [44]. A few authors described the presence of a “sweetness” taste in Italian [9,16] and Spanish [21,26] sausages, which can be a result of the carbohydrate addition in the manufacture process.

21.2.1.2 Sensations and Texture Descriptors

The most commonly evaluated sensation descriptors were “aftertaste” [15,18,26] (also called “persistence” [19]), “fat mouth feel” [28], and “astringency” [25]. The “aftertaste” sensation may be due to the presence of nucleotides, nucleosides, and free glutamic acid that is commonly generated during processing [45].

Owing to the initial acidification during the fermentation stage, the texture of dry-fermented sausages is considerably affected by coagulated proteins at acid pH. Afterwards, dehydration during ripening, together with a reduction of the water-retention capacity of proteins, contributes to the firmness of the sausage [46]. Therefore, the most common texture descriptors are “hardness” and “juiciness” [11,15,26,28]. Other texture descriptors have been used, including “chewiness” [12,19,22,27], “cohesiveness” [14,22], “elasticity” [13,14], “softness,” “fibrousness” [26], and “firmness” [16]. These textural characteristics are usually correlated to ripening time, moisture content, sausage diameter, and initial grinding size [47].

21.2.1.3 Appearance Descriptors

The main appearance descriptors used to evaluate dry-fermented sausages relate to color, using descriptive terms such as “color homogeneity” [12–14,19,23,27,28] and “red intensity” [16], which is due to the reaction of nitrite with myoglobin, producing the red cured color [48]. However, other appearance descriptors are also used, including “fat content” [11,19,27,28], “visual cut appearance,” “presence of crust” [11], “particle size” [27], and “slice cohesion” [15,16].

21.3 Dry-Cured Ham

21.3.1 Sensory Methods Applied to Dry-Cured Hams

Dry-cured ham is a typical cured product processed over a very long time, generally from 3 to 24 months. It is typical in the Mediterranean area (Spanish Serrano or Iberian, French

Bayonne, and Italian Parma dry-cured hams) as well as in China (Jinhua ham) and the United States (country-style ham). Generally, dry-cured ham processing consists of three stages—salting, postsalting, and ripening/drying. In the salting stage, the salt containing nitrate and nitrite is rubbed onto the surface of the ham and maintained at refrigerated temperature over several days for salt penetration. Postsalting consists of washing the ham, which is then left at refrigerated temperatures for salt diffusion. Finally, the ripening/drying stage is performed by increasing the temperature and decreasing the relative humidity, favoring the enzymatic activity for flavor development [49].

The sensory profiling methods applied to dry-cured hams are primarily generic descriptive analyses, followed by QDA, free choice profiling, and the spectrum method (Table 21.5). The generic descriptive analysis method was used to describe the sensory characteristics of American, French, Spanish, and Italian dry-cured hams. The number of trained or semitrained panelists used was between 6 and 16, and in most studies the training process was not indicated or briefly described. The sensory descriptors evaluated were mainly appearance, flavor, and texture descriptors. The most common scale used was an unstructured quantitative scale from 0 to 10.

The QDA methodology was mainly applied to Spanish and Italian dry-cured hams (Table 21.2), using between 5 and 14 trained panelists. However, the training process was not indicated, and in only a few cases was previous panel experience indicated. A free choice profiling method was applied to Italian dry-cured ham [50]. Finally, only one study applied the spectrum method to Spanish Serrano dry-cured ham [51]. This methodology is characterized by the use of a universal intensity scale in which references different from the product are used to anchor the intensity values (Table 21.6).

Apart from the use of the universal intensity scale using specific references different from the product [51], only two other works used attribute references [52,53]. A product-specific scale of different dry meats was used to evaluate specific texture descriptors; their intensities corresponded to the upper extreme of the scale [52]. In the evaluation of Italian dry-cured ham, different attribute-specific references were used [53]. However, these attribute references [52,53] were included only during the training process to help the panelists describe their perceptions.

Other sensory analyses have been applied to dry-cured hams, primarily acceptability tests (Table 21.7) used to evaluate the overall acceptability of Spanish dry-cured hams. However, for Italian dry-cured ham another method called “different from control” was applied to evaluate the differences in aspect, aroma, and taste from a control sample [54].

21.3.1.1 *Flavor Descriptors*

The descriptors developed to describe dry-cured ham flavor are varied, as reflected in Figure 21.2. The process-related flavor descriptors are those related to the ripening/drying stage, as with the terms “aged” [68,78], “dry ham” [53,57], and “cured” [71–76,63–67]. There is not a specific attribute reference for the descriptor “cured.” It has been defined as the complex flavor generated during dry-cure processing and can include aroma notes such as buttery, cheesy, etc. [52].

The meat-related flavor descriptors define terms related to the major ham components, lean meat and fat, and their changes during processing. Therefore, the terms used were “fat” [51,53,57,69–72], “rancid” [51,53,56–58,61,63–67,71,72,74,75], “buttery” [58,78], “meaty” [56,79], “pork” [51,78], etc. Several of these descriptive terms have specific attribute references, as shown in Table 21.6 for “fat,” “rancid,” and “buttery.” The attribute references consisted of food products or, in a few cases, a volatile compound.

Table 21.5 Methods of Sensory Profiling Applied to Dry-Cured Hams

No. of Panelists	Attributes	Dry-Cured Ham	Qualitative Attribute References	Training	Scale	Reference
Generic Descriptive Analysis						
10	Texture and flavor	American country ham	No references	Not indicated	Unmarked anchored lines	55
—	Flavor	French	No references	Not indicated	Continuous scoring scale (from 0 to 100)	56
12	Texture, taste, and aroma	French	No references	Not indicated	Ungraduated (10 cm long)	57
12	Appearance, texture, and flavor	Bayonne (French)	No references	Not indicated	Scale from 0 to 10	58
12	Appearance, odor, flavor, and texture	French	No references	Not indicated	7-point discrete scale	59
6	Texture	Spanish	Maximum scale point anchored by specific food product	Previous experience (7 years)	Unstructured quantitative (from 0 to 10)	52,60
14	Appearance, aroma, flavor, and overall quality	Serrano (Spanish)	No references	Not indicated	Unstructured interval	61
16	Appearance, flavor, and texture	Serrano (Spanish)	Reference pictures	Not indicated	Unstructured interval (from 0 to 10)	62
14	Appearance, color, aroma, texture, flavor, and acceptability	Spanish	No references (hams used as references only during training)	6 (1 h sessions)	Structured 9-point scale	63–67
5	Flavor and texture	Spanish	No references	Not indicated	Unstructured quantitative (from 0 to 10)	68,69
12	Appearance, texture, aroma, and flavor	Corsican (Italian)	References for describing attributes	3 sessions	Unstructured scale of 10 cm	53
5	Appearance	Tuscan (Italian)	No references	Not indicated	4 point scale	70

(Continued)

Table 21.5 (Continued)

No. of Panelists	Attributes	Dry-Cured Ham	Qualitative Attribute References	Training	Scale	Reference
QDA						
12–14	Appearance, texture, aroma, and flavor	Iberian (Spanish)	No references	120 h of training	Unstructured 10-cm line	71–75
6	Appearance, odor, flavor, texture, and overall acceptability	Spanish	No references	Not indicated	Rating scale from 0 to 10	76
5	Texture, flavor, and odor	Spanish	No references	5 years experience	Rating scale from 0 to 10	77
5	Flavor	Italian	No references (hams used as references only during training)	6 (1 h sessions)	Rating scale from 0 to 5	78
9	Flavor	Parma (Italian)	No references	Not indicated	Unstructured intensity scale ranging from 0 to 15	79
Free Choice Profiling						
10	Flavor	Parma (Italian)	No references	Untrained	0–9 scale	50
Spectrum Method						
13	Flavor	Serrano (Spanish)	References for anchoring different from the product	50 h	Universal intensity scale	51

Table 21.6 References Used in the Sensory Evaluation of Dry-Cured Hams

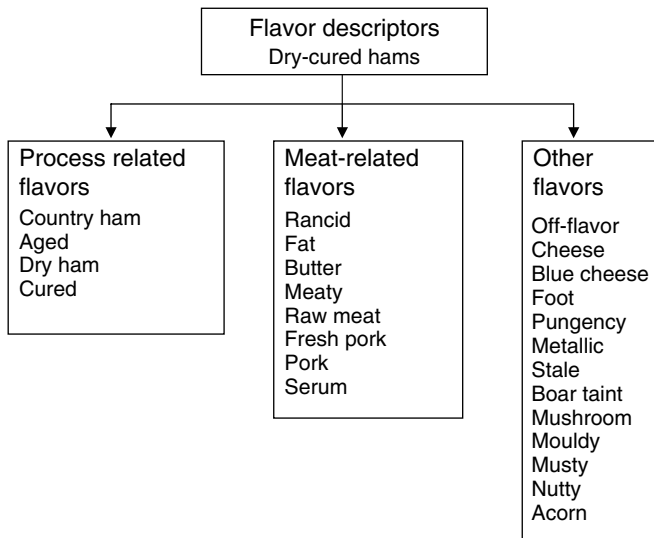
Flores et al. [51]		Guerrero et al. [52] ^a		Coutron-Gambotti et al. [53]	
Attribute	Universal Intensity Scale (Spanish) ^b	Attribute	Product-Specific Scale (Spanish)	Attribute	Attribute-Specific (French)
Sweet	1% Sucrose solution (1)	Hardness	2-mm-thick slice obtained from <i>longissimus lumborum</i> muscle (30 g of added NaCl per kg of muscle) undergoing a 60% weight loss (moisture 30% and NaCl content 6.5%) during the drying process	Fatty	Fresh pork fat
Cooked wheat	Wheat thins (1)	Pastiness and crumbliness	2-mm-thick slice obtained from <i>longissimus lumborum</i> muscle (30 g of added NaCl per kg of muscle) containing 1 g of papain (Profix 100P, 15–20%, Quest Int., Spain) per kg of muscle and undergoing a 30% weight loss during the drying process (at 4°C and HR 65–75%)	Rancid	Rancid pork fat
Oil	Frito Lay potato chips (2)	Adhesivity	External surface of <i>longissimus lumborum</i> muscle (30 g of added NaCl per kg of muscle) containing 5 g of papain (Profix 100P, 15–20%, Quest Int., Spain) per kg of muscle applied to the loin surface and undergoing a 30% weight loss during the drying process (at 4°C and HR 65–75%)	Mushroom	1-Octen-3-ol
Buttery	Land-O-Lakes margarine (3)			Buttery	2,3-Butanedione
Grape	Grape Kool-Aid (4.5)			Hazelnut	Hazelnut powder
Apple	Mott's natural apple sauce (5.0)			Blue cheese	Isovaleric acid
Orange	Minute Maid orange juice (7.0)			Fruity	Isoamyl acetate (banana)
Grape	Welch's grape juice (10.0)				
Salt	Vlasic kosher dill pickle (12.0)				
Sweet	Frosted pop-tarts (raspberry) (16.5)				
Sweet	Frosted pop-tarts (blueberry) (18.0)				

^a Corresponds to the maximum value of the scale.

^b References anchoring attributes intensities showed in parenthesis.

Table 21.7 Other Sensory Analyses Applied to Dry-Cured Hams

<i>Method</i>	<i>No. of Panelists</i>	<i>Attributes</i>	<i>Dry-Cured Ham</i>	<i>Scale</i>	<i>Reference</i>
Acceptability					
Acceptability test	268	Overall acceptability	Spanish	Hedonic	80
Acceptability test	18	Overall acceptability	Spanish	Hedonic	81
Acceptability test	30	Overall acceptability	Spanish	9-point hedonic scale	65
Acceptability test	106	Overall acceptability	Spanish	9-point hedonic scale	82
Method “difference from control”	21	Aspect, aroma, and taste	Parma (Italian)	Linear scale, not different (0) to extremely different (100)	54

**Figure 21.2 Main flavor descriptors used in the sensory analysis of dry-cured hams.**

Other flavors described in dry-cured hams are related to off-flavors such as “blue cheese” [53], “foot” [58], “boar taint” [51,61,79], metallic [57,68,76], and moldy [63,74,75]. The “boar taint” flavor has been defined as the hormone-like aroma associated with boar meat [51]. A positive flavor has been described as “nutty” [51,61,79], “hazelnut” [53], or “acorn” [63,71–73].

The major taste descriptor used in the sensory evaluation of dry-cured hams is “salty” [51,53,56–58,61,63–68,71–76], but “acid” [51,53,57,61,78] and “bitter” [51,60,61,68,71–76,78] are also used. As indicated previously, the salty taste is due to the sodium chloride added in the salting stage, whereas the bitter taste is generated due to the high proteolysis, which generates the free hydrophobic amino acids and peptides responsible for this bitter taste. On the other hand,

the sour taste found in dry-cured hams for which there is no fermentation stage originates from amino acids and short free fatty acids produced during the proteolysis and lipolysis [2].

21.3.1.2 Sensations and Texture Descriptors

The most frequently evaluated sensation descriptor was “aftertaste” [51,61,71–75], which was generally applied to Spanish dry-cured hams in which the long processing time applied produces a high concentration of glutamic acid, which, together with nucleotides, is responsible for this “aftertaste,” also called “umami” [51,61]. Also, a “piquant” descriptor has been studied in French [56] and Spanish [60,68,76] dry-cured hams. Finally, a sensation called “metallic” was studied in Spanish dry-cured hams [51]; however, this was classified as a flavor descriptor in French [57] and other Spanish [68,76] hams. This “metallic” sensation was defined as a feeling on the tongue described as flat, and was associated with iron and copper [51].

The acceptability of dry-cured hams is highly dependent on texture parameters. The most frequently evaluated descriptor was “hardness” [52,60,63–68,71–77]; other terms used were “tenderness” [55,58] and toughness [59]. Also, “dryness” was a common descriptor [53,57,59,71–75]; other texture descriptors studied were “fibrousness” [53,57,58,63–68,71–76], “pastiness,” “crumbliness,” and “adhesiveness” [52,60,63–68,76,77]. To evaluate these texture descriptors in Spanish dry-cured hams, several references were defined by Guerrero et al. [52], using dry-cured salted meat processed under special conditions to give the maximum intensity values of these texture descriptors (Table 21.6).

21.3.1.3 Appearance Descriptors

The main appearance descriptors used to evaluate dry-cured hams are related to the appearance of the lean meat and fat. The cured color generated by the reaction of nitrite with myoglobin [48] is characteristic in hams. About the color of the lean meat the descriptive terms most frequently used were “color homogeneity” [51,58,61,63], “redness” [53,71,72], and “cured color” [64–67]. Regarding the fat portion of the ham, many different papers defined the presence of intramuscular fat as “marbling” [51,53,58,59,61,63,71–75] or “fat color” [58,59,63], although other authors described the “brightness” [53,60] of the hams. On the other hand, a few authors have described the presence of “tyrosine crystals” [51,61] formed in the ham due to the long ripening period, which favors the precipitation of the amino acid tyrosine [51], which is responsible for these white spots.

The sensory quality of dry-cured hams is highly affected by premortem factors (genetic, animal species, sex, etc.), meat quality, curing salt composition, and rate and extent of the curing process [49]. The sensory evaluation of dry-cured ham is a difficult task due to the presence of different muscles in the ham and to tissue heterogeneity. In this sense, the texture and appearance is highly affected by the slice location, while the flavor is more affected by the length of the process [72].

Up to now the sensory descriptors used in descriptive analysis of dry-cured meat products have many similarities among the bibliographic references. In addition, a few attribute references have been used in descriptive analysis of dry-cured meat products and with different reference systems. In summary, the variety of sensory descriptors depends highly on traditions that affect the specific characteristics of typical dry-cured meat products.

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SAFETY

IV

Chapter 22

Spoilage Detection

Isabel Guerrero-Legarreta

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22.1 Introduction

Meat is considered to be spoiled when it is unsuitable for human consumption. Spoilage can be caused by a wide variety of factors, such as improper handling, exposure to air and high temperature, or conditions that trigger chemical reactions or microbial contamination, although the most common cause is the presence of microorganisms together with metabolite production. Spoiled meats and meat products are inedible mainly due to off-odor and flavor, but consumer rejection is also due to discoloration, blown packages, souring, surface slime, and other alterations of meat quality. However, meat may also contain pathogens without showing signs of deterioration [1].

Owing to the diversity of chemicals present, a wide variety of microorganism can be present (bacteria, yeasts, and molds). Intrinsic and extrinsic parameters determine the microflora that are able to grow [2]. Pre- and postslaughter handling are the main factors affecting microbial type and levels; temperature, humidity, and time are also determinant causes of selective microbial growth. As meat is converted into processed products, ecology changes due to processing and storage conditions, such as heat treatments, freezing and chilling, inclusion of additives into product formulations, and gas atmospheres; these determine possible changes in the proportion of the various microbial populations present as compared to raw meat. Therefore, spoilage in processed meats takes different pathways than in unprocessed meats [3].

22.2 Effect of Intrinsic and Extrinsic Parameters

Microorganisms involved in meat spoilage mainly originate in the rumen, intestines, skin, ganglions, and feathers, and sometime faces, contaminating the carcass surfaces [4]. However, handling and contact with workers' clothing, floors, walls, and instruments are also sources of contamination. Intrinsic, extrinsic, and processing conditions (processing methods, storage conditions such as temperature, gas atmosphere, and film permeability in packed items) are the main causes of the selection, growth, and metabolic activity of microorganisms present in meat and poultry products [5].

22.2.1 Oxygen Availability

Aerobic bacteria have an absolute requirement for oxygen, which limits their growth on the meat surface. Anaerobic bacteria grow within the meat, because they need the absence of oxygen. Facultative anaerobes can grow slowly without oxygen, but grow more quickly in its presence. The most important spoilage bacteria (*Pseudomonas* spp.) are aerobes. Aerobic spoilage by bacteria and yeasts usually results in slime formation, undesirable odors and flavors, mold growth resulting in a sticky surface, musty odors, alcohol flavors, and creamy, black, or green discoloration. Redox potential, related to oxygen concentration in the gas environment, is relatively low (-50 mV), although it increases at the surface, where strict or facultative aerobes are the most frequently found microorganisms. As meat is processed, redox potential changes; it can reach $+20$ mV, depending on the degree of comminution and addition of additives [6].

22.2.2 Water Activity

Water is required for microbial growth; therefore, reducing available water below the optimum level will prolong the shelf life. If meat is stored at relative humidity below 95%, moisture is lost

from the surface. Since most spoilage bacteria, being aerobic, can grow only on the surface, its drying extends the shelf life [1]. Molds are able to grow in drier conditions than bacteria, hence desiccation has a selective effect on microbial populations [7].

22.2.3 pH

In the living muscle, pH is near 7.0, but it falls to 5.4–5.6 within 24 h after slaughtering. High final pH values result when animals are exhausted during transportation; in this type of meat, spoilage bacteria multiply and shorten the shelf life [8].

22.2.4 Temperature

Keeping the meat at low temperature after carcass evisceration and cleaning is the most important requirement to achieve a desirable shelf life, as it controls microbial type and growth rate [9]. Bacteria relevant to meat, meat products, and other foods are divided into three groups according to the temperature range within which they can grow: mesophiles (10–45°C), psychrophiles (0–28°C), and psychrotrophs (10–45°C). Mesophiles do not grow below 10°C, but psychrotrophs, of which *Pseudomonas* are the most important in meat, can grow even at 0°C. The nearer to 0°C, the slower the grow rate and the longer the shelf life. Many mesophiles cause spoilage, but because meat is generally kept under refrigeration, most spoilage is due to psychrophiles.

22.2.5 Presence of Bacteriostatic Compounds

Most meat products also include bacteriostatic compounds, such as nitrites in cured products, and phenols, alcohols, and acid in smoked products. Some herb extracts such as rosemary, garlic, or onion also contain these types of compounds; therefore, the inclusion of selected plant extracts can delay, but does not completely inhibit microbial growth.

22.3 Effect of Initial Microbial Load

The main factors determining the time for microbial proliferation in meat and meat products are substrate availability and initial microbial counts. As meat is a highly nutritious food for humans as well as for microorganisms, all nutrients for microbial proliferation are present. However, glucose is the main nutrient determining the type and rate of microbial growth [5].

Microbial populations that could be beneficial in certain products, such as lactic acid bacteria that promote acidification in fermented sausages, can be highly undesirable in other products, such as cooked ham or wieners, where they cause souring. Therefore, the term spoilage depends on the meat product [1].

Shelf life, to a large extent, is determined by the initial microbial load, although certain microorganisms had a higher relationship to changes in quality characteristics than others due to the presence of specific enzymes altering the substrate. High total viable counts (TVCs), resulting from severe contamination during slaughter or processing, considerably shorten the shelf life, even in ideal conditions of processing and storage. They also indicate poor hygiene practice; therefore, contamination with food-poisoning bacteria is also likely [1].

Viable microbial populations, expressed as organisms per square centimeter or as organisms per gram of fresh meat of a meat product, set a limit to shelf life. Meat spoils at TVCs about 10^6 colony-forming units (CFU)/ cm^2 due to off-odor production; slime and discoloration appear at 10^8 CFU/ cm^2 . Type and amount of microbial populations can predict shelf life to a certain extent, but spoilage indicators are not evident until changes have proceeded too far. On high pH meat (>6.0) spoilage proceeds at lower microbial loads than in meat with normal pH (<5.8); in this case, microbial counts are not a suitable method to detect spoilage [10].

22.4 Microorganisms Involved in Meat Spoilage

Microorganisms first grow on the meat surface, as all nutrients required for growth are present in abundance. They first utilize low molecular weight nutrients such as glucose, glucose-6-phosphate, ribose, glycerol, amino acids, and lactate, altering mainly flavor, odor, and general appearance [5]. Only when the glucose utilization rate is higher than its diffusion from the inner part are amino acids utilized [1]. Several reviews report the growth requirements of microorganisms, according to their ability to degrade the meat substrate [1,4,5,7,10,11].

Once the carcass is eviscerated, cleaned, and placed under refrigeration, the “native” microflora is selected, depending on the previously mentioned intrinsic and extrinsic parameters. In raw meat, the native microflora is mainly composed of yeasts, bacilli, micrococcus, staphylococci, corynebacteria, *Brochothrix thermosphacta*, *Moraxella*, *Acinetobacter*, *Carnobacterium* spp., Enterobacteriaceae, *Lactobacillus* spp., *Leuconostoc* spp., *Salmonella* spp., *Pseudomonas* spp., *Shewanella putrefaciens*, and *Listeria* spp. [12]. However, the microorganisms mainly involved in meat and meat products spoilage are *Pseudomonas* ssp., *B. thermosphacta*, Enterobacteriaceae, and lactic acid bacteria [9].

Pseudomonas ssp. are strict aerobe microorganisms. Although these are the main organisms responsible for putrid odors, the volatiles produced appear only when the metabolized substrate changes to amino acids [7,13], producing bad odor, esters, and acid [4,14]. *B. thermosphacta* is a Gram-positive non-spore-forming facultative anaerobe, reported to be one of the most important spoilage microorganisms in meat and meat products. Glucose is the only substantial component of meat that supports its growth [4]; under anaerobic conditions, its spoilage potential is very low, producing lactic acid and small amounts of volatiles. The result is a slight off-odor [4,13,15]. In an aerobic complex medium such as meat, it produces highly odoriferous compounds such as acetoin, acetic, isobutyric, and isovaleric acids, and their aldehydes and alcohols [4,15–17]. When *B. thermosphacta* counts are higher than *Pseudomonas* ssp., large amounts of end products are detected. However, some *Pseudomonas* strains can utilize compounds produced by *B. thermosphacta*, such as diacetyl, acetoin, propylene, and butylene glycols, as carbon sources. As a result, *Pseudomonas* populations become the dominant population [18].

Enterobacteriaceae predominates in poor refrigeration conditions (above 10°C); off-odor occurs when the population is above 10^7 CFU/g. This is a wide range of facultative anaerobes that preferentially utilize glucose, although some utilize glucose-6-phosphate; their metabolism produces catabolic repression on amino acid degradation [19].

Finally, lactic acid bacteria (LAB) are a broad group of anaerobic or aero tolerant Gram-positive, non-spore-forming rods and cocci that utilize carbohydrates; they are divided into homofermentative and heterofermentative LAB. Homofermentative LAB produce almost entirely lactic acid from hexoses, but may produce lactic and acetic acids from pentoses. Heterofermentative LAB follow a different pathway to breakdown hexose, yielding 50% lactic

acid, and CO₂, as well as a mixture of end products including acetic acid, acetaldehyde, and ethanol [20]. LAB generally do not produce off-flavors or odors; their spoilage effect is mainly due to souring, unless sulfide-producing strains are present [21]. LAB only become dominant in meat after other spoilage microorganisms such as *B. thermosphacta* or Enterobacteriaceae are detected [22].

22.5 Microbial Spoilage of Raw and Processed Meat

22.5.1 Raw and Ground Meat

Microbial growth in ground meat mainly occurs on the surface; however, grinding spreads the microflora into the inner part; it also increases meat temperature, encouraging microbial growth. Pathogens can also be incorporated at this stage. For this reason, ground meat products have a considerably low shelf life. The initial alteration is presence of off-odors, and later slime production as well as protein degradation due to massive proteolysis, mainly caused by pseudomonads, although limited to the surface [23]. The inner part of ground products is colonized by Gram-positive bacteria such as *B. thermosphacta* and LAB [14,19].

Raw meat is a selective habitat for yeast bacilli, micrococcus, staphylococcus, corynebacteria, and bacteria such as *Moraxella*, *Acinetobacter*, flavobacteria, Enterobacteriaceae, *E. coli*, *Salmonella* spp., *Shewanella putrefaciens*, and *Listeria* spp. [1]. Homofermentative lactobacillus and leuconostoc in cold-stored beef promote sensory and chemical changes due to the formation of acetate, formate, ethanol, and H₂S [23]. *Lactobacillus* spp. cause a rapid decrease in sensory quality when the maximum bacterial count is reached, whereas *Leuconostoc* have the same effect before reaching maximum bacterial count [24]. Specific spoilage microorganisms in poultry have been isolated by Geornaras et al. [25], the most abundant being *Micrococcus* spp., Enterobacteriaceae, *Acinetobacter*, *Aeromonas/Vibrio*, LAB, *Corynebacterium*, and *Micrococcus* spp. On the other hand, prolonged refrigerated storage of raw pork and beef inhibits mesophile growth, psychrotrophs becoming responsible for carcass deterioration [11,26] mainly due to the growth of *B. thermosphacta*, *Carnobacterium* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Weissella* spp., Enterobacteriaceae, *Pseudomonas* spp., and *Shewanella putrefaciens* [11], as well as *Acinetobacter* and *Psychrobacter immobilis* [14,27]. Alteration is due to off-flavor, gas and slime production, discoloration, and souring. At 0°C slime develops after 10 days of storage; whereas at 5°C it occurs after 3 days, and at 16°C after 24 h [28]. At refrigeration and high humidity conditions, slime and off-odor production is evident due to aerobic psychrotroph Gram-negative bacilli, mainly *Pseudomonas* spp., *Acinetobacter* and *Psychrobacter immobilis* [14,27,29]; pseudomonads are the most abundant, such as *Pseudomonas fragi*, *P. lundensis*, and *P. fluorescens* [1]. Meat freezing at -18°C or less does not alter the microbial population present before freezing, although several molds have been identified as causing black spots, such as *Cladosporium cladosporioides*, *C. herbarum*, *Penicillium hirsutum*, *Chryso sporium pannicola*, *Cryptococcus*, *Trichosporon*, and *Candida* [30,31]. A succession of LAB in beef strip loins stored at -1.5°C were identified by Jones [32] as *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*.

22.5.2 Cured Meats

Curing is a process generally applied to most raw meat used in processed meat products. Inclusion of curing salts (nitrate, nitrite, salt, phosphates, extracts, and flavorings) reduces the growth of

microflora in the raw meat. The addition of sodium lactate reduces water activity (a_w) that, in turn, selectively decreases the growth of Gram-positive bacteria (*B. thermosphacta*, LAB, and *Staphylococcus aureus*), as well as some lactate-sensitive Gram-negative bacteria [33]. Cured products usually undergo a_w reduction, although bacterial spoilage can occur before considerable decrease prevents microbial growth. Mold growth on the finished product surface causes a highly unpleasant appearance and, in some cases, off-odors. This can be avoided by vacuum or modified atmosphere packaging. In products such as cured and cooked ham, spoilage originates from bacteria in the raw meat, such as enterobacteria and *Clostridium* spp.; these microorganisms grow in the inner part of meat pieces, before salt concentration is high enough, and before temperature is low enough to prevent bacterial proliferation [34]. In general, cured meat products such as wieners, pâtés, and bologna require refrigerated storage, because they undergo spoilage at temperatures higher than 10°C. Salt, nitrite, and vacuum packaging reduce *B. thermosphacta* populations. Greening in cured products is a consequence of sulfhemoglobin formation, due to the reaction between H_2S , produced by *Shewanella putrefaciens*, enterobacteria, and *Lactobacillus* spp., and oxymyoglobin [12,35,36].

22.5.3 Sausages

Sausages generally include a wider variety of microorganisms than other meat products, due to spices and other ingredients in the formulation that carry their own microflora. If the finished sausage is stored at high humidity and temperature, the main spoilage microorganisms are yeasts and bacteria; several authors [11,35,37] regard *B. thermosphacta* as the microorganism causing the largest extent of spoilage.

Souring is due to heat-tolerant microorganisms surviving heat processing [37]. It starts inside the casing due to growth of lactobacilli (mainly *Lactobacillus sake* and *L. curvatus*), enterococci, and related microorganisms, which possibly originate in dairy solids added to the formulation for sugar utilization [38]. Mucilage, on the other hand, is formed on the casings by yeasts or LAB (*Lactobacillus*, *Enterococcus*) and *B. thermosphacta* [39]; it can be removed by hot water washing without deteriorating the product. Greening is promoted by H_2O_2 -producing microorganisms; it tends to accumulate due to the low oxidoreduction potential in packed sausages. The microorganisms involved are *Lactobacillus viridescens*, *L. fructivorans*, and *L. jensenii*, leuconostocs, *Enterococcus faecium*, and *E. faecalis* [1,40,41]. However, greening can also be due to chemical deterioration of meat pigments [42]. In general, processed meats at pH about 6 are good substrates for *B. thermosphacta* and other Gram-positive bacteria that promote pH increase to ever higher values. Smoked Vienna sausages undergo blowing, souring, and exudate formation mainly due to homofermentative lactobacilli and leuconostocs, proliferating at the expense of heterofermentative lactobacilli, but enterobacteria, yeasts, enterococci, and staphylococci remain at low levels (10^3 CFU/g) [43,44]. If this type of product is stored at high temperatures or is not rapidly cooled after processing, spoilage can be also due to bacilli and mesophilic clostridia [45].

Molds rarely grow in sausage, except when the surface is relatively dry, but large-format cured and emulsified products, such as mortadella and bologna, can be spoiled by molds, as spores invade raw meat during refrigeration. The initial alteration is observed on the raw batter surface due to high humidity; spoilage develops later, during storage. The most commonly found genus is *Mucor*, although *Penicillium*, *Rhizopus*, and *Aspergillus* spp. are also present.

22.5.4 Cooked Meats

Cooked meat products must be quickly refrigerated to avoid a long period at warm temperatures (20–60°C) when microorganisms can grow. Warm products can condensate water in the package, allowing bacterial growth. Cooking destroys LAB populations on the product surface, but recontamination can take place during chilling from exposure to airborne microorganisms [38]. Microflora of cooked and sliced meat, stored in air at refrigeration temperatures, is mainly composed of psychrotrophic Gram-negative bacteria, such as pseudomonas and enterobacteria. In vacuum-packed cooked products, microflora predominantly consists on LAB and *B. thermosphacta* [46]. LAB reported as the main spoilage microorganisms in cooked meat are *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Lactococcus lactis* ssp. *Lactis*, and *Leuconostoc citreum*, reaching up to 10⁸ CFU/g after 7–12 days of spoilage at 10°C [47], and *Lactobacillus sake* [48]. *Leuconostoc carnosum* is the specific spoilage organism in vacuum-packaged sliced cooked ham, showing spoilage after 3 weeks of storage [49]. In high-pH cooked meat, microflora mainly consists of *Yersinia enterocolitica*, *Serratia liquefaciens*, *Shewanella putrefaciens*, and *Lactobacillus* spp. [50]. Low-salt cooked products are mainly spoiled by *Shewanella putrefaciens* [51]. Surface softening and off-odor formation are due to oxygen-dependent *Bacillus cereus* and *B. licheniformis* [52].

22.5.5 Dry Sausages

Microbial stability in dry meats depends on a_w ; molds and yeasts may develop during storage, especially if the products absorb humidity from the environment. Water activity reduction in meat products is achieved by long periods of drying. It can also involve a_w decrease by salt treatments, such as in Spanish “salazones,” or salt and nitrate inclusion in their formulation [42]. The main LAB found in dry sausages is *Leuconostoc carnosum*, reported to colonize low-humidity Spanish meat products, such as “morcilla” and “fiambre de magro” [53].

22.5.6 Canned Meat

Most canned meats do not require refrigeration; they undergo drastic heat treatments, calculated to inhibit pathogens as well as most spoilage microorganisms. If occurring, spoilage in canned meats is due to errors in process calculation, or to recontamination after heat treatment [54]. Microorganisms can find their way to the can interior through defects in the seals, producing blowing or souring without gas production. Spoilage microflora mainly consists in sporulated bacteria; when the cans are not completely exhausted (air is not entirely removed from the can), *Bacillus subtilis* and *B. mycoides* are present [54]. Schafer et al. [55] reported the production of *n*-butyric acid and D(-)2,3-butanediol in canned beef; these metabolites seem to be produced by spore-formers when the product is under-processed. Mild heat treatment, such as scalding or pasteurization, does not destroy heat-resistant psychrotrophs, such as *Lactobacillus viridescens*; the surviving cells can promote bitter flavor, gas, and greening. In the same way, enterococci surviving heat treatment alter products stored as low as 5–7°C [56].

22.5.7 Vacuum or Modified-Atmosphere Packaged Meats

Gas atmosphere modification, including vacuum packaging, notably increases food shelf life. However, the gas composition directly affects physicochemical and biochemical properties of the

product, as well as the microflora. Modified-atmosphere packaged meats (MAPs) mainly include the use of oxygen, CO₂, and nitrogen. Growth of aerobic microflora is encouraged by oxygen, although color is improved as oxymyoglobin develops. Conversely, anaerobes are inhibited in the absence of oxygen, but the bright red color does not develop. Microorganisms such as *B. thermosphacta* do not promote evident spoilage in oxygen-free atmospheres, but at small air concentration odoriferous compounds such as acetic, isovaleric, and isobutyric acids and their aldehydes are produced [9].

Meat packaged in films semipermeable to oxygen are colonized by *Aeromonas*, *Enterobacter*, *Hafnia*, *B. thermosphacta*, *Pseudomonas*, and *Morganella morganii* [57]. Psychrotrophic clostridia such as *Clostridium estertheticum* produce hydrogen and carbon dioxide in anaerobic conditions, promoting blowing as well as production of butanol, butanoic acid, ethanol, acetic acid, and sulfur-containing compounds [16,15,34].

High-pH meat stored in CO₂-enriched atmospheres also supports the growth of LAB. If the atmosphere is not saturated with CO₂, or the film is permeable to this gas, enterobacteria and *B. thermosphacta* produce off-odors and flavors, mainly putrid and sour ones [16,19]. Refrigerated meat stored under aerobic conditions encourages the growth of psychrotrophs; the most abundant is *Pseudomonas* spp. and off-odors are mainly due to acetoin and diacetyl [11,58]. Clostridia are also responsible for off-odors in vacuum-packaged meat. MAPs and anaerobic packaging of cooked meat can promote biogenic amine production (putrescine, tyramine, histamine, cadaverine, spermine, and spermidine) after prolonged storage due to the presence of decarboxylase-producing bacteria (Enterobacteriaceae, Bacillaceae, certain LAB) [59]. Processed meats packed in low-permeable films stored at less than 10°C produce bitterness, discoloration, milky exudate, and slime and gas production, mainly by LAB. Slime is mostly due to dextran production by *Leuconostoc* and *Lactobacillus* spp. Greening in the inner part of the product is due to *Lactobacillus viridescens* [60].

Vacuum-packaged meat microflora is mainly catalase-negative, including *Leuconostoc mesenteroides*, and heterofermentative and homofermentative lactobacilli [35,61]. Spoilage was described by Dainty [10] as due to the presence of H₂S; in vacuum-packed ham, it is caused by enterobacteria, due to the lack of proper hygiene. In addition, vacuum-packed meat at pH 5.6 is colonized by lactobacilli and other LAB. *Lactobacillus* spp. dominate the flora of vacuum-packaged meat; the maximum cell densities depend on substrate availability, but do not exceed 10⁸ CFU/cm². However, spoilage only becomes evident after maximum numbers are present [7]. Presence of volatile fatty acids, such as *n*-butyric, 3-methylbutyric, 2-methyl-butyrac-propionic, and valeric acids, is an indication of clostridia contamination, causing off-odors [10]. Vacuum-packaged beef, stored in chilled conditions, shows package blowing; off-odors (sulfurous, fruity, solvent-like, and strong cheese) are evident just after opening the package [15].

22.6 Spoilage Detection

Fast and accurate detection of spoilage, even before evident signs appear, is necessary to prevent losses during production, distribution, and storage of meat products. Microbial analysis by traditional methods evaluates freshness, spoilage, and safety of meat and meat products; these are precise but time-consuming methods. A similar situation occurs with the usually lengthy sensory analysis methods. Various authors report the advantages of analyzing the chemical compounds related to spoilage, mainly of microbial origin [11,62,63]. Methods such as the electronic nose, biosensors, and fluorescence spectroscopy provide accurate and fast tools for spoilage detection.

Finally, molecular techniques present a new opportunity to determine the type and load of spoilage microorganisms [64–67].

22.6.1 *Microbial Counts*

Traditional methods make use of several techniques in analyzing microbial load related to spoilage, depending on the particular meat product. The contaminant microflora is initiated during raw meat handling at the abattoir and throughout storage; later, the growth is encouraged or decreased by processing conditions. The analysis of a particular population must use selective culture media.

Indicator microbial population, expressed as organisms per square centimeter or as organisms per gram, sets a limit to shelf life. Differential culture media and specific culture techniques are applied to determine the type and amount of spoilage microorganisms involved in the deterioration of the product. Specific spoilage microflora in several types of meat products are discussed in previous sections of this chapter. In general, meat spoils with a TVC of $10^6/\text{cm}^2$ due to the production of off-odors. Slime and discoloration appear at $10^8/\text{cm}^2$. The main factors determining the time taken for TVC to reach these levels are the initial count and subsequent conditions of time, temperature, pH, and relative humidity during distribution and storage [1,7,45]. Extensive literature is published in this field.

22.6.2 *Predictive Microbiology*

These methods consider intrinsic and extrinsic parameters in a given food product, and the microbial growth response to these parameters in terms of mathematical models. In this way onset and type of spoilage can be predicted more accurately. It is important to note that intrinsic and extrinsic factors interact at different levels and intensities [68].

Predictive models are classified according to several criteria, including description of microbial response to time (population loads, consumed substrate, or indirect responses such as absorbance or turbidity), or mathematical models including microbial parameters such as growth rate and lag time. Variations on these parameters as a function of limiting factors, and the prediction of microbial variations, are generated by the use of computer models [28].

The most commonly used model in predictive microbiology is growth curves; among these, the Gompertz model is widely applied [69]. Other models such as thermal destruction curves, including calculation of D, Z, and F values, are applied to overall process calculations [54]. Non-thermal inactivation curves describe the survival rate and lag times before microbial destruction [70], as well as the growth probability and time model [71], whereas the limit models describe the limit to microbial growth [68]. Perhaps the most widely used method in predictive microbiology is the response surface, which considers several limiting factors, as it correlates microbial parameters to various levels of interactions [72].

22.6.3 *Chemometrics*

Because volatile compounds are responsible for meat odor and flavor, any factor affecting their production will determine meat quality. Each microbial population produces a particular metabolite or metabolites related to spoilage, with respect to a given microbial load. However, chemical deterioration, such as auto-oxidation, also produces spoilage-related compounds.

Chemical analysis recognizes a given compound as an indicator of spoilage (microbial or chemical). This is based on high correlations between substrate consumption and production of quantifiable metabolites [73]. Chemical analyses have been designed to target the main metabolite produced, or the one responsible for evident spoilage. Consumption of specific substrates has also been proposed to measure growth of spoilage microorganisms. Fabrer and Idziak [74] and Nychas et al. [5] reported glucose being the main nutrient involved in growth of spoilage microorganisms; glucose concentration in meat decreases with fourfold increase of glucose dehydrogenase [74]. However, the analysis of indicator metabolite concentration is a more common approach.

22.6.3.1 Biogenic Amines

A group of compounds considered to be indicators of meat bacterial spoilage are biogenic amines [5,75,76]. An in-depth discussion of this topic is presented in the chapter on amines. They are produced in foods by bacterial decarboxylases; the most abundant biogenic amines in meat products are: cadaverine (pentamethylene diamine), putrescine (1,4-diaminobutane), spermidine [*N*-(3-aminopropyl) butane-1,4-diamine], histamine [2-(3H-imidazol-4-yl)ethanamine], tryptamine [3-(2-aminoethyl) indole], agmatine (β -phenyl-ethylamine), ornithine (2,5-diaminovaleric acid), tyramine (4-hydroxy-phenethylamine), and spermine [*N,N'*-bis(3-aminopropyl)butane-1,4-diamine] [77].

The main decarboxylase-producing microorganisms involved in meat spoilage are Enterobacteriaceae, Bacillaceae, and species of *Lactobacillus*, *Pediococcus*, and *Streptococcus* [76,78]. *Pseudomonas* and *B. thermosphacta* showed no evidence of production; however, *Pseudomonas aeruginosa* can transform arginine to putrescine, through agmatine production. Lysine decarboxylation to cadaverine was also reported by *Pseudomonas cepacia* and *P. maltophilia*, normally not present in meats [62]. Putrescine formation requires the growth of arginine-utilizing lactic acid bacteria by ornithine production, and subsequent decarboxylation by Enterobacteriaceae [79,80].

Biogenic amine production has been analyzed on specific culture media. Niven et al. [81] developed a histidine-containing medium for quantitative detection of histamine-producing bacteria associated with scombroid fish poisoning outbreaks, utilizing color change of the medium adjacent to the colonies due to change in pH. Choudhury et al. [82] reported a modified decarboxylase assay medium containing histidine, lysine, ornithine, and tyrosine as precursors of the respective biogenic amines. Sumner and Taylor [83] developed an enzyme detection system for histamine-producing bacteria. The isolated bacteria were inoculated in Mann-Rogosa-Sharpe (MRS) medium; the resulting histamine reacts with diamine oxidase, which catalyzes histamine oxidation to imidazole acetaldehyde, ammonia, and hydrogen peroxide. The hydrogen peroxide was then detected by the formation of crystal violet from a white base in the presence of horseradish peroxidase. The liquid culture medium containing the bacteria produced <1200 mmol histamine per milliliter, and developed a positive purple color.

Chromatographic methods are also used to analyze biogenic amine concentrations; low- and high-pressure chromatography makes use of ion exchange columns [84]. Other analyses include a method to quantify trimethylamine in chicken broth using ion mobility spectrometry, which separates and detects electrically charged particles sorted according to the speed they travel through an electric field; this method can detect concentrations as low as 0.6 ng [85].

An American patent [86] describes a colorimetric sensor based on a molecular imprinted polymer, developed by The Johns Hopkins University Applied Physics Lab. The polymeric sensor

selectively binds biogenic amines (putrescine, cadaverine, and histamine) and undergoes color change; it is sensitive to amine concentrations as low as 20 ppm. Lou et al. [87] reported the analysis of cadaverine and putrescine by the use of chemiluminescence-flow injection using photomultiplier detectors. Chemiluminescence was initiated by hydrogen peroxide produced by enzymatic oxidation of biogenic amines in a putrescine oxidase/peroxidase reaction. The authors reported high correlations between these measurements and bacterial counts up to 10^7 CFU.

Biosensors have been also used as indicators of the presence of biogenic amines. Yano et al. [88] reported the use of a biosensor composed of Ag/AgCl and platinum electrodes, coupled to immobilized putrescine oxidase or xanthin oxidase. Putrescine and hypoxanthin were detected by voltage changes.

22.6.3.2 Volatile Metabolites

Microbial populations related to meat spoilage generally produce a particular metabolite or metabolites; most of them are highly volatile or can be derivatized by relatively simple chemical methods to increase volatility. Therefore, analyzing these products can be an index of meat spoilage. Several excellent reviews on the relationship between microbial populations in meats and the specific chemicals produced have been published [5,10,89].

Dainty et al. [79] described a sequence of volatile production by spoilage microorganisms. It starts with a dairy/butter/fatty/cheesy odor due to acetoin, and follows with diacetyl butanal and propanal produced by *B. thermosphacta* when this population reaches 10^8 CFU/g. Later, sweet/fruity odors due to esters of short-chain fatty acids are produced by *Pseudomonas* spp.; when pseudomonad reaches 10^9 CFU/g, sulfide odors appear along with slime formation. Acetic acid production increases with LAB populations, whereas butyric acid production is associated with *Leuconostoc* [32]. The multivariate approach, based on chemical compound spectra, is also helpful in analyzing spoilage correlation to microbial populations [11].

Mayr et al. [90] related microbial populations, mainly Gram-negative aerobic rod-shaped bacteria, to meat spoilage. *Pseudomonas* spp. were the dominant, but high concentrations of Enterobacteriaceae, *Enterococcus*, and LAB also have an important role; the authors related these populations to 22 volatile organic compounds using a proton transfer reaction-mass spectrometry system. The authors considered this to be a fast and reliable technique. A similar approach was applied by Huis In't veld [2], although this author also includes in the analysis biochemical changes occurring during spoilage. Ellis et al. [73] reported the use of Fourier transformed infrared spectroscopy (FT-IR) to quantitatively analyze microbial spoilage; the authors report that this noninvasive technique gives biochemical "fingerprints" of meat spoilage that correspond to metabolites produced by spoilage microorganisms. Interpretation of FT-IR spectra was carried out by statistical analysis techniques, estimating the bacterial loads and considering the extent of proteolysis.

Diacetyl is formed by *Pseudomonas* [91], as well as being a lipid oxidation product. Diacetyl aroma is not accepted in meats, because it is associated with dairy products. Although diacetyl cannot be used as an indicator of growth of LAB, because other spore-forming microorganisms also produce these compounds [22], it is widely used as an indicator of spoilage by other microbial populations. The U.S. patent 5663072 [92] describes a detection method that monitors diacetyl production by exposing an aromatic ortho-diamine (3,4-diaminobenzophenone or 3,3-diaminobenzidine), complexed with nickel chloride at low pH, to an environment containing diacetyl; up to 10 ppm diacetyl can be detected.

Volatile analysis is also carried out by instrumental methods such as gas chromatography (GC), GC/mass spectrometry (MS), and capillary GC/MS [53]. Volatile compounds such as diacetyl and hexanal, other indicators of bacterial spoilage, are present in the headspace of packaged meat products undergoing spoilage; samples are taken from the headspace and directly injected into the chromatograph [93]. Headspace analysis by solid-phase microextraction (SPME) is based on adsorption of spoilage indicator compounds, such as hexanal or diacetyl, from the headspace to a polymer-coated silica fiber, allowing direct injection of the analyte (hexanal, diacetyl, or other volatile) to a gas chromatograph; it is then desorbed into the injection port of the chromatograph; the rest of the analysis is carried out in a routine fashion [94]. Extraction methods include the use of Freon 11, reported by Tracey and Britz [95] to identify 35 volatile metabolites produced by LAB.

The electronic nose quantifies volatiles by a combination of GC/MS and sensory analysis. It consists of two chemical sensors (usually gas sensors) and a pattern-recognition algorithm. The sensor array “sniffs” the vapors from a sample and provides a set of measurements; the pattern-recognizer compares the pattern of the measurements to a library of known chemical compounds. Electronic noses have been used to describe the odor and flavor of a number of compounds; therefore, it can be also used to detect spoilage [96–99].

22.6.4 Molecular Techniques

Sensitivity in analyzing spoilage populations has been improved by molecular techniques, such as deoxyribonucleic acid (DNA) probes, allowing fingerprinting of spoilage-related microorganisms even at the subspecies level [100]. Microorganisms such as *Lactobacillus sake* strains associated with ropy slime [101], *Leuconostoc* spp. [49], *Carnobacterium* [65], *Clostridium* spp. [66], *Pseudomonas* spp., *Sphingomonas* spp., *Alcaligenes* spp., *Serratia* spp., and *Microbacterium* spp. have been accurately detected by the use of 16S rDNA-directed primers [67,102]. Although expensive, molecular techniques have proven to be highly valuable for spoilage strain identification.

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Chapter 23

Microbial Foodborne Pathogens

Marios Mataragas and Eleftherios H. Drosinos

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23.1 Introduction

Prevention of foodborne infections and intoxications are of paramount importance today. Hazard analysis and critical control point (HACCP)-type food safety management systems are applied by food enterprises to achieve this goal. Validation of all control measures requires, among other activities, microbiological testing of food and environmental samples. The presence of pathogenic bacteria on raw meat (beef, lamb, and pork) and poultry is the result of their contamination from the live animal, equipment, employees, and environment. *Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Escherichia coli* (mainly *E. coli* O157:H7), *Campylobacter jejuni*, and *Clostridium perfringens* often occur on raw meat and poultry. These pathogens have been implicated in foodborne outbreaks associated with the consumption of meat and poultry. *C. jejuni* frequently occurs on poultry meat, whereas *E. coli* is rarely found on this type of meat. However, beef has been implicated in many foodborne outbreaks associated with *E. coli*. *Salmonella* and *L. monocytogenes* may be found on all types of meat, including beef, lamb, pork, and poultry, and *Y. enterocolitica* is usually present on pork meat surfaces [1,2]. Psychrotrophic pathogens such as *L. monocytogenes* and *Y. enterocolitica* are of great concern because they are able to reach high numbers at refrigerated temperatures, especially when products are kept under abused temperatures (>7–8°C) for extended periods of time [3]. *S. aureus* and *C. perfringens* are also of great concern due to toxin production in food as a result of their growth. For more detailed information on the protocols and the culture media (including their preparation), for both cultural and rapid microbiological methods, reference works should be consulted [1,4–6]. The analytical essentials of microbiological examination of foods, as documented by the late Professor Mossel, are important elements of background information for the person performing the analysis [7].

23.2 Cultural Methods

Cultural or traditional methods are simple and relatively inexpensive, but they are time consuming. A food sample (usually 25 g) is homogenized in a stomacher bag with 225 mL of diluent using a stomacher machine to prepare a 1:10 dilution. Diluent must be correctly prepared in terms of buffer capacity and osmotic pressure (saline peptone water [SPW], 0.1% peptone and 0.85% NaCl); otherwise the microbial cells of the target microorganism may be stressed, influencing the final result. The sample withdrawn for microbiological analysis should be representative and randomly selected from different areas of the food to assure, in some degree, detection of the target microorganism if this is not uniformly distributed in the food, which very often is the case for solid foods. Information on the statistical basis of sampling plans and practical aspects of sampling and analysis are provided by Jarvis [8]. Further decimal dilutions may be required depending on

the population level of the target microorganism present in the food. An adequate volume of sample from the appropriate dilution is spread (0.1 mL), poured (1.0 mL), or streaked on selective agars to differentiate or enumerate the target microorganism. Nonselective agars may also be used to perform confirmatory biochemical and serological tests. In some cases, an enrichment and, if it is necessary, a preenrichment step may be included to suppress the growth of other microorganisms, allowing at the same time the recovery of injured cells of the target microorganism.

Laboratory media used to subculture the microorganisms present in the food sample are divided into three categories: elective, selective, and differential [9]. Elective media are those that contain agents (e.g., microelements) that support the growth of the target microorganism but do not inhibit the growth of the accompanying microflora. The latter is achieved by the use of the selective media, which contain inhibitory agents, such as inorganic salts, triphenylmethane dyes, surface-active agents, and antibiotics. These agents inhibit the growth of the nontarget microorganisms as well as, in some cases, the growth of the microorganism under examination but in lesser degree. Differential media contain agents that allow the differentiation of the microorganisms (e.g., chromogenic media). These media contain chromogenic ingredients that produce a specific color or reaction due to bacterial metabolism. These agents react with the colonies, changing the color of the media. Usually, the media contain all the preceding agents to ensure proper identification of the target microorganism. For instance, the chromogenic media Agar *Listeria* Ottavani & Agosli (ALOA) agar [10] and RAPID' L. mono *Listeria* Agar (RAPID' L. mono) [11] use the following properties to differentiate *Listeria* spp. and *L. monocytogenes* from the other *Listeriae* species. ALOA contains a chromogenic compound which colors the *Listeriae* colonies due to its degradation from the enzyme β -glucosidase. This enzyme is produced from all *Listeria* species. The differentiation of pathogenic *Listeria* from the nonpathogenic species is based on the formation of phosphatidylinositol phospholipase C (PI-PLC). This compound hydrolyzes a specific substrate added to the growth medium, resulting in a turbid halo (ALOA) or a specific color of colonies (RAPID' L. mono) [12].

Petrifilm method (3M, Minneapolis, Minnesota) is another method that uses a plastic film together with the appropriate medium in dried form. It is used mainly for coliforms (red colonies with gas bubbles) and *E. coli* (blue colonies with gas bubbles). One milliliter of sample is added directly to the plates to rehydrate the medium. Plates are then incubated and counted. Validation and collaborative studies have found the Petrifilm method to be not significantly different from the traditional methods [6,13,14].

23.2.1 Enumeration Methods

In general, two enumeration methods are used most often—the plate count and most probable number, the latter method being used for certain microorganisms, such as coliforms [15] and *E. coli* [16].

23.2.1.1 Plate Count

Plate count is the most popular cultural enumeration method. The procedure involves homogenization of the food sample, dilution, plating on various media, and incubation at selected temperatures according to which microorganism is under examination. After incubation for a sufficient period of time, counting of the specific colonies of the target microorganism is performed. If confirmation of the target microorganism is required, then a number of randomly selected colonies are obtained. The ratio of the colonies confirmed as the target microorganism to the total colonies tested should be calculated to ascertain the number of viable cells per gram

of food sample. For instance, if the mean number of presumable *C. perfringens* colonies from two pour agar plates is 20 at the second dilution (10^{-2}) and the confirmed *C. perfringens* colonies of 10 randomly selected (5 per plate) are 8, then the number of viable *C. perfringens* cells per gram of food sample will be $20 \times 10^2 \times (8/10) = 1.6 \times 10^3$ [1]. A recent critical review of the uncertainty in the enumeration of microorganisms in foods is given by Corry et al. [17].

23.2.1.2 Most Probable Number

The number of viable cells in a food sample is assessed based on probability tables. The food sample is diluted (10-fold dilutions), and then samples from each dilution are transferred to three tubes containing a growth medium (broth). After incubation of tubes, turbidity is measured and the tubes showing turbidity (growth) are compared to probability tables to find the population level of the target microorganism present in the food [1].

23.2.2 Detection Methods

Detection methods are used to determine the presence or absence of a specific pathogen. These methods include additional steps (for example, preenrichment and enrichment) to allow the increase of pathogens to a detectable population and recovery of injured cells, because the target microorganism may be present in very low levels in comparison with the population levels of the dominant microflora.

Sublethal exposure of microbial cells during processing of foods may lead to the inability of the microorganisms to form visible colonies on plate count agars. Although cells may remain undetected on selective agars, they are still viable (but not culturable), and under conditions that favor their growth may recover and become active. This is of great importance for foodborne pathogens that may lead to a food poisoning outbreak. Therefore, additional steps such as the previously mentioned enrichment steps are included in the analytical procedures to allow the resuscitation/repairing of the injured cells. There are many factors that influence the resuscitation of injured cells, such as composition and characteristics of the medium and environmental parameters [18]. Therefore, the analytical methods for the detection of the microorganisms are constructed in such a way as to allow maximum performance (recovery of stressed cells).

Usually, 25 g of food sample is aseptically weighted in a stomacher bag, homogenized in an enrichment broth (225 mL), and incubated for a certain period of time at a known temperature. After incubation, a sample from the broth is streaked on a selective agar plate using a bacteriological loop. If the examined microorganism is present, it is indicated by its characteristic colonies forming on the agar. To confirm the microorganism at strain level, some additional biochemical or serological tests may be needed. These tests are performed on a pure culture; therefore, colonies from the selective agar plates are purified (streaking) on nonselective agar plates, for example, nutrient agar or brain heart infusion (BHI) agar.

23.3 Alternative or Rapid Microbiological Methods

Rapid microbiological methods are much faster, but one disadvantage is that they are expensive. Thus, a careful look at the requirements of a laboratory or a food industry is required before the adoption of a method. These methods also include an enrichment step called a concentration step,

aiming to separate and concentrate the target microorganism or toxin. In this way, the detection time is made shorter and specificity is improved.

23.3.1 Methods with a Concentration Step

Methods that concentrate the target microorganism or toxin are

1. The *immunomagnetic separation* (IMS), in which antibodies linked to paramagnetic particles are added and the target microorganism is trapped because of the interaction between antigen and antibody. Commercial kits are available for IMS of various foodborne pathogens, such as *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7 (Dynabeads™, Dynal Biotech, Oslo, Norway). The IMS for *Salmonella* (10-min duration) has been proved to successfully replace the enrichment step (overnight incubation) of the standard procedure for the detection of *Salmonella*, shortening the time needed to obtain results.
2. The *metal hydroxide–based bacterial concentration* technique, in which metal (hafnium, titanium, or zirconium) hydroxide suspensions react with the opposing charge of the bacterial cells. The cells are then separated by centrifugation, resuspended, and plated.
3. The *hydrophobic grid membrane filter*, which is a filtration method similar to the method used for water. The food sample first is filtered to remove large particles (>5 µm) and then is filtered through a grid membrane on which the microorganisms are retained. The membrane is placed on a selective agar and after an appropriate incubation period, the colony counts are calculated.
4. The *direct epifluorescent technique* (DEFT), used for enumerating viable bacteria in milk and milk products. Microorganisms' cells are concentrated through filtration on a membrane and then retained microorganisms are colored, usually with acridine orange (fluorescent dyes) and counted. Viable cells are red (acridine orange fluoresces red with ribonucleic acid [RNA]) and nonviable green (acridine orange fluoresces green with deoxyribonucleic acid [DNA]) [6,14,19,20].

23.3.2 Detection and Enumeration Methods

Some of the most widely used methods for the identification and detection of foodborne pathogens are the following:

1. *Polymerase chain reaction (PCR)–based methods coupled to other techniques*—most probable number counting method (MPN-PCR) [21], surface plasmon resonance, and PCR acoustic wave sensors [22], LightCycler real-time PCR (LC-PCR), PCR enzyme-linked immunosorbent assay (PCR-ELISA) [23], sandwich hybridization assays (SHAs), and fluorescent *in situ* hybridization (FISH) detection test [24]. From these methods, ELISA has been widely used for pathogen detection and identification, especially for *Salmonella* spp. and *L. monocytogenes*. The detection limit is 10⁴ colony forming units (CFU)/g; therefore, a cultural enrichment step is required before testing. Specific antibodies for the target microorganism, contained in microtiter plates, react with the antigen, which is detected using a second antibody conjugated to an enzyme (horseradish peroxidase or alkaline phosphatase) to give a colorimetric reaction after the addition of substrate.
2. *Adenosine triphosphate (ATP) bioluminescence*, which can be used as an indicator of microbial contamination in foods and processing plants. This method detects the presence of

bacterial ATP. In a buffer containing magnesium, luciferase is added to a sample along with luciferin. The latter is oxidized (oxyluciferin) and the photons of light produced are measured by a luminometer. A standard curve is made to calculate the contamination level; the sensitivity of the method is 10^4 cfu/mL.

3. *Reversed passive latex agglutination*, which is used for the detection of toxins such as shiga toxins from *E. coli*. Latex beads containing antibodies (rabbit antiserum) specific for the target microorganism react with the target antigen if present. The particles agglutinate and a V-shaped microtiter well has a diffused appearance. If the antigen is not present, then a dot will appear.
4. *Impedance or conductance technique*, frequently used for enumeration. This method rapidly detects the growth of a specific microorganism based on the production of charged metabolites (direct method) or based on the carbon dioxide liberation (indirect method). In the first method, detection is measured by the change in the conductivity of the culture medium because of the accumulation of various products produced by the microorganism, such as organic acids. These changes are recorded at constant time intervals. "Time to detection" is the time needed in order for the conductance value to be changed. Because the time to detection is dependent on the inoculum size, a calibration curve is made for a known wide range of population levels of the desired microorganism. Using this calibration curve, the calculation of the population level of an unknown sample is simple after the automatic determination of the time to detection by the equipment. In the other method, the sample is distinguished from the potassium hydroxide bridge by a headspace in the test tube. The carbon dioxide produced during the microbial growth in the headspace reacts with potassium hydroxide, forming potassium carbonate, which is less conductive. Conductance decrease is the recorded parameter [6,14,20].

Genotypic, molecular methods are useful in identifying bacteria either as a complement or an alternative to phenotypic methods; besides enhancing the sensitivity and specificity of the detection process, they reduce much of the subjectivity inherent in interpreting the results. DNA is invariant throughout the microbial life cycle and after short-term environmental stress factors. This is the reason that molecular methods targeting genomic DNA are generally applicable [25]. Restriction fragment length polymorphism (RFLP) of total genomic DNA represents a technique belonging to the first-generation molecular methods [26] widely used in microbial differentiation. Southern blot hybridization tests, which enhance the result of agarose gel electrophoresis by marking specific DNA sequences, have also been used. Second-generation molecular techniques (known as PCR-based technologies), such as PCR-RFLP and randomly amplified polymorphic DNA-PCR (RAPD-PCR), have been used for differentiation and identification of microbial isolates [25]. Recent advances in PCR technology, namely real-time PCR [27], enable results to be obtained within a few hours [28]. Quantification of microorganisms is of major importance, especially in the case of toxigenic bacteria, since their concentration determines toxin production [25]. Biosensor technology promises equally reliable results in much shorter times, and is currently gaining extreme interest. Many biosensors rely on either specific antibodies or DNA probes to provide specific results [28].

The current trend is toward culture-independent PCR-based methods, which, unlike the previously mentioned ones, are believed to overcome problems associated with selective cultivation and isolation of microorganisms from natural samples. The most commonly used method among the culture-independent fingerprinting techniques is PCR followed by denaturing gradient gel electrophoresis (DGGE). PCR-DGGE provides information about the variation of the PCR products of

the same length but with different sequences on differential mobility in an acrylamide gel matrix of increasing denaturant concentration [25,29].

23.4 *Listeria monocytogenes*

L. monocytogenes is widely distributed in the environment and can be found in many food commodities [3,30]. It is a very persistent microorganism that survives on surfaces and equipment of food processing units in conditions of insufficient cleaning [31–35]. Postprocessing contamination from the plant environment (equipment, personnel, floors, etc.) is the most frequent reason for its presence on meat surface. Cross-contamination may also occur at the retail outlet, as well as in the home, especially when the products have been mishandled and improper hygiene practices have been followed [35–36]. Various foods have been associated with *L. monocytogenes* outbreaks. Milk and dairy products (e.g., cheese), meat (including poultry) and meat products, vegetables, and fish and fish products have been implicated in outbreaks of foodborne *L. monocytogenes* [37]. The pathogen is usually killed during cooking, but it is capable of growing in foods stored at refrigeration temperatures (psychrotrophic microorganism) [38–39]. High salt concentrations and acid conditions do not permit *L. monocytogenes* growth [39]. However, it may survive even under these stressful environmental conditions [40–41]. Therefore, consumption of raw products or manufacturing of products without a killing step (e.g., cooking) with products that support pathogen growth—those with, for example, high initial pH, low salt content, or high water activity—or that are stored at refrigeration temperatures for a long period of time may increase the potential of listeriosis infection involving *L. monocytogenes* [39,42]. *L. monocytogenes* is a significant hazard, particularly for the elderly, immunocompromised people, infants, and pregnant women.

23.4.1 *Detection of Listeria monocytogenes*

The method for cultural detection of *L. monocytogenes* in raw meat and poultry is shown in Figure 23.1 [43]. Two enrichment steps are employed in the method to detect *Listeria* presence. With enrichment, it is feasible to detect low numbers of *Listeria*, as few as one cell per 25 g of food, because the microorganism is allowed to grow to a level of ca. 10^4 – 10^5 cfu/g. The first enrichment step includes half Fraser broth (half-concentrated Fraser broth) containing only half concentration of the inhibitory agents (antibiotics), because these agents may have a negative effect on stressed or injured *Listeria* cells [44,45]. Antibiotics (acriflavin and nalidixic acid) are used to suppress the growth of the accompanying microflora, which may outgrow *Listeria* due to its slow growth. *Listeria* presence on the selective agar plates is observed by the formation of characteristic colonies. They are gray-green with a black center surrounded by a black zone on PALCAM agar [46]. Aesculin and ferrous iron are also added to the Fraser broth in conjunction with antibiotics to allow detection of β -D-glycosidase activity by *Listeria*, causing blackening of the medium [45].

Molecular methods that monitor the incidence of *Listeria* spp. in foods are also applied. Suggested techniques include fluorescent antibody assay, enzyme immunoassay, flow cytometry (FCM), and DNA hybridization [47]. DNA hybridization is the simplest molecular method used for the detection of *Listeria* spp. and *L. monocytogenes* in foods. The presence of a target sequence is detected using an oligonucleotide probe of a sequence complementary to the target DNA sequence, containing a label for detection. Radioactive isotopes, biotinylated probes, probes

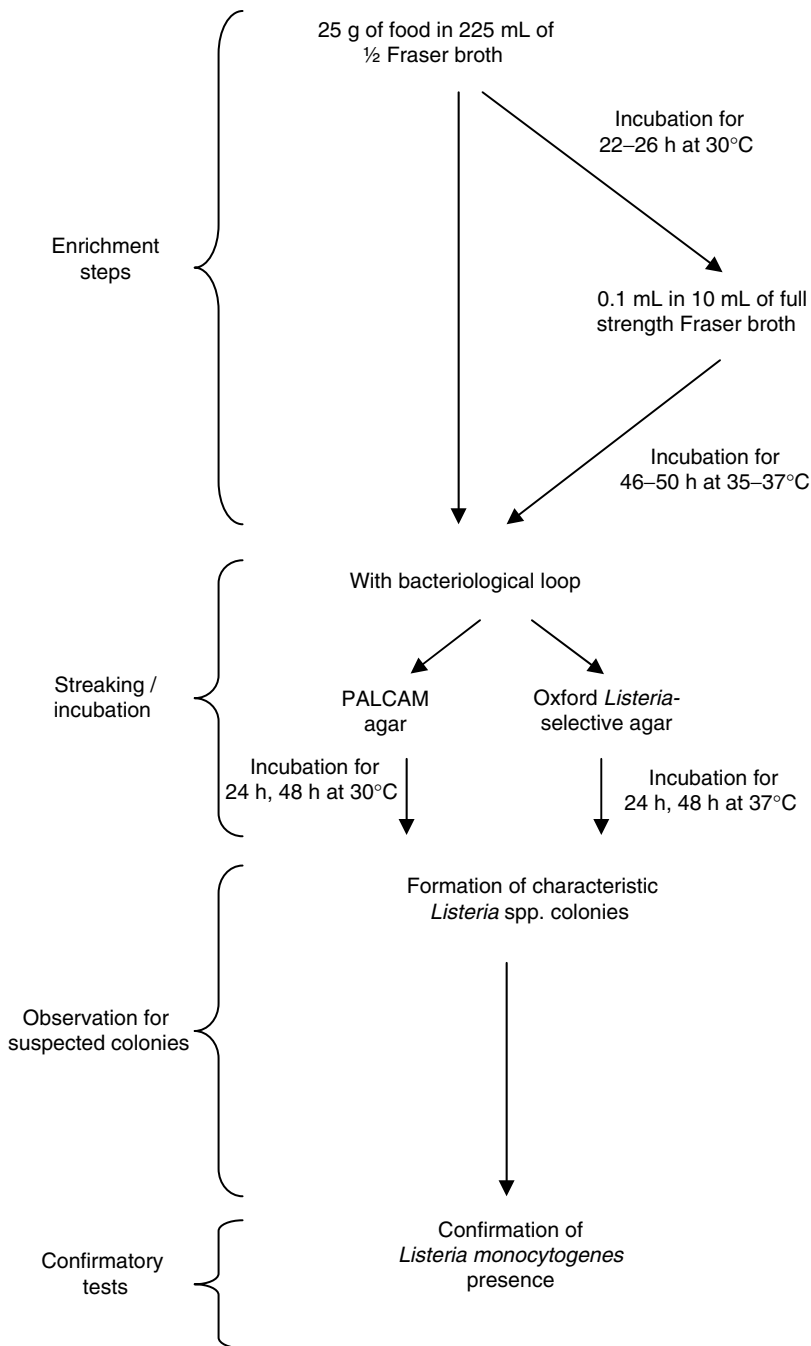


Figure 23.1 Cultural detection scheme of *L. monocytogenes* based on ISO standard. (Based on ISO. 1996. International Standard, ISO 11290-1: *Microbiology of food and animal feeding stuffs—Horizontal method for the detection and enumeration of Listeria monocytogenes—Part 1: Detection method*. Geneva: International Organization for Standardization.)

incorporating digoxigenin, or fluorescent markers allow detection of target sequences [45]. PCR combined with DNA hybridization in a microtiter plate is a convenient and highly sensitive and specific approach for detection of *Listeria* spp. in a high-throughput 96-well format [48]. Commercially available DNA hybridization tests are routinely used for food testing and have been proven to be extremely sensitive and accurate. In contrast to DNA hybridization, in which large amounts of DNA or RNA are necessary for detection, PCR provides amplification results starting from very small amounts of target DNA [45]. Detection using PCR is carried out after selectively enriching samples for 24–48 h. Multiplex PCR allows the simultaneous detection of more than one pathogen in the same sample, such as *L. monocytogenes* and *Salmonella* [49–50] or *L. monocytogenes* and other *Listeria* species [51–52]. This approach is most attractive for food analysis, where testing time, reagents, and labor costs are reduced. To detect only living pathogens, RNA can be used instead of DNA. The presence of specific RNA sequences is an indication of live cells. When an organism dies, its RNA is quickly eliminated, whereas DNA can last for years, depending on storage conditions. Klein and Juneja [53] used reverse transcription-PCR (RT-PCR) to detect live *L. monocytogenes* in pure culture and artificially contaminated cooked ground beef. DNA microarrays are a recent technique that has found applicability in the detection of *L. monocytogenes*. Call et al. [54] used probes specific for unique portions of the *16S rRNA* gene in *Listeria* spp. to demonstrate how each *Listeria* species can be differentiated by this method. In this procedure, PCR is first performed using universal primers to amplify all the *16S rRNA* genes present in a sample. The various amplified DNA fragments bind only to the probes for which they have a complementary sequence. Because one of the oligonucleotides used in the PCR contains a fluorescent label, the spots where the amplified DNA has bound fluoresce. Pathogens are identified by the pattern of fluorescing spots in the array [55]. Lampel et al. [56] and Sergeev et al. [57] claim that in pure culture the detection limit of the array is 200 *L. monocytogenes* cells. Sergeev et al. [57] also noted that the array is appropriate for detection of pathogens in food and environmental samples. Microarrays are able to identify a number of pathogens or serotypes at once, but they still require culture enrichment and PCR steps to improve sensitivity and specificity of detection [55].

23.4.2 Enumeration of *Listeria monocytogenes*

Cultural enumeration method of *L. monocytogenes* based on the International Organization for Standardization (ISO) method [58] is displayed in Figure 23.2. The method has a detection limit ≥ 100 cfu/g. If numbers of *Listeria* lower than 100 cfu/g are expected, then the following procedure might be applied, which allows detection equal to or above 10 cfu/g. One milliliter of sample from the first 1:10 dilution is spread on three PALCAM agar plates (0.333 mL on each agar plate) and after incubation the colonies on all three plates are measured as a single plate. However, if even lower *Listeria* concentration is expected (1 cfu/g), then the first dilution is made with 1 part of food sample and 4 parts of diluent (1:5) (SPW or half Fraser broth). SPW (0.1% peptone and 0.85% NaCl) or half Fraser broth have large buffer capacity, which favors the growth and repair of stressed or injured cells.

Traditional PCR methods are able to detect the presence of a pathogen but are not able to quantify the level of contamination. One way to approach this problem is the use of competitive PCR. In this method, a competitor fragment of DNA which matches the gene to be amplified is introduced into the sample. In general, the competitor fragment is synthesized as a deletion mutant that can be amplified by the same primers being used to amplify the target DNA. The

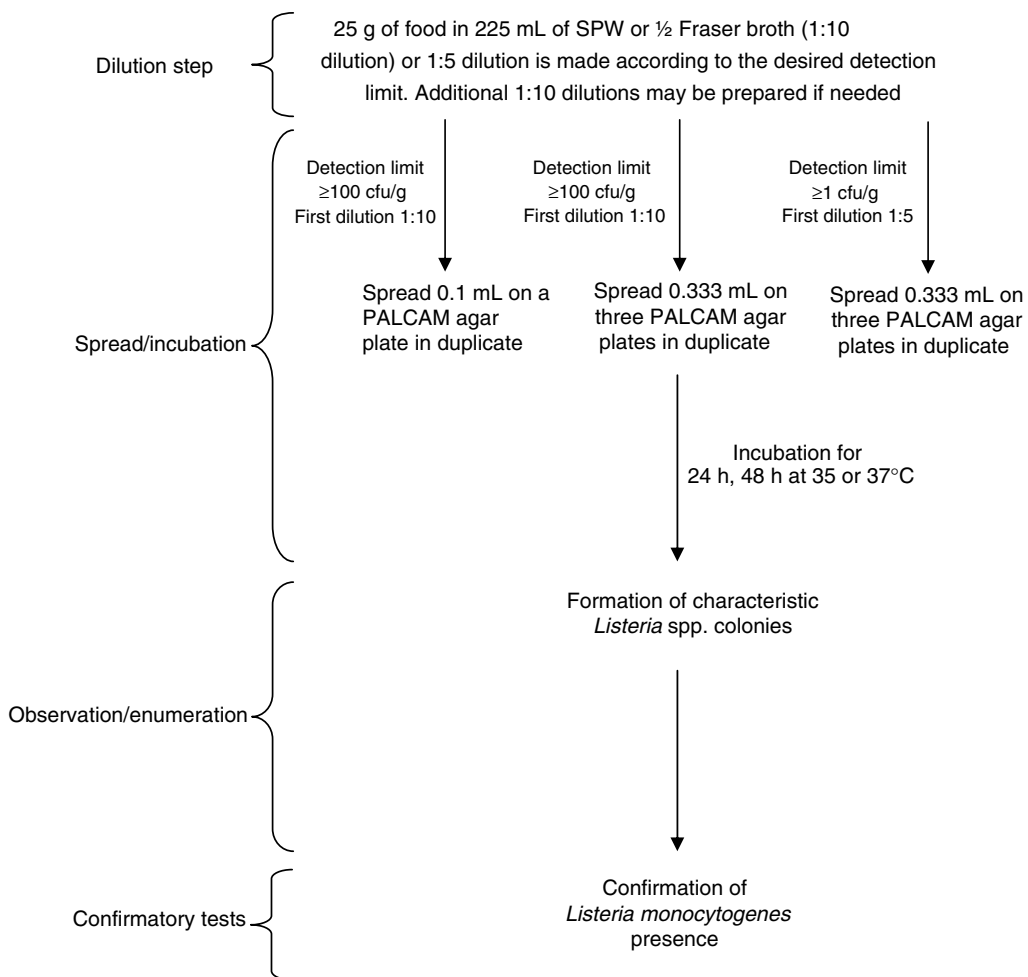


Figure 23.2 Cultural enumeration of *L. monocytogenes* based on ISO method. (Based on ISO. 1998. International Standard, ISO 11290-2: *Microbiology of food and animal feeding stuffs—Horizontal method for the detection and enumeration of Listeria monocytogenes—Part 2: Enumeration method*. Geneva: International Organization for Standardization.)

competitor fragment is distinguished from the pathogen gene fragment by its smaller size [55]. To determine the level of pathogen contamination, DNA purified from the food sample is serially diluted and added to a constant amount of competitor DNA. PCR is performed and the intensity of the pathogen's gene signal is compared to that of the competitor DNA on an agarose gel. The number of cells in the original sample can be estimated by comparing the intensity of the two DNA fragments (target versus competitor) using a standard curve [59]. Choi and Hong [60] used a variation of competitive PCR based on the presence of a restriction endonuclease site in the amplified gene for *L. monocytogenes* detection. The method was completed within 5 h without enrichment and was able to detect 10^3 cfu/0.5 mL milk using the *hlyA* gene as target. The detection limit could be reduced to 1 cfu if culture enrichment for 15 h was conducted first.

23.4.3 Confirmation of *Listeria monocytogenes*

L. monocytogenes presence is confirmed by the use of various biochemical tests. The tests are performed on purified cultures. From the PALCAM or Oxford agars, five suspected and randomly chosen colonies are isolated and streaked on tryptone soya agar containing 0.6% yeast extract (TSYEA). *Listeria* species are easily identified by Gram staining, motility, catalase, and oxidase reactions. *Listeria* spp. is Gram-positive, small rods, motile, catalase-positive, and oxidase-negative. The motility test should be performed in a semisolid TSYEA tube (TSYE broth or TSYEB supplemented with 0.5% agar) incubated at 25°C because at incubation temperatures above 30°C the motility test is negative (nonmotile). The tube is inoculated by stabbing and is observed for growth around the stab (a characteristic umbrella-like shape of turbidity is formed) [61]. Sugar fermentation, hemolysis, and the Christie–Atkins–Munch–Petersen (CAMP) test may be used to differentiate the *Listeria* species (Figure 23.3). *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* are β-hemolytic species on horse or sheep blood agar. The CAMP test distinguishes the three species

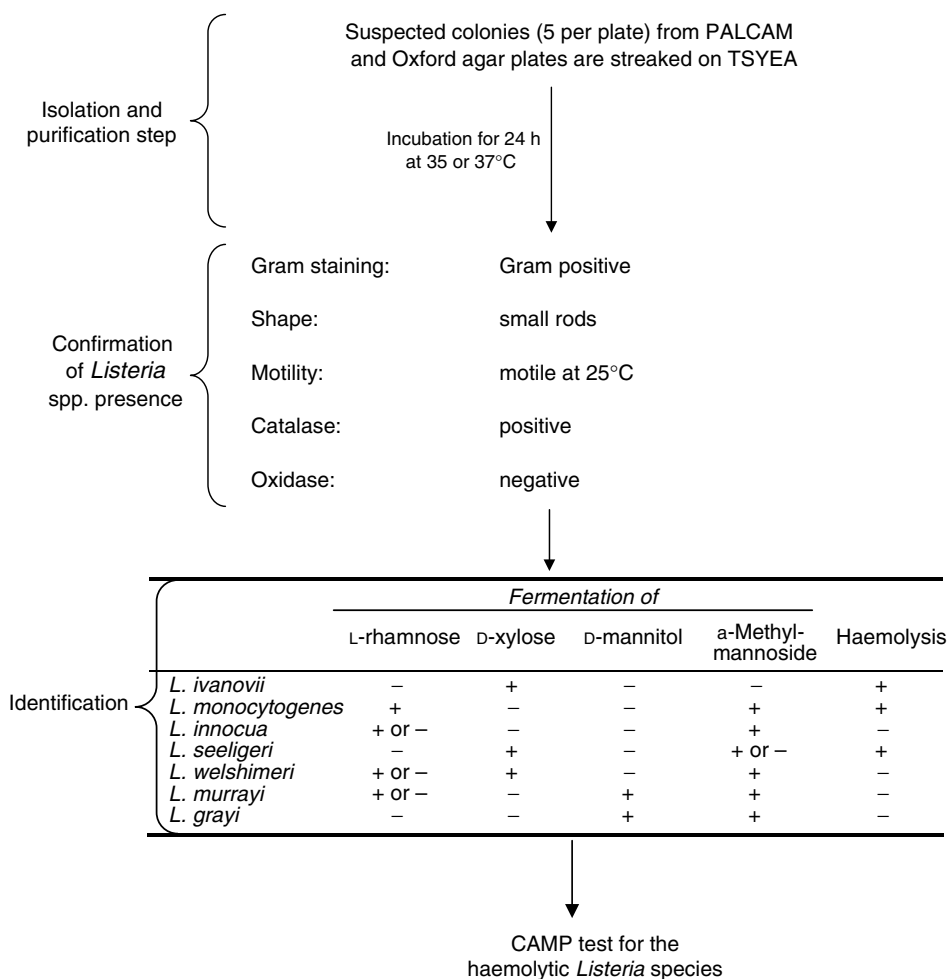


Figure 23.3 Confirmation scheme of *L. monocytogenes*.

of *Listeria* and should be done on sheep blood agar. An enhanced β -hemolysis zone is observed close to *S. aureus* NCTC 1803 when either *L. monocytogenes* or *L. seeligeri* are streaked on blood agar. *L. seeligeri* shows a less enhanced β -hemolysis zone than *L. monocytogenes*. *L. ivanovii* shows a wide enhanced β -hemolysis zone with *Rhodococcus equi* NCTC 1621. The plates are incubated at 37°C for no longer than 12–18 h. The *Listeria* isolates streaked on blood agar for the CAMP test are derived from the hemolysis plates used to examine the β -hemolysis property. The *Listeria* streaks should not touch the streaks of the *S. aureus* and *R. equi* control strains. The control strains are streaked parallel to each other and the suspected *Listeria* isolated in between the two streaks [45,61]. Alternatively, various commercial identification kits such as API 10 *Listeria* (BioMerieux, Marcy Etoile, France) might be used instead of traditional biochemical tests, which are time consuming. Finally, the previous selective agars, PALCAM and Oxford, may be substituted by other selective chromogenic media such as ALOA agar and RAPID' L. mono, as mentioned earlier in Section 23.2, which allow the direct differentiation between *Listeria* species by specific reactions on the agar plates [12,62]. In this way, the direct detection or enumeration of a specific *Listeria* species is feasible from the dilutions of the original sample.

23.5 *Escherichia coli* O157:H7

Pathogenic *E. coli* includes a variety of types having different pathogenicity based on the virulence genes involved. The different types of pathogenic *E. coli* are the enteropathogenic *E. coli* (EPEC), the enteroinvasive *E. coli* (EIEC), the enterotoxigenic *E. coli* (ETEC), the enteroaggregative *E. coli* (EAEC), and the enterohemorrhagic *E. coli* (EHEC) [63]. The latter belongs to verocytotoxigenic *E. coli* (VTEC), which produces verocytotoxins or shiga toxins. VTEC *E. coli* are of great concern because they include the most predominant foodborne pathogen *E. coli* O157:H7. The letters and numbers, for example, O157:H7, refer to the microorganism serogroup. The somatic antigens are designated with the letter “O” and the flagella antigens with the letter “H” [64]. *E. coli* O157:H7 can be found on raw and processed meat [65–68]. Most often it has been isolated from beef, which is believed to be the main vehicle for outbreaks associated with pathogenic *E. coli* O157:H7. The source of contamination of meat is usually the bovine feces or the intestinal tube during slaughtering. Their contact with muscle tissue results in meat contamination [64]. Heat treatment and fermentation processes are sufficient for producing a safe finished product. However, if these processes are not adequate, then *E. coli* O157:H7 may survive during manufacturing if the microorganism is present in the raw material [69–71]. Factors other than process may play significant roles in producing safe products, including the implementation of good manufacturing practices (GMP) or good hygiene practices (GHP) to avoid postprocess contamination [35,36,71]. For the detection of EPEC, EIEC, ETEC, and EAEC there is no standard sensitive procedure and usually the food sample is diluted in BHI broth, incubated at 35°C for 3 h to allow microbial cells to resuscitate. Then an enrichment step (at 44°C for 20 h) in tryptone phosphate broth and plating on Levine eosin–methylene blue agar and MacConkey agar are performed. Lactose-positive (typical) and lactose-negative (nontypical) colonies are collected for characterization using various biochemical, serological, or PCR-based tests [13].

23.5.1 Detection of *Escherichia coli* O157:H7

The cultural method for detecting and identifying *E. coli* O157:H7 [72] is shown in Figure 23.4. Pathogenic *E. coli* O157:H7 does not ferment sorbitol and does not possess β -glucuronidase,

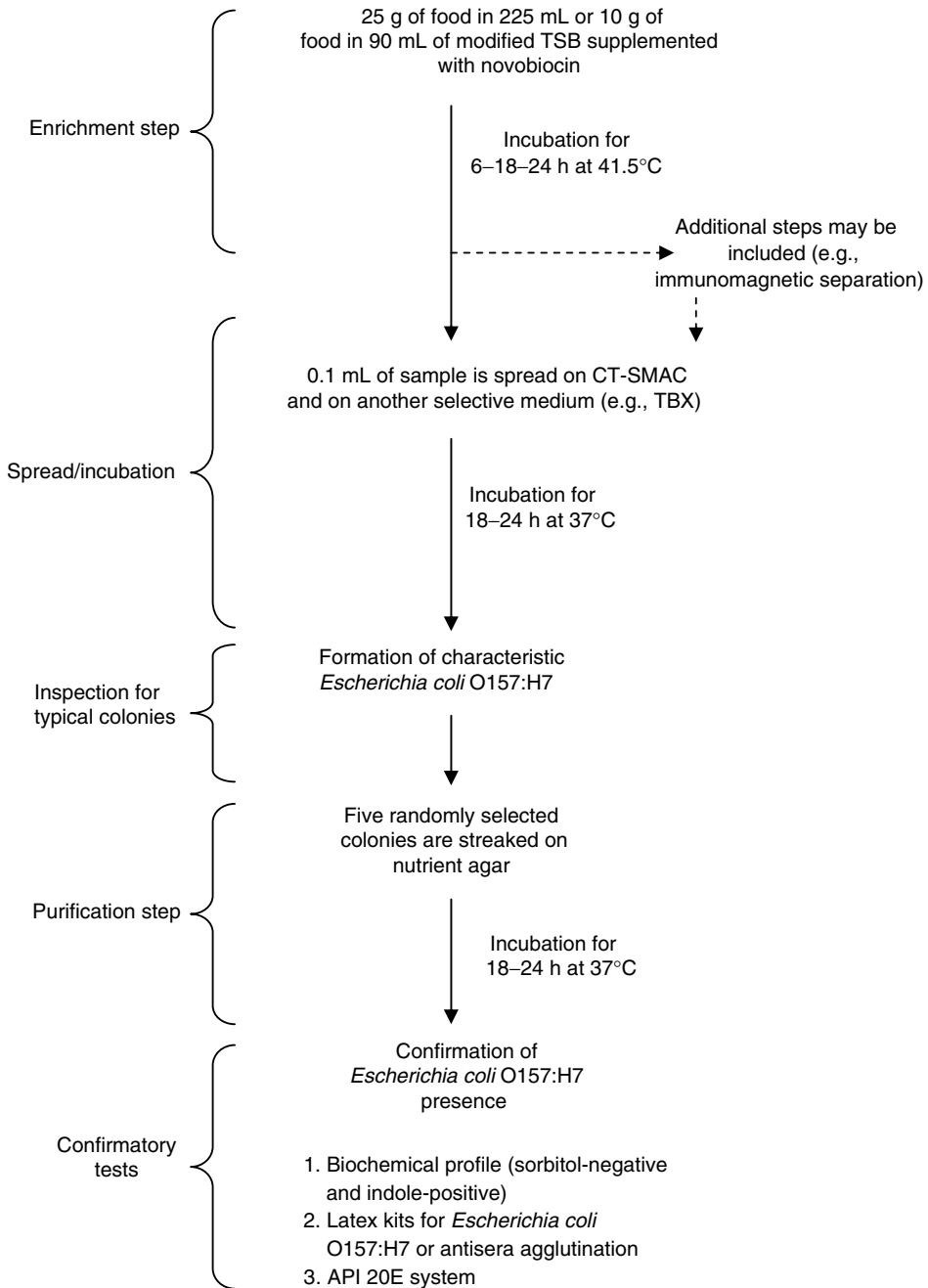


Figure 23.4 Cultural detection of *E. coli* O157:H7 based on ISO method. (Based on ISO. 2001. International Standard, ISO 16654: *Microbiology of food and animal feeding stuffs—Horizontal method for the detection of Escherichia coli* O157. Geneva: International Organization for Standardization.)

produced by almost all other *E. coli* strains [73]. The selective media exploit these attributes to distinguish the pathogenic *E. coli* O157:H7 from other, nonpathogenic *E. coli* strains. The method includes an enrichment step using a selective enrichment broth (tryptone soya broth [TSB] supplemented with novobiocin) to resuscitate the stressed cells and suppress the growth of the background flora.

Before plating onto agar plates, intermediate steps may be involved. The cell antigen O157:H7 is characteristic of the microorganism pathogenicity and therefore the IMS method (manufacturer instructions are followed to implement this technique) increases the detection of *E. coli* O157:H7 [74]. *E. coli* O157:H7 are captured on immunomagnetic particles, washed with sterile buffer, resuspended using the same buffer, and a sample of the washed and resuspended magnetic particles is inoculated on a selective medium to obtain isolated colonies.

The selective agar used to subculture the sample is the modified MacConkey agar containing sorbitol instead of lactose, as well as selective agents such as potassium tellurite and cefixime (CT-SMAC) [75] and the tryptone bile glucuronic medium (TBX) [63,64]. Because sorbitol-negative microorganisms other than *E. coli* O157:H7 may grow on the agar plates (such as *Proteus* spp. and some other *E. coli* strains), the addition of cefixime (which inhibits *Proteus* spp. but not *E. coli*) and tellurite (which inhibits *E. coli* strains other than *E. coli* O157:H7) substantially improves the selectivity of the medium [13]. CT-SMAC agar medium has been found the most effective for the detection of shiga toxin-producing *E. coli* O157:H7 [76]. Typical *E. coli* O157:H7 colonies are 1 mm in diameter and are colorless (sorbitol-negative) or pellucid with a very slight yellow-brown color. However, because sometimes *E. coli* O157:H7 forms colonies similar to other *E. coli* strains (pink to red surrounded by a zone), further purification (streaking) on nutrient agar and confirmation of the typical and nontypical colonies is required.

Biochemical methods require time; hence, PCR-based protocols, including multiplex PCR (MPCR), have been developed. Detection of STEC strains by MPCR was first described by Osek [77]. A protocol was developed using primers specific for genes that are involved in the biosynthesis of the O157 *E. coli* antigen (*rfb* O157), and primers that identify the sequences of shiga toxins 1 and 2 (*stx1* and *stx2*) and the intimin protein (*eaeA*) involved in the attachment of bacteria to enterocytes [25]. The different strains were identified by the presence of one to four amplicons [77]. More protocols have been developed and applied in the detection and identification of *E. coli* in feces and meat (pork, beef, and chicken) samples [78,79]. Later, Kadhum et al. [80] designed an MPCR to determine the prevalence of cytotoxic necrotizing factors and cytolethal distending toxin-producing *E. coli* on animal carcasses and meat products, from Northern Ireland, in a preliminary investigation into whether they could be a source of human infection.

23.5.2 Enumeration of *Escherichia coli* O157:H7

The cultural enumeration method of *E. coli* O157:H7 based on the ISO standard method [81] is presented in Figure 23.5. The key step in the case of stressed cells is the additional incubation period required (at 37°C for 4 h) before incubation at 44°C for 18–24 h. Typical *E. coli* O157:H7 colonies have a blue color, and plates with colonies (blue) less than 150 and less than 300 in total (typical and nontypical) are counted. The detection limit of the method is a population of 10 cfu/g.

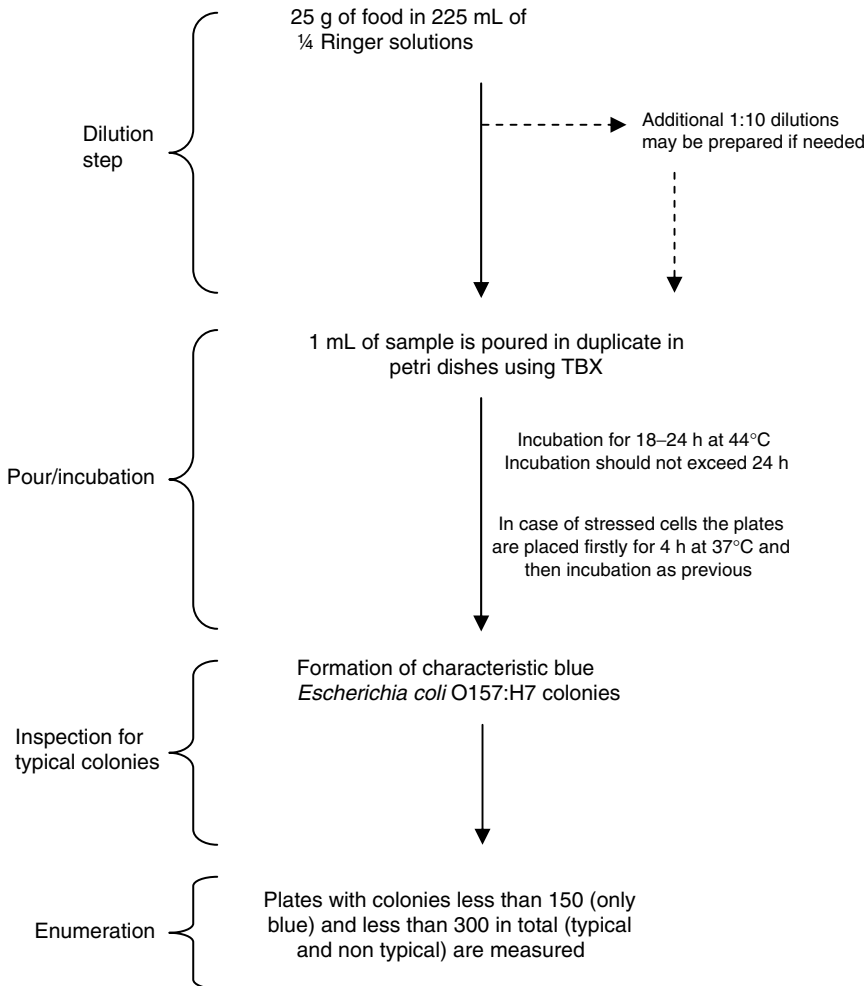


Figure 23.5 Cultural enumeration of *E. coli* O157:H7 based on ISO standard. (Based on ISO. 2001. International Standard, ISO 16649-2: *Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli—Part 2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide*. Geneva: International Organization for Standardization.)

23.5.3 Confirmation of *Escherichia coli* O157:H7

To confirm the presence of *E. coli* O157:H7, the following tests should be carried out. *E. coli* O157:H7 is negative to sorbitol, unlike most nonpathogenic *E. coli* strains, and indole positive. After defining the biochemical profile of the suspected colonies, latex kits for *E. coli* O157:H7 or antisera agglutination can be used to confirm *E. coli* O157:H7. Commercial kits such as API 20E (BioMerieux, Marcy Etoile, France) constitute an alternative for *E. coli* O157:H7 confirmation. *E. coli* O157:H7 toxins can be detected using reversed passive latex agglutination and cultured vero cells. Polymyxin B may be used in the culture to facilitate shiga toxin release [6].

23.6 *Salmonella* spp.

Salmonella spp. has been isolated from all types of raw meat including poultry, pork, beef, and lamb. All these products have been implicated in outbreaks of *Salmonella* spp. Most often, however, *Salmonella* spp. occurs in poultry and pork meat. The main source of contamination of the raw meat is the transfer of the microorganism from feces to the meat tissue during slaughtering and the following processing [82]. Postprocess contamination may also occur and, therefore, the GHP regarding equipment and personnel are essential.

23.6.1 Detection and Confirmation of *Salmonella* spp.

The cultural method for detecting and identifying *Salmonella* spp. [83] is depicted in Figure 23.6. The microbiological criterion for *Salmonella* spp. is “absence in 25 g.” The method includes two enrichment steps—a preenrichment step to allow injured cells to resuscitate and a selective enrichment step to favor the growth of *Salmonella* cells. In the first step, a nonselective but nutritious medium is used (buffered peptone water); in the second step, the selective medium contains selective agents to suppress the growth of accompanying microflora. Two different selective media are used in the second step because the culture media have different selective characteristics against the numerous *Salmonella* serovars [20]. Time and temperature of incubation during the preenrichment and selective enrichment steps play a significant role in the selectivity of the media. One of the selective media used in the second enrichment step has historically been a selenite cystine broth that contains a very toxic substance (sodium biselenite), and for this reason its use has been replaced by other media such as a Müller-Kauffmann tetrathionate/novobiocin (MKTn) broth. Rappaport-Vassiliadis soya peptone (RVS) broth is the standard Rappaport-Vassiliadis (RV) broth but with tryptone substituted by soya peptone because it has shown better performance than the standard broth [13]. The next step is plating of the samples on selective differential agars containing selective agents such as bile salts and brilliant green, which have various diagnostic characteristics (e.g., lactose fermentation, H₂S production, and motility) to differentiate *Salmonella* spp. from the other microflora such as *Proteus* spp., *Citrobacter* spp., and *E. coli*. The Oxoid Biochemical Identification System (OBIS) *Salmonella* test (Oxoid, Basingstoke, U.K.) is a rapid test to differentiate *Salmonella* spp. from *Citrobacter* spp. and *Proteus* spp. The principle of the test is based on the determination of pyroglutamyl aminopeptidase (PYRase) and nitrophenylalanine deaminase (NPA) activity, to which *Salmonella* spp. is negative, *Citrobacter* spp. is PYRase-positive and NPA-negative, and *Proteus* spp. NPA-positive and PYRase-negative. Selective agars differ in their selectivity toward *Salmonella*, and for this reason a number of media are used in parallel (xylose lysine desoxycholate [XLD] or xylose lysine tergitol-4 [XLT-4] and phenol red/brilliant green agar). The last steps include biochemical and serological confirmation of suspected *Salmonella* colonies to confirm the identity and to identify the serotype of the isolates [13,84]. *Salmonella* spp. is lactose-negative, H₂S-positive, and motile. However, lactose-positive strains have been isolated from human infections, and an additional selective medium agar may therefore be needed. Bismuth sulfite agar is considered as the most suitable medium for such strains [13,85,86].

The most frequently isolated serovars from foodborne outbreaks are *S. typhimurium* and *S. enteritidis*. Traditional phenotypic methods such as biotyping, serotyping, and phage typing of isolates, as well as antimicrobial susceptibility testing, provide sufficient information for

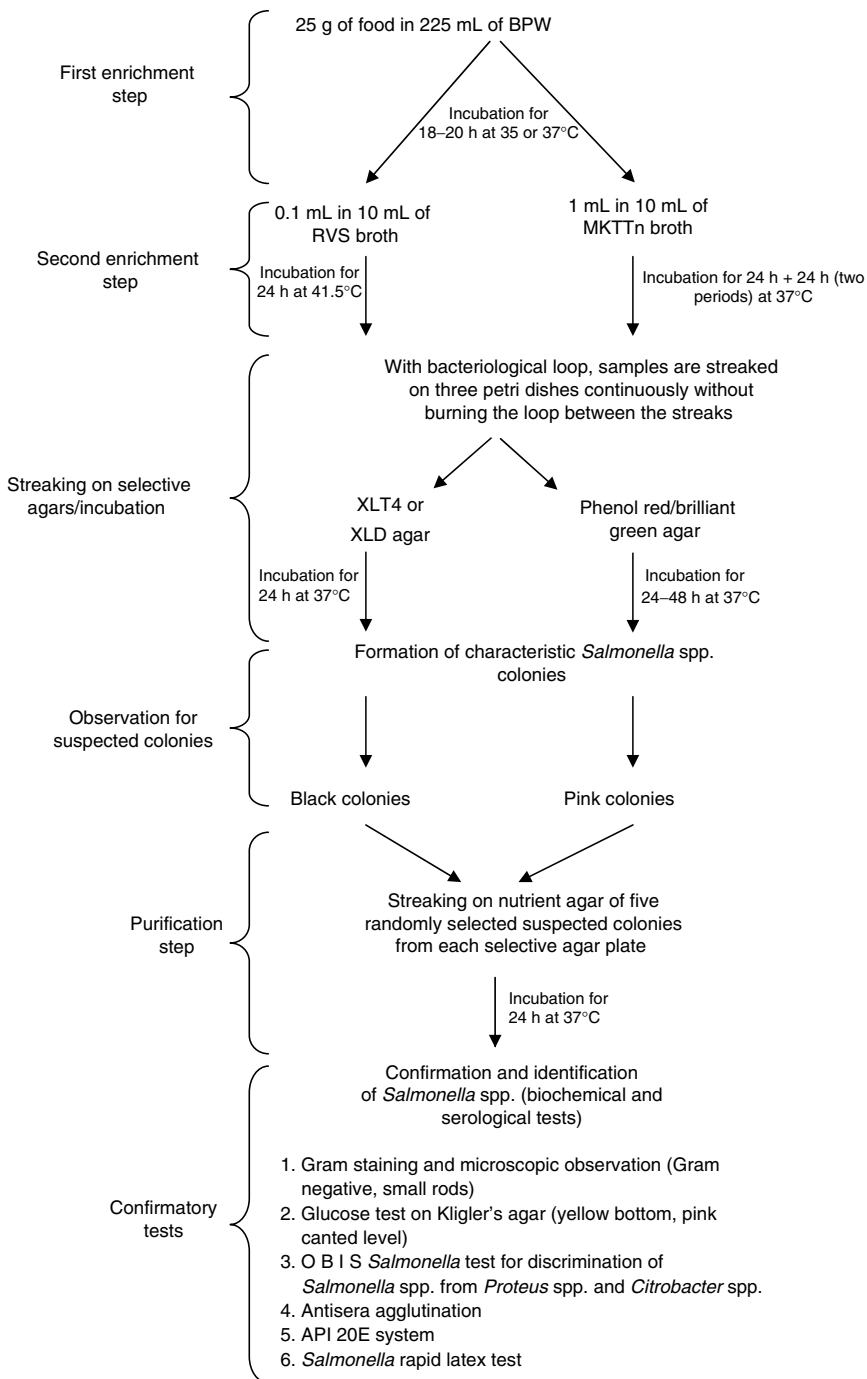


Figure 23.6 Cultural detection and identification scheme of *Salmonella* spp. based on ISO method. (Based on ISO. 1993. International Standard, ISO 6579: *Microbiology of food and animal feeding stuffs—Horizontal method for the detection of Salmonella spp.* Geneva: International Organization for Standardization.)

epidemiological purposes. Molecular genetic methods have revolutionized the fingerprinting of microbial strains. However, not all of them have been internationally standardized, and problems in interpreting the results of different laboratories might occur. Nevertheless, the accuracy and speed at which results are obtained have rendered them more and more applicable.

The assay generally used to identify *Salmonella* serovars is represented by a serological method which requires the preparation of specific antibodies for each serovar and is thus extremely complex and time consuming [25]. Plasmids are characteristic of *Salmonella* and therefore plasmid analysis can often be used to differentiate strains [87]. A faster alternative involves PCR approaches. On the basis of primers designed for detecting O4, H:i, and H:1,2 antigen genes from the antigen-specific genes *rfbJ*, *fliC*, and *fljB* (coding for phase 2 flagellin), respectively, Lim et al. [88] described an MPCR for the identification of *S. typhimurium*, whose presence was associated with the appearance of three amplification products. MPCR targeted to the *tyv* (CDP-tyvelose-2-epimerase), *pri* (paratose synthase), and *invA* (invasion) genes were designed to identify *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A by the production of three or two bands, respectively [89]. PCR amplifications of the 16S–23S spacer region of bacterial rRNA as well as specific monoclonal antibodies to the lipopolysaccharide of *S. typhimurium* DT104 have been used [90].

23.7 *Staphylococcus aureus*

Reservoirs of the *S. aureus* microorganism are the animals in which it is part of their normal microflora. Food contamination with *S. aureus* may occur through humans, who also carry staphylococci. Food poisoning by *S. aureus* is the result of ingestion of food containing staphylococcal enterotoxin(s). Enterotoxin is a heat-stable substance, and high cell numbers are required to produce sufficient amounts of toxin. Temperatures above 15°C favor the rapid growth of the microorganism and the production of enterotoxin. The minimum temperatures for microorganism growth and enterotoxin production are 7 and 10°C, respectively. Attention is required in the implementation of GMP and GHP to minimize the contamination of raw materials with *S. aureus* and to avoid postprocess contamination of processed meat products since staphylococci are part of the natural microflora of humans and animals [91].

23.7.1 Enumeration and Confirmation of *Staphylococcus aureus*

The cultural enumeration method of *Staphylococcus* spp. based on ISO [92] is shown in Figure 23.7. The method has a detection limit ≥ 100 cfu/g. If lower numbers of staphylococci than 100 cfu/g are expected, then the procedure followed for *L. monocytogenes* enumeration may be applied. Low numbers of *S. aureus* are of little significance because extensive growth is needed in order for the microorganism to produce sufficient amounts of enterotoxin, and therefore an enrichment step is not required for its isolation. The most widely used and accepted medium for *S. aureus* is the Baird-Parker (BP) agar [93] (egg yolk–glycine–potassium tellurite–sodium pyruvate). Sodium pyruvate assists the resuscitation of stressed cells, while potassium tellurite, glycine, and lithium chloride enhance the medium's selectivity. *Staphylococcus* spp. forms black colonies (tellurite reduction), and *S. aureus* colonies are also surrounded by a halo (clearance of egg yolk due to lipase activity). Plates having 15–300 colonies in total (*Staphylococcus* spp. and *S. aureus*, if present) are measured. A coagulase test, reversed-passive latex agglutination test, or ELISA methods for

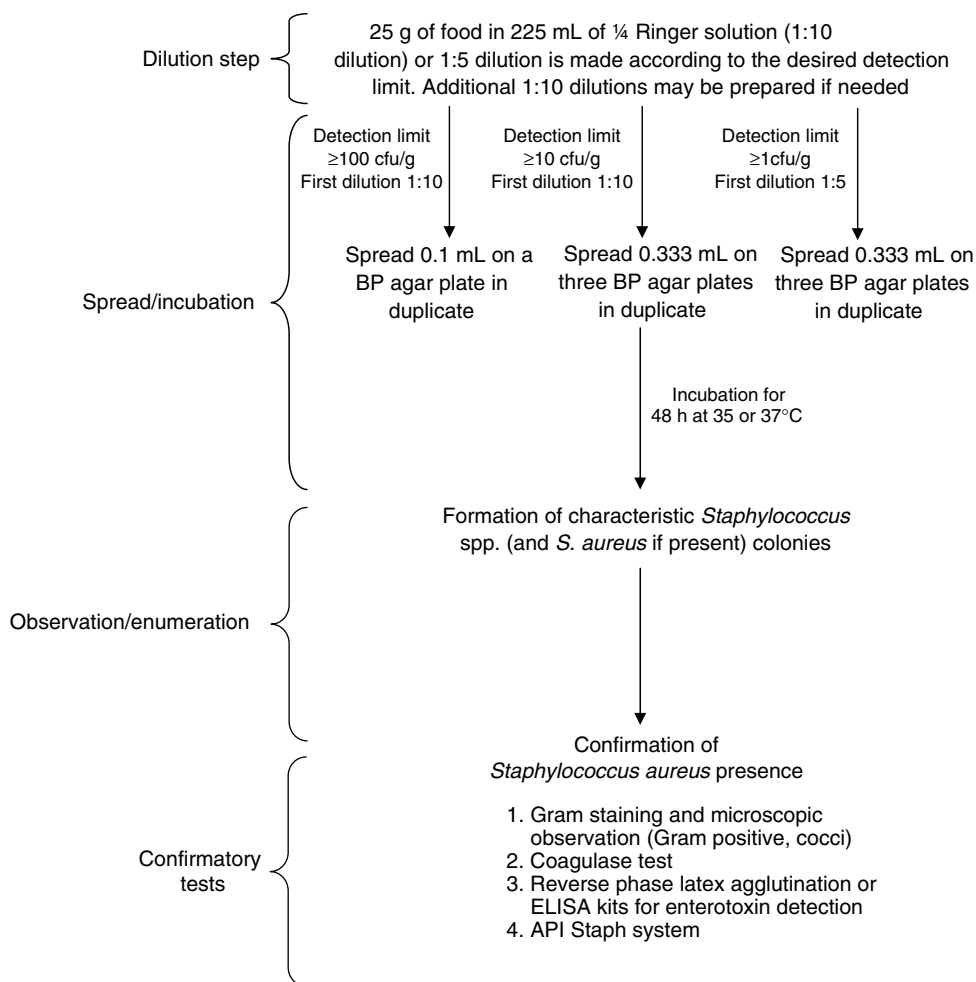


Figure 23.7 Cultural detection and confirmation of *S. aureus* presence based on ISO method. (Based on ISO. 1999. International Standard, ISO 6888-1: *Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species)—Part 1: Technique using Baird-Parker agar medium*. Geneva: International Organization for Standardization.)

enterotoxin detection may be used as confirmatory tests for *S. aureus* presence. The coagulase test is considered positive for enterotoxin presence only in case of a strong positive reaction. API Staph (BioMerieux, Marcy Etoile, France) may be also used to identify the isolated colonies from the agar plates [94].

Molecular techniques have been applied in the case of *S. aureus* to quickly determine its presence and identification. Occasionally, isolates of *S. aureus* give equivocal results in biochemical and coagulase tests [95]. Most *S. aureus* molecular identification methods have been PCR-based. Primers targeted to the nuclease (*nuc*), coagulase (*coa*), protein A (*spa*), *femA* and

femB, *Sa442*, 16S rRNA, and surface-associated fibrinogen-binding genes have been developed [96,97].

S. aureus food poisoning is caused by ingestion of preformed toxins (*Staphylococcus aureus* enterotoxins [SEs]) produced in foods. It has been reported that nearly all SEs are superantigens and are encoded by mobile genetic elements including phages, plasmids, and pathogenicity islands [98,99]. Several methods for SE detection from isolated strains and foods have been described in the recent years; these include biological, immunological, chromatographical, and molecular assays [100,101]. The four SEs originally described can be detected with commercial antisera or by PCR reactions [102,103].

Detection and identification of methicillin-resistant *S. aureus* (MRSA) has gained great attention since in immunocompromised patients it can cause serious infections which may ultimately lead to septicaemia. Since MRSA strains mainly appear in nosocomial environments, most of the techniques developed for their detection are focused on clinical or blood isolates [104]. Such techniques include DNA probes [27,105], peptide nucleic acid probes [106], MPCR [97], real-time PCR [107–109], LightCycler PCR [108,109], and a combination of fluorescence *in situ* hybridization and FCM [110]. Recent advances include the development of segment-based DNA microarrays [104]. Although, as mentioned earlier, MRSA strains are mainly encountered in nosocomial environments, food can be considered an excellent environment for introducing pathogenic microorganisms in the general population, especially in immunocompromised people and in the intestinal tract, transfer of resistant genes between nonpathogenic and pathogenic or opportunistic pathogens could occur [111]. A community-acquired case was reported in 2001, in which a family was involved in an outbreak after ingesting MRSA with baked port meat contaminated by the handler [112]. Therefore, the techniques applied in different samples might have applicability in food products.

23.8 *Yersinia enterocolitica*

Infections with *Y. enterocolitica* involve meat and meat products. In particular, pork meat has been implicated in *Y. enterocolitica* outbreaks (yersiniosis). Not all *Y. enterocolitica* strains cause illness. The most common serotypes causing yersiniosis are the serotypes O:3, O:9, O:5,27, and O:8. Because contamination of meat with high numbers of *Y. enterocolitica* may occur during pre-process (e.g., slaughtering), precautionary measures such as GHP are essential [113]. Contamination with *Y. enterocolitica* is a serious concern due to its ability to grow at refrigerated temperatures (4°C) [13,91].

23.8.1 Detection and Confirmation of *Yersinia enterocolitica*

The cultural method for detecting *Y. enterocolitica* [114] is presented in Figure 23.8. The method involves elements of the methods from Schiemann [115,116], the Nordic Committee on Food Analysis [117], and Wauters et al. [118]. If specific serotypes are considered (e.g., O:3), then two isolation procedures are proposed to run in parallel [13]. The procedure involving enrichment with irgasan–ticarcillin–potassium chlorate (ITC) broth is selective for serotype O:3 and possibly O:9. However, poor recovery of the serotype O:9 from ground pork using ITC has been found by De Zutter et al. [119]. After enrichment with ITC, plating of the samples should be done on *Salmonella–Shigella* sodium deoxycholate calcium chloride (SSDC) instead of cefsulodin irgasan

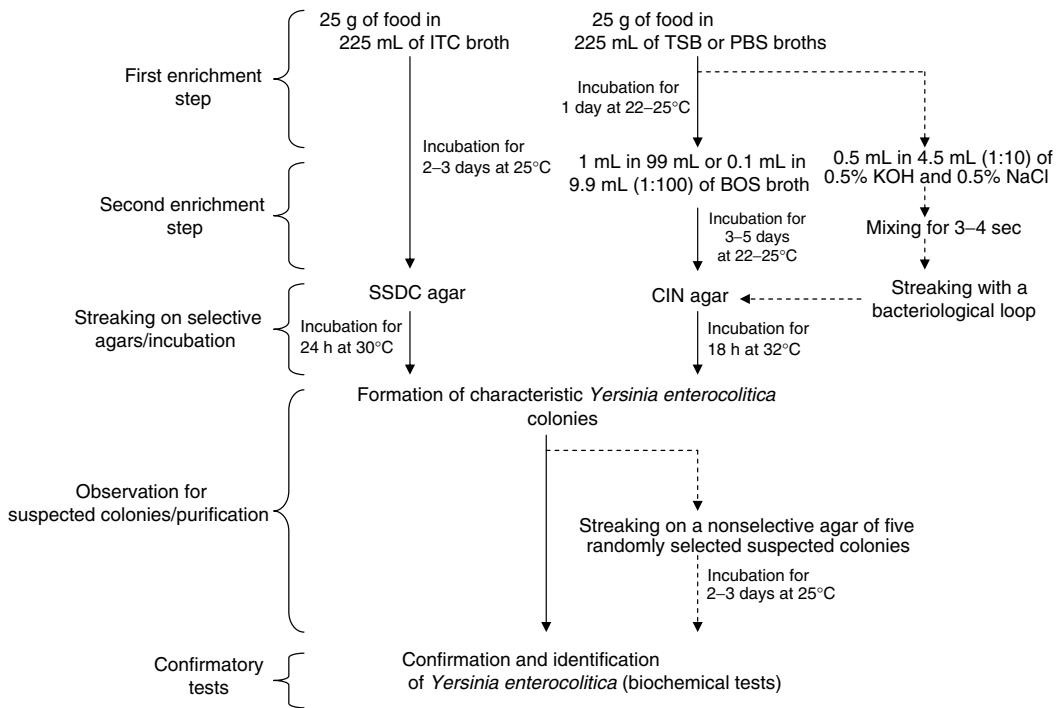


Figure 23.8 Cultural detection of *Y. enterocolitica* based on ISO method. (Based on ISO. 1994. International Standard, ISO 10273: *Microbiology of food and animal feeding stuffs—Horizontal method for the detection of presumptive pathogenic Yersinia enterocolitica*. Geneva: International Organization for Standardization.)

novobiocin (CIN) because the latter medium is inhibitory for the serotype O:3. Furthermore, the isolation and identification of *Y. enterocolitica* from ground meat on CIN medium agar has been proved to cause problems because many typical *Yersinia*-like colonies may grow [120]. After enrichment (primary) with TSB or peptone sorbitol bile salts (PBS) broth (peptone buffered saline with 1% sorbitol and 0.15% bile salts), an alkali treatment (potassium hydroxide [KOH]) may be used to increase recovery rates of *Yersinia* strains instead of secondary enrichment with bile oxalate sorbose (BOS) [121]. This method should not be used with the procedure involving the ITC broth as a selective enrichment step [122]. On SDC agar, the *Yersinia* colonies are 1 mm in diameter, round, and colorless or opaque. On CIN agar, the colonies have a transparent border with a red circle in the center (bull's eye).

Yersinia strains and *Y. enterocolitica* serotypes may be distinguished using biochemical tests. *Y. enterocolitica* may be identified using urease and citrate utilization tests, and fermentation of the following sugars: sucrose, raffinose, rhamnose, α -methyl-D-glucoside, and melibiose. *Y. enterocolitica* is urease and sucrose positive, but negative in the other tests. The most frequently used tests to identify pathogenic *Y. enterocolitica* strains are calcium-dependent growth at 37°C, Congo red binding on Congo red magnesium oxalate (CR-MOX) agar, or low-calcium Congo red BHI agarose agar (CR-BHO), which determine the Congo red dye uptake, pyrazinamidase activity, and salicin–esculin fermentation [115,122–126]. Because the last two tests are not plasmid dependent

as are the other tests, the pyrazinamidase, salicin, and esculin tests are considered the most reliable biochemical screening tests for pathogenicity because plasmids may be lost during subculture. Before testing, suspected colonies may be subcultured on a nonselective medium incubated at 25°C to reduce the risk of plasmid loss [122]. Pathogenic strains are negative to these three tests. Esculin fermentation and pyrazinamidase activity tests should be conducted at 25°C, whereas salicin fermentation is conducted at 35 or 37°C. Commercial kits for *Y. enterocolitica* identification such as API 20E (BioMerieux, Marcy Etoile, France) also may be used as an alternative that has been proved to be suitable for routine laboratory diagnostics [120].

From a food hygiene point of view, *Y. enterocolitica* is of major importance and is a very heterogeneous species. Nonpathogenic strains may contaminate food products to the same extent as pathogenic *Y. enterocolitica*, and a principal goal for nucleic acid–based methods has been to separate this group of pathogenic bacteria. Both polynucleotide and oligonucleotide probes, as well as PCR-based methods, have been applied for its detection and quantification in meat and meat products [127,128]. Nested-PCR has also been developed for its detection in meat food products and can satisfactorily detect pathogenic *Y. enterocolitica* even in the presence of a high background of microflora [129]. Comparative genomic DNA (gDNA) microarray analysis has recently been developed to differentiate between nonpathogenic and pathogenic biotypes [130].

23.9 *Bacillus cereus*

B. cereus can be found in meat and especially in dishes containing meat. Outbreaks attributed to *B. cereus* infections have also been associated with cooked meats. Its presence in food is not considered significant since high numbers ($>10^5$ – 10^6 cfu/g) are needed to cause a diarrheal or emetic syndrome. The two types of illness are caused by an enterotoxin (diarrheagenic or emetic) produced by the microorganism. Because other *Bacillus* species are closely related physiologically to *B. cereus*, including *B. mycoides*, *B. thuringiensis*, and *B. anthracis*, further confirmatory tests are required to differentiate typical *B. cereus* (egg yolk reaction, inability to ferment mannitol) from the other species [131].

23.9.1 Enumeration and Confirmation of *Bacillus cereus*

The presence of low numbers of *B. cereus* is not considered significant, and thus an enrichment step is not needed unless *B. cereus* growth is likely to occur (Figure 23.9). However, if enrichment must be applied, this can be done using BHI broth supplemented with polymyxin B and sodium chloride [132]. To enhance selection of *B. cereus*, the following attributes of the microorganism are employed: its resistance to the antibiotic polymyxin, the production of phospholipase C causing turbidity around colonies grown on agar containing egg yolk, and its inability to ferment mannitol. The media used for selection are usually the mannitol–egg yolk–polymyxin (MYP) [133] and the Kim-Goepfert (KG) agars [134]. Because of the similarity in composition and functionality of the KG medium with the polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) [132,135], the latter medium may be used instead of KG [131].

Colonies on MYP agar have a surrounding precipitate zone (turbidity) and both colonies and zone are pink (no fermentation of mannitol). On PEMBA agar, the colonies are peacock blue with a blue egg yolk precipitation zone. Finally, on KG agar the colonies are translucent or white

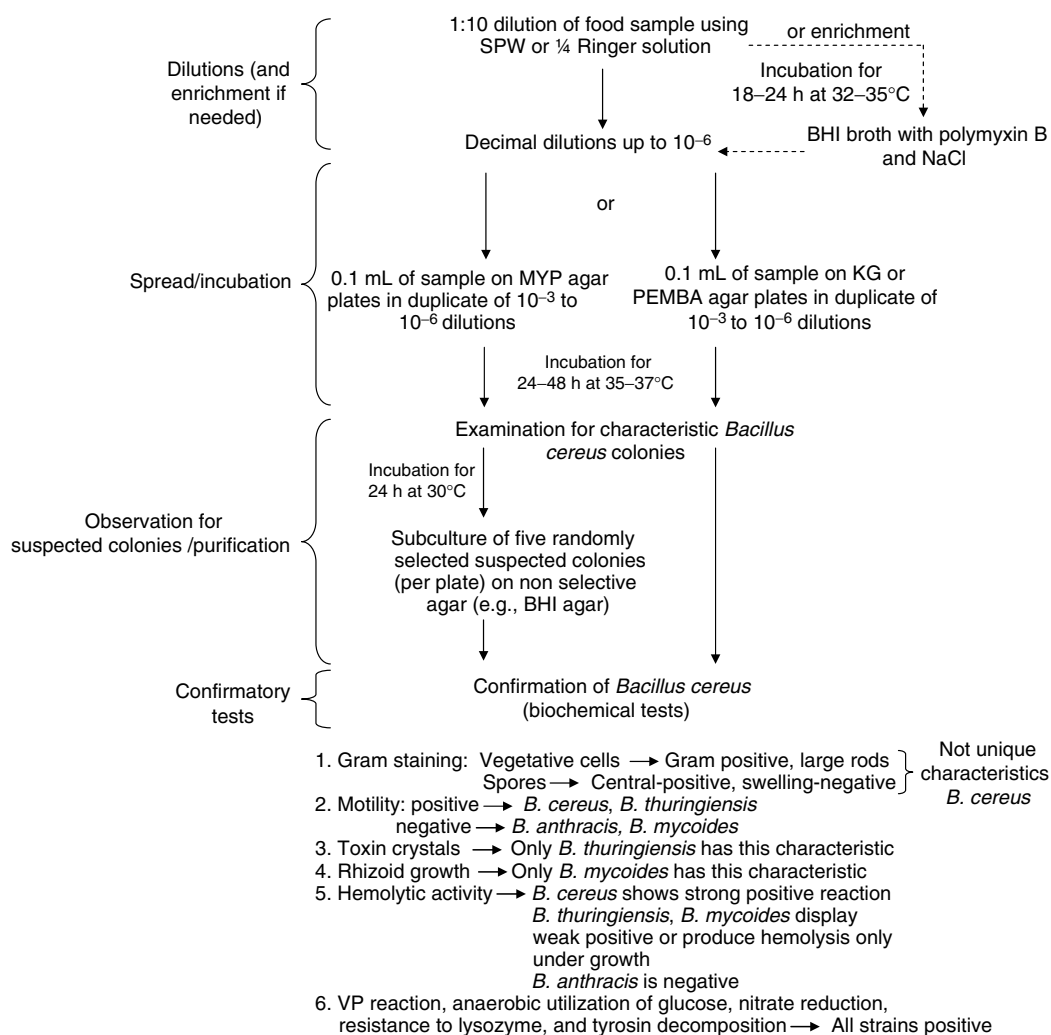


Figure 23.9 Cultural detection and identification of *B. cereus*.

cream. Plates having 10–100 colonies per plate are counted instead of 30–300 colonies per plate because turbidity zones may overlap each other and measurement of the colonies with a precipitation zone may be difficult. For low numbers (<100–1000 cfu/g) of *B. cereus* in the food sample, the MPN technique may be used. A suitable medium for this purpose is the trypticase soy polymyxin broth. Each of three tubes of 1:10, 1:100, and 1:1000 is inoculated with 1 mL of sample and the tubes are incubated at 30°C for 48 h and examined for tense turbidity. Confirmation of *B. cereus* presence is required before determining the MPN [131–132]. If only spores are to be counted, the sample is heated (the initial 1:10 dilution is heated for 15 min at 70°C) or treated with alcohol (1:1 initial dilution in 95% ethyl alcohol for 30 min at room temperature) to kill the vegetative cells, and the detection and identification scheme is followed (Figure 23.9). Potential emetic strains can be identified using the identification kit from BioMerieux called API 50CHB

(BioMerieux, Marcy Etoile, France) [136]. Before testing isolated colonies for *B. cereus* identity, the culture should be purified on a nonselective agar (e.g., BHI agar) to promote sporulation. Isolated colonies grown on KG agar, used as a selective agar, may be tested directly because KG medium favors sporulation.

ELISA and reverse passive latex agglutination (RPLA) tests are commercially available for *Bacillus* diarrheal enterotoxin. No tests have been developed for emetic enterotoxin due to purification problems, although tissue culture assay using HEp-2 cells may be useful for the detection and purification of the emetic toxin [131,136].

Several molecular techniques have also been developed for the detection and characterization of *B. cereus* derived from food products. Immunological methods for semiquantitative identification of enterotoxins are available (ELISA, RPLA), which demand at least 2 days to obtain a result, since enterotoxin expression during growth is necessary [137]. Although genetic probes are also applied for detection of *B. cereus*, the information provided would involve the presence of the gene and not the level of enterotoxin production. It seems that the production of enterotoxins from enterotoxin-positive strains is too low to cause food poisoning [138]. A good choice for the detection of *B. cereus* would be the use of probes directed to the phospholipase C genes, which are present in the majority of the strains. Different confirmatory tests exist for *B. cereus*. For enterotoxic *B. cereus*, molecular diagnostic (PCR-based) [139,140], biochemical, and immunological assays [139,141,142] are commercially available. Three methods for detection of the emetic toxin have been described during the past years—a cytotoxicity assay, liquid chromatography-mass spectrometry (LC-MS) analysis, and a sperm-based bioassay [143,144]. They have, however, proved difficult to use for routine applications and are not specific enough. Recently, a novel PCR-based detection system has been developed based on the emetic toxin cereulide gene [145].

The latest trend is toward the development of molecular tools that would be able to characterize virulence mechanisms of bacterial isolates within minutes [146]. The next generation assays, such as biosensors and DNA chips, have already been developed [147]. They can be classified in high-density DNA arrays [148] and low-density DNA sensors [149]. An automated electrochemical detection system, which allows simultaneous detection of presently described toxin-encoding genes of pathogenic *B. cereus* [146], and a nanowire labeled direct-charge transfer biosensor capable of detecting *Bacillus* species have also been developed [150].

23.10 *Clostridium perfringens*

Foods usually associated with *C. perfringens* infections are cooked meat and poultry. Its presence in raw meats and poultry is not unusual. The illness (diarrhea) is caused by a heat-sensitive enterotoxin produced only by sporulating cells. Usually, large numbers of the microorganism are required to cause illness. As a consequence, the microorganism is enumerated using direct plating without enrichment. Also, *C. perfringens* does not sporulate in food and therefore there is no need to heat the sample before enumerating the microorganism [151].

23.10.1 *Enumeration and Confirmation of Clostridium perfringens*

The selective media used for enumeration of *C. perfringens* contain antibiotics to inhibit other anaerobic microorganisms, along with iron and sulfite because *Clostridia* reduce the latter to sulfide, which reacts with iron to form a black precipitate (black colonies) characteristic of clostridia.

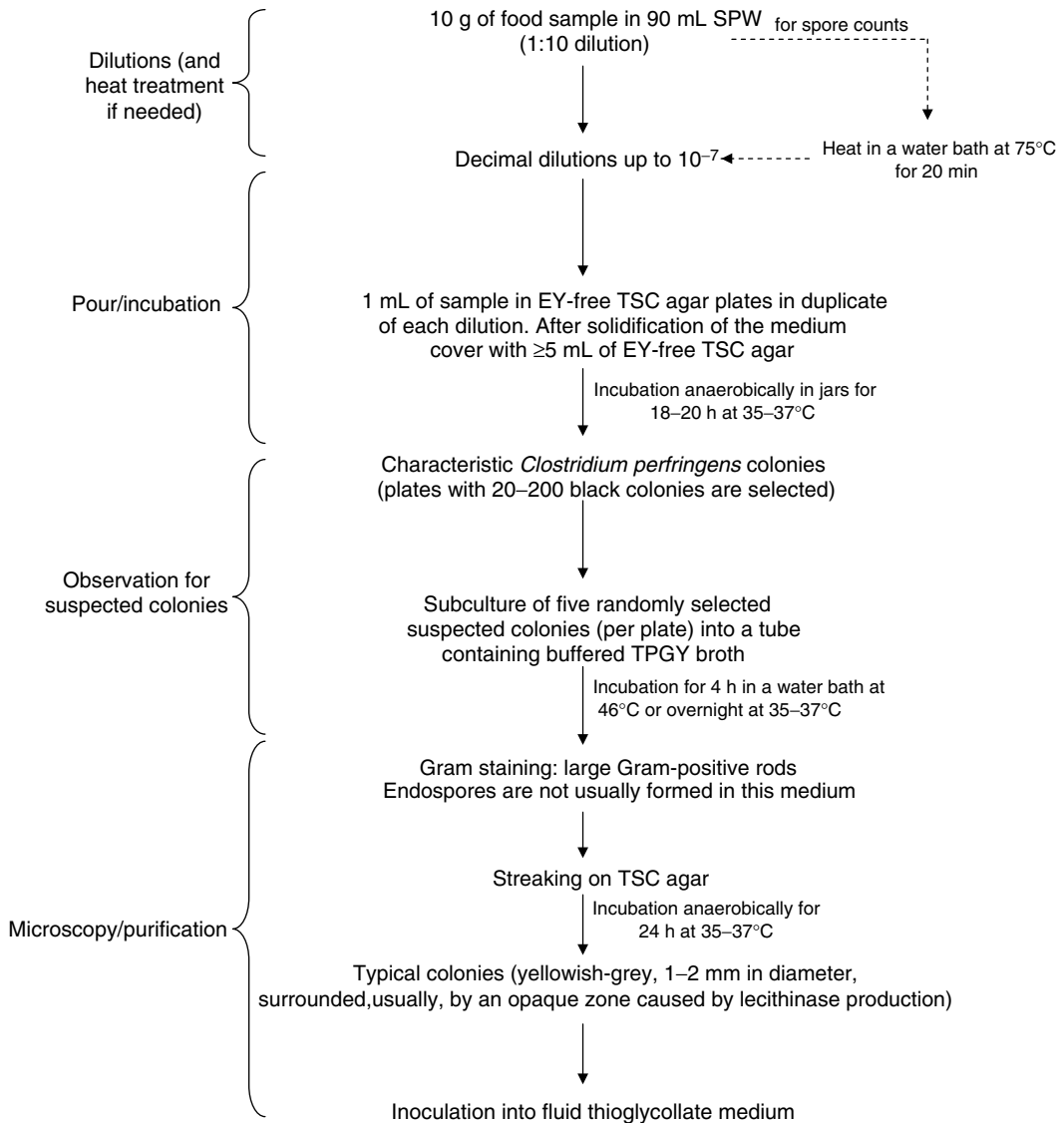


Figure 23.10 Cultural detection of *C. perfringens*.

The most commonly used and useful medium to recover *C. perfringens* is the egg yolk free tryptose sulfite cycloserine (EY-free TSC) agar (Figure 23.10) [152]. EY-free TSC agar is used in pour plates. Cycloserine is added to inhibit growth of *Enterococci*. Because other sulfite-reducing clostridia that produce black colonies may grow on EY-free TSC agar, further confirmatory tests are needed to identify the presence of *C. perfringens* (Figure 23.11). If low numbers are expected, the MPN technique or enrichment using buffered trypticase peptone glucose yeast extract (TPGY) broth may be used. Two grams of food sample is inoculated into 15–20 mL of medium in a tube. The tube is incubated at 35–37°C for 20–24 h. With a bacteriological loop a sample from the

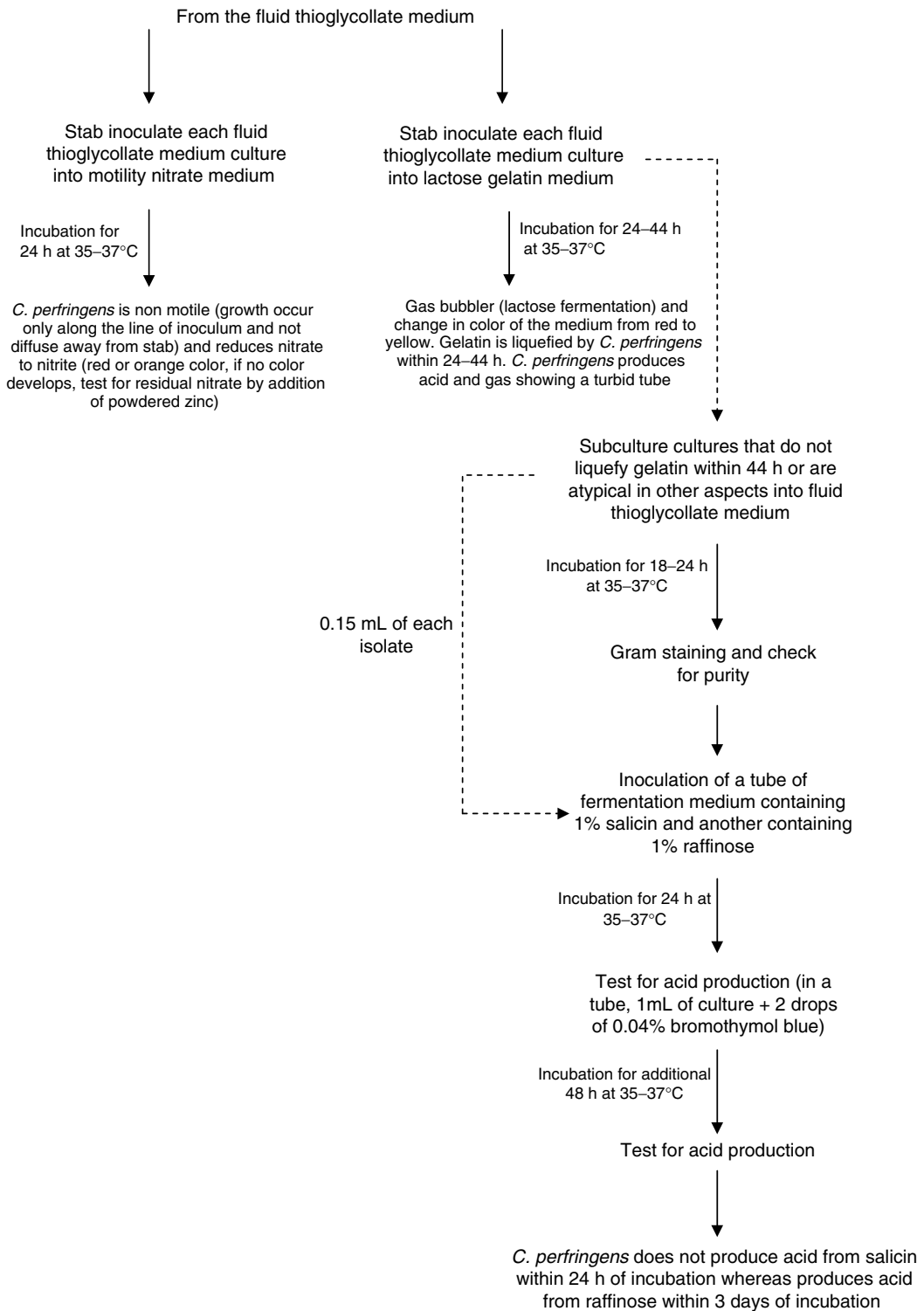


Figure 23.11 Identification scheme of *C. perfringens*.

positive tubes (turbidity and gas production) is streaked on EY-free TSC agar plates [151]. Enterotoxin of *C. perfringens* can be detected using commercial kits such as ELISA and RPLA.

A nonisotopic colony hybridization technique has been developed for the detection and enumeration of *C. perfringens*; this proved to be more sensitive than the conventional culture methods [153]. It provides quantitative assessment of the presence of potentially enterotoxigenic strains of *C. perfringens* as determined by the presence of the enterotoxin A gene, and the results are acquired within 48 h. A multiplex PCR assay has also been developed for the detection of *C. perfringens* type A [154] and has been evaluated in relation to American retail food by Wen et al. [155]. Methods similar to the ones described earlier for *B. cereus* [137] have also been applied.

23.11 *Campylobacter jejuni*

Campylobacter species are part of intestinal tract microflora of animals and thus may contaminate foods such as meat, poultry, and their products. The most frequent *Campylobacter* species implicated in illnesses is *C. jejuni*. The microorganism is Gram-negative, motile, and oxidase-positive, forming curved rods. Poultry is considered the most important vehicle of *Campylobacter* illness; several outbreaks have been associated with poultry [156,157]. *C. coli* and *C. lari* have also been isolated from poultry and recognized as potential hazards to human health, causing illness, though less frequently than *C. jejuni* [158].

23.11.1 Detection and Confirmation of *Campylobacter jejuni*

In general, *Campylobacter* species are sensitive microorganisms and are stressed during processing, and therefore an enrichment step is needed to resuscitate injured cells. Also, the microorganism fails to grow under normal atmospheric conditions since *Campylobacter* is microaerophilic and capnophilic, and gas jars should be used to provide the right gas atmosphere (5% oxygen, 10% carbon dioxide, and 85% nitrogen). Because of its sensitivity to oxygen, food samples should be kept before analysis in an environment without oxygen (100% nitrogen) with 0.01% sodium bisulfite and under refrigeration. Wang's medium may be used for this purpose [159].

The cultural detection of *Campylobacter* spp. [160] is shown in Figure 23.12. Usually, 10 g of food sample (ground beef) are added to 90 mL of enrichment broth. Sampling of poultry carcasses and large pieces of foods may be performed by the surface rinse technique. The sample is placed in a sterile stomacher bag with 250 mL of Brucella broth and the surface is rinsed by shaking and massaging. The broth (rinse/suspension) is filtered and centrifuged at $16,000 \times g$ for 10 min at 4°C. The supernatant fluid is discarded and the pellet is suspended in 2–5 mL of enrichment broth. After enrichment or during the direct plating without enrichment, two selective agars are used, specifically, Karmali agar and one of the following agars: Butzler agar, Campy-BAP or Blaser agar, *Campylobacter* charcoal differential agar (CCDA)-Preston blood-free agar, and Skirrow agar. It has been found that CCDA-Preston blood-free medium has excellent selectivity and is good for quantitative recovery of *C. jejuni* [159]. The oxygen tolerance of *Campylobacter* may be enhanced by adding to the growth media 0.025% of each of the following: ferrous sulfate, sodium metabisulfite, and sodium pyruvate (FBP supplement) [161].

Purification of the culture is made as follows for conducting confirmatory tests: Colonies from the selective agar plates are transferred to a Heart Infusion agar with 5% defibrinated rabbit blood (HIA-RB), and plates are incubated at 42°C for 24 h under microaerophilic conditions. The culture is transferred to 5 mL of HIB and the density of the cells is adjusted to meet the

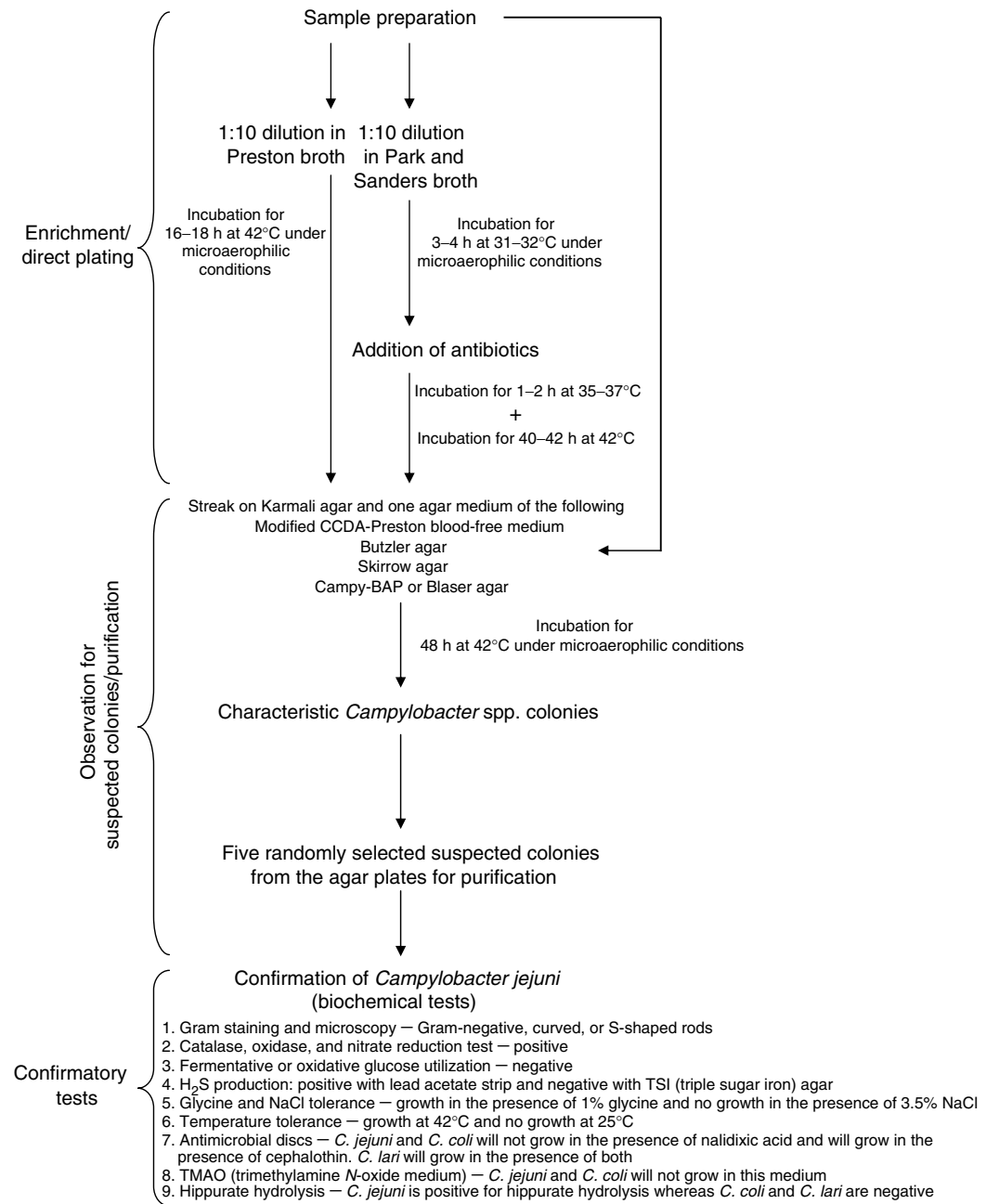


Figure 23.12 Cultural method for detecting and identifying *C. jejuni* based on ISO standard. (Based on ISO. 1995. International Standard, ISO 10272: *Microbiology of food and animal feeding stuffs—Horizontal method for detection and enumeration of Campylobacter spp.* Geneva: International Organization for Standardization.)

McFarland no. 1 turbidity standard (BioMerieux, Marcy Etoile, France). This cell suspension is used further for biochemical testing in tubes or on agar plates [159]. Finally, the commercial kit API Campy (BioMerieux, Marcy Etoile, France) may be used as an alternative for differentiation of *Campylobacter* spp.

Polynucleotide and oligonucleotide probes have been used for the detection of *C. jejuni*; they are reviewed by Olsen et al. [137]. A rapid and sensitive method based on PCR for the detection of *Campylobacter* spp. from chicken products, described by Giesendorf et al. [162], provided results within 48 h with the same sensitivity as the conventional method. Konkel et al. [163] developed a detection and identification method based on the presence of the *cadF* virulence gene, an adhesin to fibronectin, which aids the binding of *C. jejuni* to the intestinal epithelial cells. This method may be useful for the detection of the microorganism in food products, since it does not require bacterial cultivation before its application. Further techniques have been developed since then with the incorporation of an enrichment step before the PCR and real-time PCR amplification, respectively [164,165]. A more recent evaluation of a PCR assay for the detection and identification of *C. jejuni* in poultry products reduced the time of analysis to 24 h or less depending on the necessity of the enrichment step [166]. This method did not seem to be appropriate for ready-to-eat products but was proven to be useful in naturally contaminated poultry samples. Further improvements and trends include multiplex PCRs, reviewed by Settanni and Corsetti [25] as well as real-time nucleic acid sequence-based amplifications with molecular beacons [167].

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Chapter 24

Mycotoxin Analysis in Poultry and Processed Meats

Jean-Denis Bailly and Philippe Guerre

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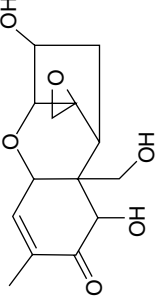
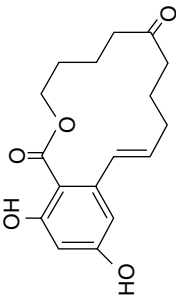
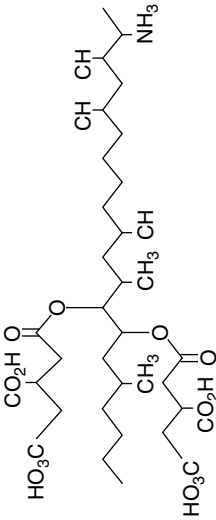
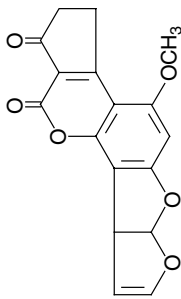
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24.1 Introduction

Mycotoxins are a heterogeneous group of secondary metabolites elaborated by fungi during their development. About 30 molecules are of real concern for human and animal health [1]. They can be found as natural contaminants of many vegetal foods or feeds, mainly cereals, but also of fruits, nuts, grains, and forage, as well as of compound foods intended for human or animal consumption. The most important mycotoxins are produced by molds belonging to the *Aspergillus*, *Penicillium*, and *Fusarium* genera (Table 24.1) [2–4].

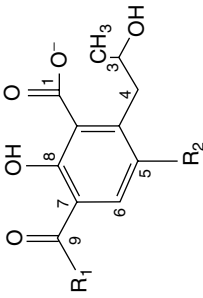
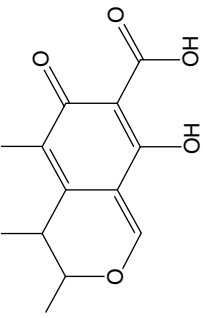
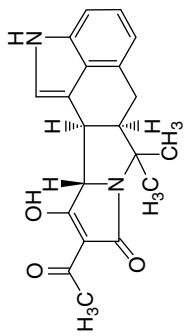
Mycotoxin toxicity is variable. Some have hepatotoxicity (aflatoxins), others have an estrogenic potential (zearalenone [ZEA]), or are immunotoxic (trichothecenes, fumonisins) (Table 24.1) [1]. Some mycotoxins are considered to be carcinogenic or are suspected to have carcinogenic properties [5]. Although some toxins display an important acute toxicity (after unique exposure to one high dose), chronic effects (observed after repeated exposure to weak doses) are probably more important in humans. Mycotoxins are suspected to be responsible for several pathological syndromes in humans, including ochratoxin A (OTA), which is associated with Balkan endemic

Table 24.1 Mycotoxins of Interest in Poultry and Processed Meat

Toxin	Main Producing Fungal Species	Toxicity	Structure
Deoxynivalenol	<i>Fusarium nivale</i> <i>Fusarium crookwellense</i> <i>Fusarium oxysporum</i> <i>Fusarium avenaceum</i> <i>Fusarium graminearum</i> <i>Fusarium solani</i>	Hematotoxicity Immunomodulation skin toxicity	
Zearalenone	<i>Fusarium graminearum</i> <i>Fusarium culmorum</i> <i>Fusarium crookwellense</i>	Fertility and reproduction troubles	
Fumonisin B1	<i>Fusarium verticillioides</i> <i>Fusarium proliferatum</i>	Lesion of central nervous system Hematotoxicity Genotoxicity Immunomodulation	
Aflatoxin B1	<i>Aspergillus flavus</i> <i>Aspergillus parasiticus</i> <i>Aspergillus nomius</i>	Hepatotoxic Genotoxic Carcinogenic Immunomodulation	

(Continued)

Table 24.1 (Continued)

Toxin	Main Producing Fungal Species	Toxicity	Structure
Ochratoxin A	<i>Penicillium verrucosum</i> <i>Aspergillus ochraceus</i> <i>Aspergillus carbonarius</i>	Nephrotoxic Genotoxic Immunomodulation	
Citrinin	<i>Aspergillus terreus</i> <i>Aspergillus carneus</i> <i>Aspergillus niveus</i> <i>Penicillium verrucosum</i> <i>Penicillium citrinum</i> <i>Penicillium expansum</i>	Nephrotoxic	
Cyclopiazonic acid	<i>Aspergillus flavus</i> <i>Aspergillus versicolor</i> <i>Aspergillus tamarii</i> <i>Penicillium camemberti</i>	Neurotoxicity Tremorgenic	

nephropathy (BEN), and fumonisin B1, which is associated with esophageal cancer. Mycotoxin exposure of human consumers is usually directly linked with alimentary habits.

For human consumers, the main source of exposure to mycotoxins is represented by cereals and cereal-based products [6–8]. However, they may also be exposed to these toxic compounds after ingestion of animal-derived products. Indeed, foods prepared from animals that have been fed with contaminated feeds may contain residual contamination and represent a vector of mycotoxins. Depending on the mycotoxins, the residues may correspond to the native toxin or to metabolites that keep all or part of the toxic properties of the parental molecule.

Among farm animals, poultry species can be exposed to several different mycotoxins, due to their breeding and feeding conditions. Moreover, given the importance of poultry meat and poultry products in the diet of many people around the world, it is very important to characterize potential transfer within tissues of edible poultry products.

The exposure of human consumers may also result from mycotoxin synthesis during ripening of products. Indeed, ripened foods are favorable to mold development, because they often participate in organoleptic improvement of such products. Therefore, the contamination with a toxigenic strain may lead to mycotoxin synthesis and accumulation in the final product [9].

At the present time, few toxins are regulated in foods (Table 24.2) [10–11]. The risk management is mainly based on controlling the contamination of vegetal raw materials intended for both human and animal consumption and limiting animal exposure through feed ingestion. It may guarantee against the presence of residual contamination of mycotoxins in animal derived products. However, a high level of contamination may accidentally lead to a sporadic contamination of products coming from exposed animals. Moreover, some toxins, mainly from *Penicillium* species, may also appear later, particularly during ripening of dry-cured meat products.

The aim of this work is to present methodology described for mycotoxin quantification in poultry and processed meats. Owing to the important structural diversity of mycotoxins and to the variations in their metabolism, it is impossible to establish general rules; each toxin and each product has to be investigated as a particular case. Therefore, we will first present the main toxins with their most important characteristics. After that, their analysis and prevalence will be presented in poultry and processed meats.

24.2 Main Mycotoxins

Depending on the fungal species that produces them, mycotoxins can be classified as “field” or “storage” toxins. The former are mainly produced by *Fusarium* fungi that develop on living plants, because a high water activity is required for their growth [12]. The later are toxins from *Penicillium* that may grow on foods and feeds during storage when moisture and temperature are favorable [13]. Between these two groups, the toxins produced by *Aspergillus* may occur both in the field and during storage, depending on climatic conditions [14]. We will now focus on the most important toxins of these three groups, based on their toxicity or their prevalence in foods and feeds.

24.2.1 Trichothecenes

24.2.1.1 Origin and Nature

Trichothecenes constitute a large group of secondary metabolites produced by numerous species of *Fusarium*, such as *F. graminearum*, *F. culmorum*, *F. poae*, and *F. sporotrichioides*. More than

Table 24.2 EU Regulation for Mycotoxin Contamination (µg/kg)

Toxin	Destination	Matrix	Maximal Concentration (µg/kg)
Aflatoxins	Aflatoxin B1	Groundnuts + grains + dry fruits	2, 5 or 8 depending on the product and the processing step
		Cereals	2 or 5 depending on the product and the processing step
		Spices	5
Aflatoxins B1 + B2 + G1 + G2	Human food	Cereal based foods for young children	0, 1
		Groundnuts + grains + dry fruits	4, 10 or 15 depending on the product and the processing step
		Cereals	4 or 10 depending on the product and the processing step
Aflatoxin M1	Animal feed	Spices	10
		Milk	0, 05
		Preparation for young children	0, 025
Aflatoxin B1	Animal feed	Raw material for animal feeds	20
		Compound feeds	5 to 20 depending on animal species

Ochratoxin A	Human food	Raw cereal grains	5
		All cereal products	3
		Dried vine fruits	10
		Coffee	3
Zearalenone	Human food	Raw cereals	100
		Cereal flours	75
		Bread, biscuits, corn flakes, snacks	50
		Baby food	20
Deoxynivalenol	Human food	Raw cereals	1250
		Durum wheat, maize	1750
		Cereal flours	750
		Bread, corn flakes, snacks, biscuits	750
		Pâtés	750
		Baby food	200
Fumonisin	Human food	Maize	2000
		Maize flour	1000
		Maize-based food	400
		Baby food	200

Source: European Union, Commission Regulation (EC) N° 466/2001 setting maximum levels for certain contaminants in foodstuffs, *Off. J. Eur. Un.*, L77, 1, 2001; and European Union, Commission Regulation n° 856/2005, toxins of *Fusarium*, *Off. J. Eur. Un.*, L143, 3, 2005.

160 trichothecenes have been identified, notably deoxynivalenol (DON), nivalenol (NIV), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), and fusarenon X. DON is the most frequently found trichothecene. Trichothecenes are frequent worldwide contaminants of cereals, mainly wheat and maize, and cereal-based products [8,15–18].

Because trichothecenes are a large family grouping many compounds of variable structure and properties, their toxicity can be very different depending on the molecule, the animal species, the dose, and the exposure period. There are many reviews available on trichothecenes toxicity [19–22]; only the main features will be presented here.

Trichothecenes are potent inhibitors of eukaryotic protein synthesis, interfering with initiation, elongation, or termination stages.

Concerning their toxicity in animals, DAS, DON, and T-2 toxin are the most studied molecules. The symptoms include effects on almost all major systems of organisms; many of them are secondarily initiated by poorly understood metabolic processes connected with protein synthesis inhibition.

Among naturally occurring trichothecenes, DAS and T-2 toxin seem to be the most potent in animals. They have an immunosuppressive effect, decreasing resistance to microbial infections [21]. They also cause a wide range of gastrointestinal, dermatological, and neurological symptoms [23]. In humans, these molecules have been suspected to be associated with alimentary toxic aleukia. The disease, often reported in Russia during the nineteenth century, is characterized by inflammation of the skin, vomiting, and damage to haematopoietic tissues [24,25]. When ingested at high concentrations, DON causes nausea, vomiting, and diarrhea. At lower doses, pigs and other farm animals display weight loss and feed refusal [21]. For this reason, DON is often called vomitoxin or feed refusal factor.

24.2.1.2 *Structure and Physicochemical Properties*

Trichothecenes belong to the sesquiterpenoid group. They all contain a 12,13-epoxytrichothene skeleton and an olefinic bond with various side chain substitutions. Trichothecenes are classified as macrocyclic or nonmacrocyclic, depending on the presence of a macrocyclic ester or an ester–ester bridge between C-4 and C-15 [26]. The nonmacrocyclic trichothecene can be classified in two groups: type A, which does not have a ketone group on C-8 (T-2 toxin, HT-2 toxin, DAS), and type B, with a ketone group on C-8 (DON, NIV, fusarenon X) [27].

Trichothecenes have a molecular weight ranging from 154 to 697 Da, but it is often between 300 and 600 Da. They do not absorb ultraviolet (UV) or visible radiations, with the exception of type D, which absorbs UV light at 260 nm. They are neutral compounds, usually soluble in mildly polar solvents such as alcohols, chlorinated solvents, ethyl acetate, or ethyl ether. They are sometimes weakly soluble in water [27].

These molecules are very stable, even if stored for a long time at room temperature. They are not degraded by cooking or sterilization processes (15 min at 118°C) [28].

24.2.1.3 *Analytical Methods*

Methods reported mainly concern the most frequently found toxins in cereals, which are DON, NIV, T-2 toxin, and HT-2 toxin [29]. Validated methods are now available for DON [30], but this is not the case for type A trichothecenes, and reference material and interlaboratory studies are still required [31].

24.2.1.3.1 Type A Trichothecenes

Extraction from solid matrixes is usually done with binary mixtures associating water and acetonitrile, water and methanol, chloroform and methanol, or methanol alone.

Purification is done with solid-phase extraction (SPE) columns working in normal phase (silica, florisil) or inverse phase (C18). Another approach, employing ready-to-use Mycosep columns (Romer Labs Inc., Union, MO), may be applied. These columns are adsorbants (charcoal, celite, ion exchange resin) mixed in a plastic tube. These multifunctional columns are increasingly popular.

Immunoassays are the main method routinely used for T-2 and HT-2 determination in cereals. Detection limits are in accordance with the contamination levels that are observed for these contaminants, and range from 0.2 to 50 ng/g for T-2 toxin [32].

Other methods have also been described, but type A trichothecenes cannot be analyzed by high-pressure liquid chromatography (HPLC)-UV due to the absence of ketone group in C-8 position. That is why gas chromatography (GC) is the most popular approach for this family of compounds. The derivatization of the native compounds by silylation or fluoroacylation is necessary to increase the sensitivity of the measure. Detection can be performed with an electron capture detector or by mass spectrometry (MS). The limits of detection of these methods are a few tens ng/g [33]. Another method was reported using HPLC with fluorescence detection after immuno-affinity columns (IAC) purification of extract and derivatization of T-2 toxin with 1-anthrolylnitrile. This procedure allowed a limit of detection of 5 ng/g [34].

24.2.1.3.2 Type B Trichothecenes

Extraction of type B trichothecenes is done with a mixture of acetonitrile–water or chloroform–methanol [35].

Many purification procedures have been reported for type B trichothecenes, such as liquid–liquid extraction (LLE), SPE, and IAC [36]. However, the use of mixed columns (charcoal–alumina–celite) is still widespread [37]. Once again, the Mycosep column is increasingly used for DON analysis.

Thin-layer chromatographic methods are still used for screening, particularly in countries where GC or HPLC are not easily available [38]. Since trichothecenes are not fluorescent, the detection of the molecules requires the use of revelators such as sulfuric acid, para-anisaldehyde, or aluminum chloride. Detection limits of thin-layer chromatography (TLC) range from 20 to 300 ng/g.

Enzyme-linked immunosorbent assay (ELISA) can also be of interest to get rapid and semiquantitative results with only minor purification of the extract. Many kits are commercially available for DON analysis in cereals [39,40].

GC coupled with an electron capture detector, a mass spectrometer, or in tandem (MS-MS) is regularly used after derivatization of the analyte [41–44]. Derivatization reactions are trimethylsilylation or perfluoroacylation. Fluoroacylation with anhydride perfluorated acid improves detection limits using an electron capture detector or MS. However, a European interlaboratory investigation of the official Association of Official Analytical Chemists (AOAC) method for DON measurement revealed that coefficient of variation between laboratories was very important (about 50%), despite the relatively high level of contamination of the material used (between 350 and 750 µg/kg). These observations increased interest in HPLC-MS methodology in trichothecene determination. This is progressively becoming the choice method [45].

24.2.2 Zearalenone

24.2.2.1 Origin and Nature

ZEA is a mycotoxin with estrogenic effect that is produced by *Fusarium* species such as *F. graminearum*, *F. proliferatum*, *F. culmorum*, and *F. oxysporum* [46,47]. Such molecules are suspected of reducing male fertility in human and wildlife populations, and is possibly involved in several types of cancer development [48]. This molecule is well known by farmers, often being responsible for reproduction perturbation, especially in pigs.

Acute toxicity of ZEA is usually considered as weak, with LD₅₀ after oral ingestion ranging from 2,000 to more than 20,000 mg/kg body weight [49,50]. Subacute and chronic toxicity of the mycotoxin is more frequent and may be observed at the natural contamination levels of feeds. The effects are directly related to the fixation of ZEA and metabolites on estrogenic receptors [51]. Affinity with estrogenic receptors is, in decreasing order: α -zearalanol > α -zearalenol > β -zearalanol > ZEA > β -zearalenol. Pigs and sheep appear more sensitive than other animal species [49,50].

ZEA induces alteration in the reproductive tracts of both laboratory and farm animals. Variable estrogenic effects have been described, such as a decrease in fertility, a decrease in litter size, an increase in embryo-lethal resorptions, and change in adrenal, thyroid, and pituitary gland weight. In male pigs, ZEA can depress testosterone, weight of testes, and spermatogenesis while inducing feminization and suppressing libido [49,50,52]. Long-term exposure studies did not demonstrate any carcinogenic potential for this mycotoxin [5].

24.2.2.2 Structure and Physicochemical Properties

The structure of ZEA is shown in Table 24.1. α - and β -zearalenol, the natural metabolites of the native toxin, correspond to the reduction of the ketone function in C₆.

ZEA has a molecular weight of 318 g/mol. This compound is weakly soluble in water and in hexane. Its solubility increases with the polarity of solvents such as benzene, chloroform, ethyl acetate, acetonitrile, acetone, methanol, and ethanol [53]. The molecule has three maximal absorption wavelengths in UV light: 236, 274, and 314 nm. The 274-nm peak is the most characteristic and commonly used for UV detection of the toxin.

ZEA emits a blue fluorescence with maximal emission at 450 nm after excitation between 230 and 340 nm [54].

24.2.2.3 Analytical Methods

Owing to regulatory limits, methods for analysis of ZEA content in foods and feeds may allow the detection of several nanograms per gram. Reviews have been published detailing the analytical methods available [45,55,56]. ZEA is sensitive to light exposure, especially when in solution. Therefore, preventive measures have to be taken to avoid this photodegradation.

Solvents used for liquid extraction of ZEA and metabolites are mainly ethyl-acetate, methanol, acetonitrile, and chloroform, alone or mixed. The mixture acetonitrile–water is the most commonly used. For solid matrixes, more sophisticated and efficient methods may be applied: for example, ultrasounds or microwaves [57,58].

In biological matrixes (e.g., plasma, urine, feces), hydrolysis of phase II metabolites is necessary before the purification procedure. It can be achieved by an enzymatic or a chemical protocol [59].

In vegetal materials, the demonstrated presence of sulfate conjugates [60] or glucoside conjugates [61] is rarely taken into account in routine methods.

Purification may be achieved using LLE, SPE, or IAC procedures. For SPE, most stationary phases may be used: inverse phase (C18, C8, or C4), normal phase (florisil, SiOH, NH₂), or strong anion exchange (SAX) [62]. The ready-to-use Mycosep column allows a rapid purification of samples without any rinsing and with a selective retention of impurities [63].

IAC columns have also been developed for ZEA and are very popular [64–70]. Although purification is very selective and extraction yields usually high, several points have to be highlighted:

- Antibody may not have the same affinity for all metabolites, some not being accurately extracted.
- Fixation capacity of columns are limited; a great number of interfering substances may perturb the purification by saturation of the fixation sites [62].
- These columns may be reused, increasing the risk of cross-contamination of samples.

For quantification of ZEA and metabolites in cereals and other matrixes, several immunological methods have been set up, including radioimmunoassay and ELISA [71–75]. The limit of quantification of these methods is several tens of nanograms per gram. ELISA kits show a cross-reactivity with α - and β -zearalenol [76].

Physicochemical methods are also widely used. They mainly include HPLC and GC, TLC being nearly withdrawn [77,78]. Many methods using C18 as stationary phase and CH₃CN/H₂O as mobile phase have been described. More specific stationary phases have also been proposed, such as molecular printing (MIP) [79]. Detectors are often fluorimeters [64–67,69,80] or UV detectors [62,66]. Sensitivity of these methods varies, depending on the metabolites, and is less important for reduced metabolites (α - and β -zearalenol).

ZEA and metabolites can also be detected by GC. However, the usefulness of this method is limited due to the time-consuming need to derivatize phenolic hydroxy groups. Consequently, only GC-MS has been applied for confirmation of positive results [81,42].

Many liquid chromatography (LC)-MS methods have also been proposed for ZEA and metabolites detection [45]. The method of chemical ionization at atmospheric pressure is most often used followed by electrospray [70,82–85]. These methods allow the detection of ZEA and metabolites at levels below 1 ng/g [45].

In an international interlaboratory study, important variations were observed between results from the participant laboratories, probably related to differences in sample preparation (LLE, SPE, or IAC) and quantification (HPLC, GC, TLC, and ELISA) [73,86].

24.2.3 Fumonisin

24.2.3.1 Origin and Nature

Fumonisin were first described and characterized in 1988 from *F. verticillioides* (formerly *F. moniliforme*) culture material [87,88]. The most abundant and toxic member of the family is fumonisin B1. These molecules can be produced by several species of *Fusarium* fungi: *F. verticillioides*, *F. proliferatum*, and *F. nygamai* [89,90]. These fungal species are worldwide contaminants of maize, and represent the main source of fumonisins [91].

One major characteristic of fumonisins is that they induce very different syndromes depending on the animal species. FB1 is responsible for equine leukoencephalomalacia characterized by

necrosis and liquefaction of cerebral tissues [92,93]. Horses appear to be the most sensitive species; clinical signs may appear after exposure to doses as low as 5 mg FB1/kg feed over a few weeks. Pigs are also sensitive to FB1 toxicity. In this species, fumonisins induce pulmonary edema after exposure to high doses (higher than 20 mg FB1/kg feed) of mycotoxins, and are hepatotoxic and immunotoxic at lower doses [94–96]. By contrast, poultry and ruminants are more resistant to this mycotoxin, and clinical signs appear only after exposure to doses higher than 100 mg FB1/kg, which may be encountered in natural conditions, but are quite rare [97–102]. In rodents, FB1 is hepatotoxic and carcinogenic, leading to the appearance of hepatocarcinoma in long-term feeding studies [103,104]. In humans, FB1 exposure has been correlated with a high prevalence of oesophageal cancer in some parts of the world, mainly South Africa, China, and Italy [105]. Finally, fumonisins can cause neural tube defects in experimental animals, and thus may also have a role in human cases [106–109]. At the cellular level, FB1 interacts with sphingolipid metabolism by inhibiting ceramide synthase [110]. This leads to the accumulation of free sphinganine (Sa) and, to a lesser extent, of free sphingosine (So). Therefore, the determination of the Sa/So ratio has been proposed as a biomarker of fumonisin exposure in all species in which it has been studied [111–114].

24.2.3.2 *Physicochemical Properties*

The structure of FB1 and related compounds is shown in Table 24.1. FB1 has a molecular weight of 722 g/mol. It is a polar compound, soluble in water and not soluble in apolar solvents. FB1 does not absorb UV light, nor is it fluorescent. Fumonisins are thermostable [115]. However, extrusion cooking may reduce fumonisin content in maize products [116].

24.2.3.3 *Methods of Analysis*

Because of their relatively recent discovery, analytical methodology for fumonisin analysis is still undergoing development. In most described methods, the food or foodstuff is corn. An HPLC method has been adopted by the AOAC and the European Committee for Standardization as a reference methodology for fumonisin B1 and B2 in maize [117–119].

An efficient extraction of fumonisins in solid matrix can be obtained with acetonitrile–water or methanol–water mixtures [120,121]. This was assessed by interlaboratory assay [122]. Increased contact time and solvent/sample ratio also increase yield of extraction step.

Purification of extracts is usually based on SPE with SAX, inverse phase (C18), or IAC [123,124].

Quantification of FB1 can be done by TLC, HPLC, or GC-MS. However, derivatization of the fumonisins is usually required. For TLC, this is usually done by spraying *p*-anisaldehyde on the plates after development in a chloroform-methanol-acetic acid mixture. It leads to the appearance of blue-violet spots that can be quantified by densitometry [115,125]. Quantification limits obtained with TLC methods often range from 0.1 to 3 mg/kg. That may be sufficient for rapid and costless screening of raw materials [126,127].

For HPLC analysis, fluorescent derivatives are formed with *o*-phthalaldehyde (OPA), naphthalene-2,3-dicarboxaldehyde, or 4-fluoro-2,1,3-benzoxadiazole [128]. OPA derivatization offers the best response, and has been generally adopted, but the derivatization product is very unstable, and analysis of samples has to be quickly performed after derivatization [129]. HPLC with fluorescence detection (HPLC-FL) methods have detection limits usually ranging from 10 to 100 µg/kg [124,128,133].

GC has also been proposed for FB1 determination. It is based on partial hydrolysis of fumonisins before reesterification and GC-MS analysis. However, this structural change does

not allow the distinction of different fumonisin molecules [130]. Another GC-MS method has been described, developing a derivatization step with trimethylsilylation coupled with detection by flame ionization [131].

The introduction of LC-MS with atmospheric pressure ionization has increased specificity and sensitivity of the detection. The majority of published fumonisin analysis with LC-MS was performed to the low parts per billion level in grains and maize-derived products. Furthermore, this methodology also appeared powerful in investigating for new fumonisin molecules, and elucidating structures and biosynthetic pathways and behavior during food processing [47].

ELISA kits are also commercially available for fumonisin quantification in vegetal matrix [132–134]. They usually offer detection limits around 500 µg/kg. However, the comparison with HPLC-FL shows that ELISA often overestimates the fumonisin content of samples. This may be due to cross-reactions between antibody and coextracted impurities [135]. This drawback could be overcome by purification of extracts before ELISA realization. This method can nevertheless be useful for rapid screening of maize and maize products. One ELISA kit has been validated by the AOAC for total fumonisin determination in corn [136].

24.2.4 Aflatoxins

24.2.4.1 Origin and Nature

Aflatoxins are probably the most studied and documented mycotoxins. They were discovered following a toxic accident in turkeys fed a groundnut oilcake supplemented diet (Turkey X disease) [137–139]. The four natural aflatoxins (B1, B2, G1, and G2) can be produced by strains of fungal species belonging to the *Aspergillus* genus, mainly *A. flavus* and *A. parasiticus* [14,140]. These are worldwide common contaminants of a wide variety of commodities, and therefore aflatoxins may be found in many vegetal products, including cereals, groundnuts, cotton seeds, dry fruits, and spices [141–146]. If these fungal species can grow and produce toxins in the field or during storage, climatic conditions required for their development are often associated with tropical areas (high humidity of the air, temperature ranging from 25 to 40°C) [147–151]. However, following extreme climatic conditions (an abnormally hot summer period), aflatoxins could be found in other parts of the world. For example, in 2003, controls on maize harvested in Europe were found contaminated by unusual AFB1 concentrations [152,153].

Aflatoxin B1 is a highly carcinogenic agent leading to primary hepatocarcinoma [154–157]. This property is directly linked to its metabolism and to the appearance of the highly reactive epoxide derivative. Formation of DNA adducts of AFB1-epoxide is well characterized [158]. Differences in AFB1 metabolism within animal species could explain the variability of the response in terms of carcinogenic potential of the mycotoxin [159,160].

AFM1, a hydroxylated metabolite of AFB1, can also be considered a genotoxic agent, but its carcinogenic potential is weaker than that of AFB1 [161]. Taking into account the toxicity of these molecules, the International Agency for Research on Cancer classified AFB1 in the group 1 of carcinogenic agents, and AFM1 in the 2B group of molecules that are carcinogenic in animals and possibly carcinogenic in humans [5].

24.2.4.2 Structure and Chemical Properties

The structures of aflatoxin B1 are presented in Table 24.1. Molecular weights of aflatoxins range from 312 to 320 g/mol. These toxins are weakly soluble into water, insoluble in nonpolar solvents,

and very soluble in mildly polar organic solvents (i.e., chloroform and methanol). They are fluorescent under UV light (blue fluorescence for AF^B and green for AF^G) [162].

24.2.4.3 Analytical Methods

Most common solvent systems used for extraction of aflatoxins are mixtures of chloroform–water [163–165] or methanol–water [166–170]. This latter mixture is mainly used for multiextraction of mycotoxins, and is not specific for aflatoxin extraction [171]. Whatever the solvent system used, the extract obtained still contains various impurities and requires further cleanup steps. The most commonly used extraction technique is SPE, which has replaced the traditional liquid–liquid partition for cleanup [165]. Stationary phase of the SPE columns used may be silica gel, C18 bonded-phase, and magnesium silicate (commercially available as Florisil) [163,172]. Antibody affinity SPE columns are also widely used.

IAC chromatography using antitoxin antibodies allowed the improvement of both specificity and sensitivity [173,174]. Indeed, methods were validated for grains [175], cattle feed [176,177], maize, groundnuts, and groundnut butter [178], pistachio, figs, and paprika [179], and baby food [180]. Analytical methods of the same kind were validated for quantification of AM1 in milk [181] and in powder milk [182,183], these methods show limits of quantification below the regulatory limit of 0.05 µg/L.

Aflatoxins are usually quantified by TLC, HPLC, or ELISA.

TLC was first developed in the early 1980s. Using strong fluorescence of the molecules, the characterization of signals with naked eyes or densitometric analysis could give semiquantitative to quantitative results (AOAC methods 980.20 and 993.17) [184]. Therefore, aflatoxin B1 could be measured in concentrations ranging from 5 to 10 µg/kg. A TLC method for quantification of AFM1 in milk was also validated by AOAC (980.21) [185] and normalized (International Standardization Organisation [ISO] 14675:2005) [186]. A method for semiquantitative analysis of AFB1 in cattle feed was also published (ISO 6651:2001) [187]. Confirmation of identity of aflatoxins B1 and M1 in foods and feeds is still classically done by TLC after bidimensional migration and trifluoric acid–hexane (1:4) spraying of plates.

HPLC allowed the reduction of detection limits together with an improvement of the specificity of the dosage [188]. Therefore, new methods were validated for aflatoxin quantification in grains (AOAC 990.33), cattle feed (ISO 14718:1998), and AFM1 in milk (ISO/FDIS 14501) [189–191]. These methods are based on the use of a fluorescence detector allowing the quantification of low levels of aflatoxins. The sensitivity can be increased by the treatment of extracts with trifluoric acid to catalyze the hydration of aflatoxins M1, B1, and G1 into their highly fluorescent M2a, B2a, and G2a derivatives.

ELISA has been developed for both total aflatoxins [192,193] and AFB1 detection in feeds and grains [194–197] and for AFM1 in milk [198]. These methods have limits of quantification in accordance with international regulations. Therefore, some commercially available kits have been validated by the AOAC, as for example the one referenced as AOAC 989.86, devoted to AFB1 dosage in animal feed. However, in spite of the development of ELISA methods for AFM1 detection [199], no ELISA kit has been validated following the harmonized protocol of ISO/AOAC/International Union of Pure and Applied Chemistry (IUPAC) for AFM1 quantification in milk. The AOAC has edited rules for characterization of antibodies used in immunochemical methods [200].

Detection limits in the low parts per trillion range can be achieved by these classical LC-fluorescence methods. Therefore, methods such as LC-MS may represent only a minor alternative or confirmation technique for already well-established methodologies [45]. It may however be useful to

confirm positive results of TLC or ELISA-based screening analysis [201]. At the present time, few quantitative methods have been published for aflatoxin determination in food and milk [202–205].

24.2.5 Ochratoxin A

24.2.5.1 Origin and Nature

Ochratoxins A, B, and C are secondary metabolites produced by several *Aspergillus* and *Penicillium* species. According to its prevalence and toxicity, only OTA will be treated in this section. This molecule can be produced by *Aspergillus* species such as *A. ochraceus* [206], *A. carbonarius* [207,208], *A. alliaceus* [209], and *A. niger* [210], although the frequency of toxigenic strains in this species appears moderate [211–213]. OTA can also be synthesized by *Penicillium* species, mainly *P. verrucosum* (previously named *P. virridicatum*) [214–215].

The ability of both *Aspergillus* and *Penicillium* species to produce OTA makes it a worldwide contaminant of numerous foodstuffs. Indeed, *Aspergillus* is usually found in tropical or subtropical regions, whereas *Penicillium* is a very common contaminant in temperate and cold climate areas [216–219]. Many surveys revealed the contamination of a large variety of vegetal products such as cereals [220,221], grapefruit [222,223], and coffee [221,224]. For cereals, OTA contamination generally occurs during storage of raw materials, especially when moisture and temperature are abnormally high, whereas for coffee and wine, contamination occurs in the field or during the drying step [219,225–227]. When ingested by animals, OTA can be found at residue level in several edible organs (see 23.3.5). Therefore, the consumption of meat contaminated with OTA has also been suspected to represent a source of exposure for humans [228]. Recent surveys done in European countries demonstrated that the role of meat products in human exposure to OTA can be considered low [6,229].

Kidney is the primary target of OTA. This molecule is nephrotoxic in all animal species studied. For example, OTA is considered responsible for a porcine nephropathy that has been studied intensively in the Scandinavian countries [230,231]. This disease is endemic in Denmark, where rates of porcine nephropathy and ochratoxin contamination of pig feed are highly correlated [232]. Because the renal lesions observed in pig kidneys after exposure to OTA are quite similar to those observed in kidneys of patients suffering from BEN, OTA is suspected to play a role in this human syndrome [233–235]. BEN is a progressive chronic nephropathy that occurs in populations living in areas bordering the Danube River in Romania, Bulgaria, Serbia, and Croatia [236,237].

24.2.5.2 Physicochemical Properties

The structure of OTA is presented in Table 24.1. OTA has a molecular weight of 403.8 g/mol. It is a weak organic acid with a pK_a of 7.1. At an acidic or neutral pH, it is soluble in polar organic solvents and weakly soluble in water. At a basic pH, it is soluble and stable in an aqueous solution of sodium bicarbonate (0.1 M; pH: 7.4), as well as in alkaline aqueous solutions in general.

OTA is fluorescent after excitation at 340 nm, and emits at 428 nm when nonionized and at 467 nm when ionized.

24.2.5.3 Methods of Analysis

Extraction of OTA is often achieved by using a mixture of acidified water and organic solvents. An IUPAC/AOC method validated for OTA determination in barley uses a chloroform–phosphoric

acid mixture [238]. For coffee or wine, chloroform is successfully used [239,240]. Mixtures of methanol–water or acetonitrile–water have also been reported [241,242]. *Tert*-butylmethylether has been used for OTA extraction from baby food, and may represent an alternative to the use of chlorinated solvents [243].

Several efficient cleanup procedures based on IAC and SPE using C8, C18, and C-N stationary phases were developed to replace, when possible, conventional LLE [244]. Stationary phases based on the principle of MIP are emerging [245,246]. The specificity of such methods is comparable to that of IAC. Although their applicability in real matrixes has not been established, they may represent alternatives to IAC and SPE methods in the future.

Many methods have been developed for separation and detection of OTA. TLC methods have been published [247–249]. However, both specificity and sensitivity of TLC are limited, and interferences with the sample matrix often occur [250]. These drawbacks may be overcome by two-dimensional TLC [251]. However, HPLC is the most commonly used method for determination of OTA [244,252].

Most described HPLC methods use a reverse-phase C18 column and an acidic mobile phase composed of acetonitrile or methanol with acetic, formic, or phosphoric acid [242,253–255]. The property of OTA to form an ion-pair on addition of a counter ion to the mobile phase has been used [256]. This led to a shift in OTA fluorescence from 330 to 380 nm and allowed an improvement of the signal. Ion-pair chromatography was also used for detection of OTA in plasma and human and cows' milk, with detection levels of 0.02 and 10 ng/mL for plasma and milk, respectively [257–258]. The major limit of the method is that small changes in composition of mobile phase may change retention time of OTA.

HPLC methods using fluorescence detection are applicable to OTA detection in barley, wheat, and rye at concentrations of about 10 µg/kg [259]. For baby foods, a quantification limit of 8 ng/kg has been reached by postcolumn derivatization with ammoniac [240,243].

Today, several validated methods have been published for OTA detection in cereals and derived products [260], in barley and coffee [261–263], and in wine and beer [264].

Immunoassays such as ELISA and radioimmunoassays have been developed [265–268], and may be regarded as qualitative or semiquantitative methods, useful for rapid screening.

Owing to its toxicity and regulatory values, OTA analysis has to be performed down to the ppb range in foods and feeds. In addition, plasma and urine samples are analyzed to monitor OTA exposure in humans and animals. In this context, methods using LC-MS may be used to confirm OTA-positive results obtained by ELISA or HPLC-FL. They may also be powerful tools to elucidate structure of *in vivo* metabolites and OTA adducts in biological fluids. Many studies have described LC-MS methods for OTA determination [47].

24.2.6 Other Toxins

24.2.6.1 Citrinin

24.2.6.1.1 Origin and Nature

Citrinin is produced by different *Aspergillus* (*A. terreus*, *A. carneus*, *A. niveus*) and *Penicillium* species (*P. citrinum*, *P. verrucosum*, *P. expansum*) [269]. It may also be produced by fungi belonging to the *Monascus* genus [270]. It has been found at levels ranging from few micrograms per kilogram to several milligrams per kilogram in barley, wheat, and maize, and also in rice, nuts, dry fruits, and apple juice [1,271–273].

Citrinin is nephrotoxic in all animal species where it has been studied, leading to a time- and dose-dependent necrosis of renal tubules [274–276]. This is mainly due to citrinin-mediated oxidative stress [277].

24.2.6.1.2 Physicochemical Properties

Citrinin is an acidic phenolic benzopyrane with a molecular weight of 250 g/mol (Table 24.1). This molecule is insoluble in water but very soluble in most of organic solvents, such as methanol, ethanol, and acetonitrile [38]. Citrinin is heat labile in acidic or alkaline solution. It easily links to proteins.

24.2.6.1.3 Analytical Methods

Several methods have been used for citrinin determination in foods and feeds. A rapid TLC method allows the detection of 15–20 µg/kg in fruits [278]. Immunological methods such as ELISA have also been developed, and present good sensitivity [272]. HPLC allows the detection of citrinin in cereals, biological fluids (urine and bile), and fermentation media [272]. It has to be noted that efficiency of HPLC methods greatly depends on the extraction step, which must not degrade the toxin. Detection is made in UV at 254 or 366 nm [38]. The detection limits in cereals are usually about 10 µg/kg. A semiquantitative fluorimetric method has also been set up to detect citrinin in fungal culture isolated from cheeses [279].

24.2.6.2 Cyclopiazonic Acid

24.2.6.2.1 Origin and Nature

Cyclopiazonic acid (CPA) was first isolated from culture of *P. cyclospium*, but has also been shown to be produced by several species of *Aspergillus* and *Penicillium*, such as *A. flavus*, *A. tamarii*, or *P. camemberti* [280,281]. Therefore, CPA has been detected in many foods, especially cheeses [282], although few cases of intoxication have been described. However, retrospective analysis of “Turkey X disease” performed in 1986 by Cole suggested that clinical signs were not all typical of aflatoxicosis. He thus tried to demonstrate a possible role for cyclopiazonic acid in this affection. For instance, opisthotonos originally described in “Turkey X disease” can be reproduced by administration of a high dose of cyclopiazonic acid but not by ingestion of aflatoxin [283]. Cyclopiazonic acid is a specific inhibitor of the Ca²⁺ ATPase pump of the endoplasmic reticulum [284], which plays a key role in muscular contraction and relaxation. Principal target organs of cyclopiazonic acid in mammals are the gastrointestinal tract, liver, and kidneys [285,286]. Main symptoms observed after acute intoxication with CPA are nervous signs, including eyelid ptosis, ataxia with hypothermia, tremors, and convulsions [287].

24.2.6.2.2 Physicochemical Properties

CPA is a tetramic indole acid with a molecular weight of 336 g/mol (Table 24.1). It is produced by the amino acid pathway and derived from tryptophane, mevalonate, and two acetate molecules.

24.2.6.2.3 Methods of Analysis

TLC is still used to quantify CPA in cereals and milk products [280]. For milk products, several methods were developed: inverse phase LC [288] and LC-ion trap electrospray MS-MS [289] allowing a detection limit of 5 ng/mL. Methods using LC with UV detection were also developed for quantification in cheese [290] and cereals and derived products [291].

Immunoenzymatic methods allow detection of CPA in maize and animal organs (muscles and plasma) [292], and also in peanuts and mixed feed [293]. Detection limits of such methods range from 1 to 20 ng/g.

24.3 Mycotoxin Analysis and Prevalence in Poultry

If many methods have been developed and validated for vegetal matrix, due to the absence of regulation, few data are available on techniques that may be used for animal-derived foods. With the exception of the detection of Aflatoxin M1 in milk and milk products [294], no official method is available for such products.

Taking into account the great structural differences that exist between mycotoxins and their distinct metabolism after absorption in animal digestive tracts, no multidetection method can be carried out; methods have to be developed specifically for each toxin and metabolite.

In this section will be presented the analytical methods used for mycotoxin quantification in poultry organs, as well as the available data concerning the metabolism of these toxic compounds in avian species and the persistence of a residual contamination after dietary exposure. These data are helpful to evaluate the real risk of mycotoxin contamination of poultry products and the subsequent possible need for development of analytical methods.

24.3.1 *Trichothecenes*

24.3.1.1 *Methods of Analysis*

Few methods have been developed for trichothecenes analysis in poultry tissues. Indeed, first experiments on the pharmacokinetics and distribution of these mycotoxins were performed using radiolabeled toxins [295–299]. Because these experiments revealed that trichothecenes were rapidly excreted and carryover of the toxins in edible parts of poultries was minimal (see 24.3.1.2), few studies were carried out to evaluate trichothecene presence in muscle and other tissues of animals after exposure to unlabeled toxins. The methods used in these works are summarized in Table 24.3 [300–309].

24.3.1.2 *Behavior and Residual Contamination of Poultry Tissues*

Oral absorption of trichothecenes is limited (<10% at 6 h) in poultry, at least for DON and T-2 toxin. For example, in laying hens, after oral administration of 0.25 mg DON/kg BW, the mean plasmatic peak was reached after 2.25 h, and average bioavailability was 0.64%, with marked individual variations [297,310,311].

As is true for other animal species, distribution of trichothecenes is wide and rapid. Maximal tissue concentrations of DON, T-2 toxin, and their metabolites were observed after 3 h in liver and kidneys, and 4–6 h in muscle, fat, and the oviduct. Higher concentrations were found in

Table 24.3 Methods for Mycotoxin Analysis in Poultry Muscle and Tissues

Toxin	Organ (species)	Extraction and Clean Up	Derivatisation–Quantification	LOD*	References
T-2	Liver, kidney, heart (chicken)	Acetonitrile Amberlite XAD-2 resin column	TFAA – tri-Sil TBT GC-MS	—	300
DON	Liver, kidney, muscle (hens)	Acetonitrile-water Alumina-charcoal column	Heptafluorobutyl imidazole Gas–liquid chromatography	10 ng/g	301
ZEA	Muscle (laying hens)	Overnight treatment with 2/0,9U b-glucuronidase/arylsulfatase Ethyl acetate IAC	HPLC fluorescence	1 ng/g	302
ZEA	Muscle (chicken)	Acetone-water Basic alumina and phosphate exchange AGMP-1 resin column	HPLC UV	4 ng/g	303
FB1	Muscle, kidney, liver (mule ducks)	Acetonitrile-methanol Fat removal with n-hexane Immunoaffinity column Column chromatography	HPLC fluorescence	25 ng/g	304
AFB1	Liver, kidney, heart, muscle (Chicken)	Immunoaffinity column Column chromatography	2D TLC fluorodensitometry	≤0.1 ng/g**	305
AFB1	Liver (Chicken)	Immunoaffinity columns	ELISA optical density	1 ng/g**	306
AFB1	Liver (Chicken)	Immunoaffinity columns	HPLC fluorescence	0.008 ng/g**	307
OTA	Muscle (Turkey, chicken)	Chloroform-orthophosphoric acid Immunoaffinity columns	HPLC fluorescence	0.04 ng/g**	308
OTA	Muscle (broiler chicks)	0.1 M Phosphoric acid-Chloroform Diatomaceous hearth column	HPLC fluorescence	0.05 ng/g	309
OTA	Muscle (broiler chicks)	Dichloromethane-citric acid	ELISA optical density	0.042 ng/g	309

Note: "**", detection limit; and "***", quantification limit.

the anterior digestive tract, kidney, liver, gall bladder, and spleen. Plasmatic distribution profiles did not show a secondary peak correlated with the enterohepatic cycle [297,310,311]. When administration was prolonged, maximal DON values in tissues were reached rapidly and remained relatively constant throughout the exposure period. The highest concentrations were detected in the same organs as described above after a single administration [311]. Residual persistence of T-2 toxin and DON, as well as of their metabolites, in muscle, liver, and kidney, in the case of single or repeated administration, is summarized in Table 24.4 [296,297,311,312]. Detected levels of contamination were on the scale of micrograms per kilogram. Prolonged administration of trichothecenes led to a higher level of contamination than a single one, indicating an accumulation of toxins or metabolites. The decrease in the residual contamination was slower.

24.3.2 Zearalenone

24.3.2.1 Methods of Analysis

Owing to metabolism of the native molecule and the very weak carryover of ZEA in edible parts of farm animals (see 24.3.2.2), few “classical” physicochemical or immunological methods have been developed for ZEA detection in edible parts of poultry species (Table 24.3) [302,313–315]. HPLC-UV or HPLC-FL are used for quantification and display detection limits near 1 ng/g.

24.3.2.2 Behavior and Residual Contamination of Poultry Tissues

Although metabolism is a key point of ZEA toxicity [316], few studies are available concerning poultry. An intracellular partitioning of reduction activity of ZEA in liver has been described, the extent varying depending on the species and on the isomer produced. *Ex vivo*, hens almost exclusively produced α -zearalenol with a microsomal fraction and β -zearalenol with a cytosolic fraction [317]. Hen hepatocytes are said to produce mainly β -zearalenol; only traces of α -zearalenol have been found [318]. These results are not in agreement with those obtained *in vivo*. In chickens, administration of a diet containing 100 mg/kg ZEA for 8 days, followed by exposure to 10^9 dpm/kg [^3H] ZEA, revealed that the kinetics of the toxin is rapid, with tissue half-life ranging from 24 to 48 h [319]. In addition to the digestive tracts and excreta (bile), most of the radioactivity was found in the liver and kidneys, and a concentration peak was reached 30 min after administration. The residue profile found in liver (GC-MS), in nanograms per gram, was the following: zearalenone 681, α -zearalenol 1200, β -zearalenol 662. After 24 h, total quantities found in liver, gizzard (without mucosa), muscle, plasma, skin, and fat were respectively 651, 297, 111, 91, 70, and 53 ng/g. These results are similar to those obtained by Maryamma et al. [320] after 20 days' administration of 10 mg/kg body weight (BW) of zearalenone to broilers. Hepatic and muscular concentrations of 207 and 170 ng/g were found 24 h after the last administration. Likewise, in turkeys, administration of feed containing 800 mg ZEA/kg for 2 weeks resulted in plasmatic concentrations of 66 ng/mL ZEA and 194 ng/mL α -zearalenol at the end of the experiment. Only traces of β -zearalenol were found [303]. All these studies were performed using very high doses of the toxin. A recent experiment in chickens using a 1.58 mg ZEA/kg feed for 16 weeks appears to confirm these results for low concentrations. Hepatic concentrations obtained at the end of the experiment were 2.1 ng/g ZEA and 3.7 ng/g α -zearalenol, mainly in conjugated forms, whereas β -zearalenol was below the detection limit (<3 ng/g) [321]. No trace of zearalenone or of its metabolites was found in muscles, fat, or eggs.

Table 24.4 Residues of Trichothecenes in Poultry Tissues (expressed as equivalent-toxin)

Toxin	Species	Route	Dose (mg/kg b.w.)	Tissues	Residues ($\mu\text{g}/\text{kg}$)							Half-life	References
					After a Single Administration								
					6 h	12 h	24 h	2 j	4 j	4 j	4 j		
DON	Hen	VO	1.3–1.7	Muscle	8.46	6.6	4.3	2.1	ND			311	
				Liver	74	56	30	13	ND		15.7 h		
T-2	Chicken	VO	0.126–1.895	Kidney	165	123	44	19	2			296	
				Muscle			17/220						
	Chicken/duck	VO	5	Liver			32/416					297	
				Kidney	30	30	<10	<10					
				Muscle	130/90	30/40	10/<10	<10					
				Liver	30	20	<10	<10					

Toxin	Species	Route	Length	Dose	Tissues	Residues ($\mu\text{g}/\text{kg}$)						References
						After a Repeated Administration						
						2 j	4 j	6 j	8 j	10 j	12 j	
DON	Hen	VO	6 j	1.3–1.7 mg/kg b.w.	Muscle	16	17	10	11	7	3	311
					Liver	37	41	39	25	15	9	
					Kidney	60	51	55	21	15	9	
Chicken	VO	28–190 j	5 mg/kg feed	Muscle			<10				312	
				Liver								
				Kidney								

Note: ND, not detectable.

24.3.3 Fumonisin

24.3.3.1 Methods of Analysis

Measurement of FB1 in poultry is poorly documented. Moreover, most of the data concern its toxicokinetic effect in animals and were obtained by using labeled molecules [322]. Finally, only one method was described concerning the determination of nonradiolabeled FB1 in duck tissues. It is based on the use of immunoaffinity columns for the extraction of the mycotoxin and quantification of derivatized FB1 by fluorescence detection after its separation by HPLC [304]. This method allowed fumonisin B1 detection in liver, kidney, and muscle, with a limit of quantification of 25 ng/g (Table 24.3).

24.3.3.2 Behavior and Prevalence in Poultry Tissues

No data are available on a possible metabolism of FB1 in poultry, and few data are available concerning its toxicokinetics.

Absorption after oral administration is reported to be very limited in laying hens (<1%), but higher in growing ducks (2.5–3.5%), close to values already described in rodents, pigs, and nonhuman primates [322,323]. Concentrations in the muscles were about 10-fold lower than in plasma, and no transfer to eggs has been reported.

24.3.4 Aflatoxins

24.3.4.1 Methods of Analysis

Techniques described for aflatoxin analysis in poultry tissues mainly use native fluorescence of these compounds after purification and separation of extract with chromatographic methods (TLC, HPLC) (Table 24.3). Since the 1980s, few studies and surveys have been carried out to characterize aflatoxin presence in poultry products [306,307]. Indeed, risk management is based on the control of animal feed quality, which may guarantee the absence of toxin residues in animal-derived products. These few surveys all demonstrated that muscle foods were not an important source of aflatoxin exposure in humans. It is, however, likely that recent alerts for unusual aflatoxin contamination of cereals produced in temperate climates and the possible consequent animal exposure may strengthen the interest of aflatoxin testing in animal-derived foods. That is why some authors investigated the possible use of ELISA for determination of aflatoxin residue in chicken livers [306].

24.3.4.2 Behavior and Prevalence in Poultry Tissues

Few data are available on aflatoxin behavior in poultry. Oral absorption seems to be comparable to that occurring in other monogastric species, and could represent 90% of the administered dose [324]. This absorption could be decreased by several adsorbants [325]. Aluminosilicates and clays are among the most effective, and a protective effect has been demonstrated in numerous studies. These studies, the first of which was performed by Phillips in the 1980s, certainly help explain the interest in these kinds of compounds in animal feed [326]. Many studies are done each year to confirm the benefit of these molecules in the case of exposure to aflatoxin.

As is true in other animal species, in poultry metabolism and liver bioactivation in AFB1-8,9-epoxyde and in aflatoxicol could play a key role in the appearance of hepatic lesions.

Bioactivation could explain the greater sensitivity of ducks to aflatoxins, whereas quails could be more resistant due to their lower metabolic capacities [327].

Persistence of aflatoxin B1 and its metabolites at the residual level appears to vary depending on the species and the study. These differences cannot all be explained by differences in metabolism processes between species; differences in the procedures used for detection, extraction, and purification of the toxin and its metabolites from the tissues are more likely to be responsible. The most conclusive results are listed in Table 24.5 [328–333]. Liver and kidney contain more toxin and metabolites than muscles, with the exception of the gizzard, which is directly exposed. Quail appears to be a more important vector for residues than the other species. Hens could be a more important vector than chickens, because excretion in the eggs is also possible, at least after exposure to high concentrations of toxins.

24.3.5 Ochratoxin A

24.3.5.1 Methods of Analysis

All previously described methods were used to analyze OTA content of animal tissues and animal-derived products. The aim of such studies was to characterize the potential carryover of the mycotoxin in animal tissues and to assess human exposure. Most studies have been set up in pigs and pig tissues, because this species appears to be the most sensitive and exposed to OTA. For poultry meat samples, the solvent extraction step cannot be avoided, and precedes the purification step. Typical procedures include extraction with acidic chloroform or acidic ethyl acetate, followed by back extraction into NaHCO_3 before cleanup on IAC or C18 columns [308,309].

It appears that detection limits exhibited by HPLC-FL are sufficient to control meat products according to existing regulations. The use of IAC for cleanup allows the reduction of the limit of quantification (LOQ) below 1 ng/g [308].

By contrast, the use of HPLC-MS does not strongly increase the sensitivity of detection, but may be used as a confirmatory method in the case of a positive result.

ELISA tests usually display LOQ higher than other methods. Nevertheless, due to their simplicity and rapidity, these tests could be useful as screening methods in slaughterhouses [309].

24.3.5.2 Behavior and Prevalence in Poultry Tissues

In poultry, oral absorption of OTA appears to occur in the same way as in other monogastric species (passive diffusion of the nonionized lipophilic form), but absorption is apparently lower: about 40% in broilers and only 6.2% in quails. The concentration peak is more rapidly reached in broilers, after 0.33 h [334].

During circulation, OTA fixes to plasmatic proteins, its affinity constant for serum albumin being of about 5.1×10^4 mol/L, which is very close to the value observed in humans [335]. Distribution of OTA in chicken tissue appears to be higher than in other avian species (above 2 L/kg). The highest tissue concentrations were observed in the following organs: kidney > liver > muscles. No residue was found in fat or skin. Transfer to eggs is minimal or nil [334].

To our knowledge, no data are available on OTA metabolism in poultry. Plasmatic half-life of OTA after oral administration ranges from 4.1 h in chicken to 6.7 h in quail. This half-life is well below that reported in most mammalian species [336].

Table 24.5 Residues of Aflatoxin in Animal Tissues (only the most demonstrative studies are reported)

<i>Animal Species</i>	<i>Dose and Duration of Exposure</i>	<i>Tissues</i>	<i>Residues ($\mu\text{g}/\text{kg}$)</i>	<i>Metabolites</i>	<i>References</i>
Poultry	50 and 150 $\mu\text{g}/\text{kg}$ feed for 11 weeks	Liver	0.02–0.009 and 0.11–0.23	AFB1 + AFM1	328
		Kidney	0.02–0.04 and 0.11–0.21	AFB1 + AFM1	
	50 and 150 $\mu\text{g}/\text{kg}$ feed for 11 weeks and 1 week with toxin free feed	Gizzard	0.04–0.16 and 0.01–0.12	AFB1 (AFM1 <0.01)	AFB1 + AFM1
		Liver	<0.01	<0.01	AFB1 + AFM1
		Kidney	<0.01	<0.01	AFB1 + AFM1
Quail	3000 $\mu\text{g}/\text{kg}$ feed for 8 days	Gizzard	0.04–1.9 and 0.09–0.24	AFB1 (AFM1 <0.01)	329
		Liver	7.83 \pm 0.49 and 5.31 \pm 0.22 22.34 \pm 2.4 and 10.54 \pm 0.42	Free and conjugated AFB1 Free and conjugated metabolites	
Duck	3000 $\mu\text{g}/\text{kg}$ feed for 8 days	Muscle	0.38 \pm 0.03 and <0.03	Free and conjugated AFB1	
			0.82 \pm 0.05 and 0.32 \pm 0.08	Free and conjugated metabolites	
		Liver	0.52 \pm 0.04 and 0.44 \pm 0.16 2.74 \pm 0.15 and 3.81 \pm 0.25	Free and conjugated AFB1 Free and conjugated metabolites	
		Muscle	<0.03 and <0.03 0.21 \pm 0.09 and 0.14 \pm 0.05	Free and conjugated AFB1 Free and conjugated metabolites	

Chicken	3000 µg/kg feed for 8 days	Liver	0.15 ± 0.09 and 0.10 ± 0.01	Free and conjugated AFB1	330
			1.54 ± 0.36 and 0.93 ± 0.04	Free and conjugated metabolites	
Hen	3000 µg/kg feed for 8 days	Muscle	<0.03 and <0.03	Free and conjugated AFB1	331
			0.11 ± 0.02 and 0.08 ± 0.05	Free and conjugated metabolites	
		Liver	0.34 ± 0.03 and 0.23 ± 0.08	Free and conjugated AFB1	
			2.38 ± 0.36 and 4.04 ± 0.1	Free and conjugated metabolites	
Laying hen Laying hen Laying hen Chicken Chicken	2500 µg/kg feed for 4 weeks	Muscle	<0.03 and <0.03	Free and conjugated AFB1	332
			0.14 ± 0.04 and 0.11 ± 0.04	Free and conjugated metabolites	
		Eggs	0.28 ± 0.1 and 0.38 ± 0.11	AFB1 and total Aflatoxicol	
			0.49 ± 0.28 and 0.2 ± 0.09	AFB1 and total Aflatoxicol	
		Kidney	0.32 ± 0.18 and 0.1 ± 0.04	AFB1 and total Aflatoxicol	
			0.08 ± 0.03	Aflatoxicol	
		Eggs	0.24 ± 0.07 and 0.25 ± 0.09	AFB1 and total Aflatoxicol	
			4.13 ± 1.95	AFB1	
		Liver	<0.5 and <0.01	AFB1 and AFM1	
			0.26 and 0.02	AFB1 and AFM1	
Liver	1.52 and <0.1	AFB1 and AFM1			

24.3.6 Other Toxins

24.3.6.1 Citrinin

If several studies evaluating citrinin toxicity in avian species, no method was specially set up for the determination of residual contamination of edible organs with this toxin, although poultry appeared sensitive to citrinin toxicity. Indeed, administration of 125–250 ppm citrinin to young chicken leads to acute toxicity with diarrhea and increase in water consumption without any mortality [337,338]. Lesions were mainly digestive hemorrhages, lipidic infiltrations in liver, kidney, and pancreas, and an increase in kidney weight for birds treated with 250 ppm [338].

Administration of labeled toxin demonstrated that citrinin is only weakly absorbed after oral administration and quickly eliminated in urine and feces, at least in rodents [339]. In poultry, the administration of a contaminated diet containing 440 ppm of citrinin did not allow the detection of residual contamination in muscles, whereas only weak amounts of the toxin were found in liver of exposed animals. Lower doses (110–330 ppm) did not lead to residual contamination of tissues [340]. Therefore, due to the natural contamination levels observed in poultry feeds [341], the risk of contamination of poultry tissues seems very low.

24.3.6.2 Cyclopiazonic Acid

Only one HPLC method was developed for CPA analysis in poultry tissues. Extraction is achieved with chloroform–methanol. Then partition into 0.1 N sodium hydroxide is done before acidification and dichloromethane extraction. The existence of an interfering compound requires cleanup with silica gel column. Mean recovery of CPA from meat samples spiked with pure toxin at levels ranging from 0.016 to 16.6 mg/kg is about 70% [342].

Tissue transfer in muscle was characterized after oral administration of 0.5, 5, and 10 mg/kg BW using this HPLC quantification. The highest levels of contamination were found in muscle 3 h after administration. For birds fed 0.5 and 5 mg/kg BW, the toxin was rapidly eliminated from meat in 24–48 h [343]. In laying hens, two studies on egg transfer were done after administration of cyclopiazonic acid at 0, 2.5, 5, and 10 mg/kg BW/day for 9 days and 0, 1.25, and 2.5 mg/kg BW/day for 4 weeks. Whatever the group of animals concerned, all eggs contained cyclopiazonic acid from the first day of exposure. The concentration of toxin was higher in albumen than in yolk (average of 100 ng/g and 10 ng/g, respectively). All birds fed 10 mg/kg BW and four of the five treated with 5 mg/kg BW died after a decrease in feed intake, in body weight, and in egg production. Other authors have reported a reduction in egg production and shell quality [344,345].

24.4 Mycotoxin Analysis and Prevalence in Processed Meats

Several studies have shown that mold species belonging to the genera *Penicillium* and *Aspergillus* could be isolated from meat products such as ripened sausages or dry-cured ham [346–348]. This mycoflora actively participates in the acquisition or improvement of organoleptic qualities of these products. However, fungal development also raises the question of a possible mycotoxin synthesis in these products, leading to the contamination of final products. Usually, fungal ferments used are selected for their lack of toxigenic potential (*P. nalgiovenses* for instance). However, many studies have demonstrated that fungal mycoflora of dry-cured meat products is usually complex and made of many fungal species, from which several may be toxinogenic, at least *in vitro*. Indeed, some of these strains were found to be able to produce aflatoxins [349,350], ochratoxins [351], citrinin, or

cyclopiazonic acid on culture medium [348,352]. Nevertheless, few studies have demonstrated the presence of mycotoxins in such processed meat. It can be linked to the lack of production of mycotoxins in this kind of substrate, to the rapid degradation of the toxins, or to both.

In this section, we will present the few available data on mycotoxin analysis in processed meat. We will focus on mycotoxins that may be produced during the ripening period. The analytical methods that may be used to evaluate the residual contamination of meat as a raw material in food making have already been presented elsewhere [353]. Therefore, fusariotoxins (trichothecenes, ZEA, and fumonisines) will not be presented. Indeed, production of these molecules cannot be observed in processed meats due to environmental conditions required for *Fusarium* development and toxinogenesis (mainly water activity) [12].

24.4.1 Aflatoxin B1

Several studies have indicated that processed meats can be contaminated with toxigenic *Aspergillus flavus* strains, especially when products are processed in countries with a hot climate [349,350, 354–356]. Moreover, it has been demonstrated that the processing conditions during aging of hams may allow aflatoxin synthesis [357]. Therefore, it is of public health importance to evaluate the possible production of aflatoxin B1 during meat processing and aging. Few studies have been carried out, but all demonstrated that the frequency of contamination of processed meat with aflatoxin B1 was low, and that the level of toxin within meat was usually below 10 ng/g [354,356]. However, it is not clear whether aflatoxin B1 was produced during meat processing or was present before at the residual level in muscles. It seems there is no relationship between the presence of toxigenic strains of *A. flavus* and aflatoxin contamination of meat samples [354]. Moreover, the frequent contamination of spices and additives used in such meat processing may also represent a source of mycotoxin [356,358]. All these studies were performed using classic methods for aflatoxin B1 analysis (see 24.2.4.3), and no special treatment was applied to samples according to their composition or process-induced changes.

24.4.2 Ochratoxin A

Many methods were devoted to the OTA analysis in processed meat; the most recent ones are summarized in Table 24.6 (Refs 359–363). They have essentially been set up in pig products because this species appears to be the most sensitive and exposed to OTA. It appears that detection limits exhibited by HPLC-FL are sufficient to control meat products according to existing regulations. The use of IAC for cleanup allows the reduction of the LOQ below 1 ng/g. However, a 10-fold OTA fluorescence enhancement obtained by using the alkaline eluent in HPLC permitted the determination of a very low level of OTA in muscle without any column purification or a concentration step [363].

However, all of these surveys essentially demonstrated the possible carryover of OTA in processed meat. Indeed, even if ochratoxigenic molds have been isolated from such foods [348,364,365], it appears that ripening and aging conditions are not favorable to toxin production [9,351].

24.4.3 Citrinin

Although citrinin-producing fungal strains have been isolated from dry-cured meat products [349,366], and it has been demonstrated that citrinin production may occur on dry-cured meat [9,367], no data are available on citrinin content in meat products, despite that this toxin has been

Table 24.6 Recent Methods for OTA Determination in Processed Meat

<i>Quantification</i>	<i>Tissue</i>	<i>Extraction</i>	<i>Clean Up</i>	<i>LOQ (ng/g)</i>	<i>References</i>
Fluorimetry	Ham	Methanol-1% sodium bicarbonate (70:30)	IAC	0.7 ^a	359
HPLC-FL	Ham	Chloroform-orthophosphoric acid	Back extraction with NaHCO ₃ , pH 7.5	0.04 ^a	360
HPLC-FL	Ham	Centrifugation	IAC	0.03	
HPIC-FL	Salami	Ethyl acetate (0.5 mol NaCl)-phosphoric acid	Back extraction with NaHCO ₃ , pH 8.0	0.2	361
HPIC-FL	Pig liver derived pâté	Acidified acetonitrile-water	C8 columns	0.84	362
HPLC-FL ^b	Dry cured pork meat	Chloroform-phosphoric acid	Back extraction with Tris-HCl pH 8.5 Addition of chloroform until 90:10 ratio	0.06	363

Note: a, limits of detection; b, mobile phase: NH₃/NH₄Cl : CH₃-CN (85:15), pH 9.8 instead of acetonitrile-water-acetic acid (99:99:2) in others.

suspected to play a role in BEN [368] and is mutagenic [369]. However, stability studies have demonstrated that this mycotoxin is only partially stable in cured ham, as already demonstrated in other animal derived foods [9,370]. Nevertheless, it may be of interest to develop methods able to quantify a possible contamination of processed meat with citrinin.

24.4.4 Cyclopiazonic Acid

As for citrinin, no survey is available concerning CPA contamination of meat products. It has been demonstrated that CPA-producing strains could be isolated from processed meats [348,352,371]. Moreover, it has been shown that toxigenic strains of *Penicillium* were able to produce the toxin on meat products, and that the toxin was stable on that substrate, with more than 80% of the initial contamination still recoverable after 8 days of incubation [9]. These results suggest that an accumulation of a relatively high level of CPA could be observed on cured meat after contamination and development of toxigenic strains. Owing to cyclopiazonic toxicity and its suspected role in “Kodua poisoning” in humans [372,373], fungal strains used in meat processing should be tested for their ability to produce cyclopiazonic acid before use in commercial products. This recommendation is in agreement with previous one concerning the use of fungal starters in cheese [374]. The development of micellar capillary electrophoresis for the detection of toxigenic mold strains may represent a useful alternative to classical analysis [375]. It has already been applied to fungal strains isolated from cured meat and allowed multidetection of mycotoxins such as CPA and also aflatoxin B1 [376]. It appears also important to develop or adapt existing analytical methods to allow the final control of processed meats.

24.5 Conclusion

Mycotoxins are widely found contaminants of cereals and other vegetal products. When contaminated feeds are distributed to farm animals, mycotoxin may be found as residues in edible parts of the animals. Owing to their breeding and feeding conditions, poultry may often be exposed to such contamination, which has consequences for the safety of edible organs. For the most important toxins, the available data on absorption, distribution within animal organism, and metabolism revealed that mainly aflatoxins and OTA may be found at significant levels in muscles and muscle foods. For these molecules, sensitive and specific methods are required to allow safety control of poultry and processed meats, because levels of contamination are usually in the low ppb range. Most commonly used methodologies are based on HPLC-FL detection of molecules. Mycotoxin contamination of meat may also result from toxigenic mold development during ripening and aging. It may lead to production and accumulation of toxins such as citrinin or cyclopiazonic acid, for which few if any methods have been established for meat control. Even if the toxicity of such molecules appears less important than the previous ones, their possible implications in human diseases or syndromes should lead to the implementation of methods able to control contamination of processed meat.

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Chapter 25

Detection of Genetically Modified Organisms in Processed Meats and Poultry

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25.1 Introduction

25.1.1 *Genetically Modified Organism Production for Food and Feed*

The advancement of biotechnologies applied to the agro-food industry has resulted, during the past few years, in an increasing number of genetically modified organisms (GMOs) being introduced into the food chain at various levels. Although the regulatory approach to this matter differs depending on the attitudes of different legislative bodies, to inform final consumers correctly and to be able to guarantee the safety of food production chains, the traceability of genetically modified products or ingredients coming from genetically modified products must be guaranteed.

GMOs can be defined as organisms in which the genetic material has been altered by recombinant deoxyribonucleic acid (DNA) technologies, in a way that does not occur naturally by mating or natural recombination. Recombinant DNA techniques allow the direct transfer of one or a few genes between either closely or distantly related organisms; in this way, only the desired characteristic should be safely transferred from one organism to another, speeding up the process of improving the characteristics of target organisms and facilitating the tracking of the genetic changes and of their effects.

The first transgenic plants obtained by recombinant DNA technologies were produced in 1984, and since then more than 100 plant varieties, many of which are economically important crop species, have been genetically modified. The majority of these GMOs have been approved, albeit with differences according to the various legislations worldwide, for use in livestock feed and human nutrition.¹

Whereas only a few crops have been modified so far to improve their nutritional value, most of the first generation of genetically modified (GM) crops (i.e., those currently in, or close to, commercialization) aim to increase yields, and to facilitate crop management. This is achieved through the introduction of resistance to viral, fungal, and bacterial diseases, or insect pests, or through herbicide tolerance. So far the majority of GM crops can be clustered according to three main characteristics:

- *Insect-protected plants.* The majority of the commercialized products belonging to this category are engineered to express a gene derived from the soil bacterium *Bacillus thuringiensis* (Bt) that encodes for the production of a protein, the delta endotoxin, with insecticidal activity. Other genes that are used in developing this category of crops encode inhibitors of digestive enzymes of pest organisms, such as insect-specific proteinases and amylases, or direct chemically mediated plant defense by plant secondary metabolites.
- *Herbicide-tolerant plants.* A variety of products have been genetically engineered to create crops in which the synthesis of essential amino acids is not inhibited by the action of broad-spectrum herbicides like glufosinate, as happens for conventional plants.
- *Disease-resistant plants.* Using gene manipulation technology, specific disease resistance genes can be transferred from other plants that would not interbreed with the crops of interest, or from other organisms; this allows the transformed crops to express proteins or enzymes that interfere with bacterial or fungal growth. GM virus-resistant crops have also been developed using “pathogen-derived resistance,” in which plants expressing genes for particular viral proteins are “immunized” to resist subsequent infection.

Other phenotypic characteristics, less common than those mentioned earlier, include: modified fatty acid composition, fertility restoration, male sterility, modified color, and delayed ripening.

According to the latest statistics available, GMO crop cultivation has been continuously growing, since its introduction in the agricultural practice, in both industrial and developing countries. “Although the first commercial GM crop (tomato) was planted in 1994, it has been in the last few years that a dramatic increase in planting has been observed, bringing the estimated global area of GM crops in 2007 to around 114, 3 million hectares, involving 12 million farmers in 23 countries worldwide, and with a global market value for biotech crops estimated to be around \$6.9 billion. As for the kinds of cultivated crops, four GM crops represent at present almost 100% of the market: GM soybean accounts for the largest share, 51.3%, followed by maize with 30.8%, cotton, 13.1%, and canola, 4.8%. These figures confirm how globally widespread GM cultivation is and how important the numbers are becoming compared to traditional crops: in particular, in 2007, GM soybean accounted for 64% of total soybean-plantings worldwide, whereas maize, cotton, and canola represented 23, 43, and 20% of their respective global plantings.²

A new wave of genetically modified products, the second generation of GM-derived food and feed, is now at the end of its developing stage or already under evaluation from the competent authorities for approval. These products mainly respond with similar approaches to the same issues addressed by the first generation (herbicide resistance, pest protection, and disease resistance). However, an increasing number of products are trying to respond to various new problems, such as removing detrimental substances, enhancing health-promoting substances, enhancing vitamin and micronutrient content, altering fatty acids and starch composition, reducing susceptibility to adverse environmental conditions, and improving carbon and nitrogen utilization. This second generation of GMOs should constitute a new class of products in an attempt to respond to the needs of consumers and of industries in the near future.

25.1.2 *Legislative Framework for Genetically Modified Organism Traceability*

The need for monitoring the presence of GM plants in a wide variety of food and feed matrices has become an important issue both for countries with specific regulations on mandatory labeling of food products containing GM ingredients or products derived from GMOs, and for countries without mandatory labeling on food products but that are required to test for the presence of unapproved GM varieties in food products.

Among the countries with mandatory labeling, the European Union (EU) has devised an articulated regulatory framework on GMOs to guarantee an efficient control on food safety-related issues and to ensure correct information to European consumers; the use and commercialization of GM products and their derivatives have been strictly regulated in both food and feedstuffs, and compulsory labeling applies to all products containing more than 0.9% genetically modified ingredients (an adventitious presence threshold of 0.5% applies for GMOs that have already received a favorable risk evaluation but have not yet been approved). Other mandatory schemes for labeling are present worldwide in various countries, including Australia and New Zealand, Brazil, Cameroon, Chile, China, Costa Rica, Ecuador, India, Japan, Malaysia, Mali, Mauritius, Mexico, Norway, the Philippines, Russia, Saudi Arabia, South Africa, South Korea, Switzerland, Taiwan,

Thailand, and Vietnam. Most of these countries have established mandatory labeling thresholds ranging from 0 to 5% of GMO content.^{3,4} In other countries in which labeling is voluntary, such as the United States, Canada, and Argentina among the most important, being able to detect GM varieties is however of great importance, e.g., to prevent unauthorized transgenes from entering the food productions chains.

25.1.3 Analytical Methods for Genetically Modified Organism Traceability

One of the main challenges related to the use of GMOs is their traceability all along the food chain. In general, to be able to correctly identify the presence of transgenic material, a three-stage approach is needed:⁵

- *Detection.* A preliminary screening is performed to detect characteristic transgenic constructs used to develop GMOs (e.g., promoter and terminator sequences in the case of DNA analysis) and to gain initial insight into the composition of the sample analyzed.
- *Identification.* This stage allows researchers to gain information on the presence of specific transgenic events in the sample analyzed. According to the specific regulation framework in which the analysis is performed, the presence of authorized GMOs should then be quantified, and the presence of unauthorized GMOs should be reported to competent authorities and the product prevented from entering the food chain.
- *Quantitation.* Transgene-specific quantification methods should be used at this stage to determine the amount of one or more authorized GMOs in the sample, and to assess compliance with the labeling thresholds set in the context of the applicable regulative framework.

All along this analytical scheme for the detection of GMOs, particular attention should be paid to the evaluation of the degradation of the target DNA/protein during sampling and processing and to the robustness of the analytical methods. Thorough knowledge and understanding of the problems associated with both the sample to be analyzed and the method for the analysis are fundamental prerequisites to obtaining reliable results.

The first two stages of this scheme of analysis can essentially be accomplished by qualitative methods, whereas semiquantitative or quantitative methods need to be used to accomplish the third stage of analysis.

At present the two most important approaches for the detection of GMOs are (i) immunological assays based on the use of antibodies that bind to the novel proteins expressed, and (ii) polymerase chain reaction (PCR)-based methods using primer oligonucleotides that selectively recognize DNA sequences unique to the transgene.

The two most common immunological assays are enzyme-linked immunosorbent assay (ELISA) based methods and immunochromatographic assays (e.g., lateral flow strip tests). Whereas the former can produce qualitative, semiquantitative, and quantitative results according to the method employed, the latter, although fast and easy to perform, produces mainly qualitative results. However, both techniques require a sufficient protein concentration to be detected by specific antibodies, and thus their efficiency is strictly related to the plant environment, tissue-specific protein expression, and, not least, protein degradation during sampling and processing.

The most powerful and versatile methods for tracking transgenes are, however, based on the detection of specific DNA sequences by means of PCR methods. These methods are reported to

be highly specific, and have detection limits close to a few copies of the target DNA sequence. Qualitative and semiquantitative detection of GMOs can easily be achieved via end-point PCR combined with gel electrophoresis, whereas quantitative detection can only be obtained by applying specific real-time PCR protocols, which rely on the quantification of fluorescent reporter molecules that increase during the analysis with the amount of PCR product.

In addition to the aforementioned methods, other detection methods based on chromatography, mass spectrometry, and near-infrared (NIR) spectroscopy have been developed⁵ and found to be suitable for specific applications, in particular when the genetic modifications create significant changes in the chemical composition of the host organism.

25.1.4 Transgenic Material in Processed Meats and Poultry

The significant increase of GM productions since the commercialization of the first genetically modified crop has generated interest and concern regarding the fate of transgenic material along the food chain. Questions have been posed both at public and at scientific levels about the potential appearance of novel proteins and recombinant DNA in products for human consumption, driven by animal products potentially containing GMOs. Considering the fact that livestock consume large amounts of plant material and that high-protein feeds are among the most common GM crops, it has become necessary to evaluate the fate of GMOs in the animals' diet and the possible consequences on human health. From a legislative point of view, however, countries that have implemented labeling regulation concerning GM feed have at present no mandatory regulations on products derived from livestock fed transgenic feed.

Although in the past few years several attempts to investigate the fate of transgenic proteins and DNA within the gastrointestinal tract of livestock fed GMOs and the incorporation of transgenic material into tissues have been reported,³ to date very few results support the feasibility of detecting traces of transgenic material in animal tissues outside the gastrointestinal tract. Indeed several factors could influence the presence and hamper the detectability of DNA and protein targets in animal tissues as a result of GM crops feeding: (i) the kind of genetic modification and the type of plant tissue in which the protein is expressed, together with environmental conditions of growth of the GM crop, could cause the content of transgenic protein to vary greatly; (ii) postharvest feed processing, such as ensiling, steeping, wet-milling, and heating, often degrade DNA and protein to an undetectable level; (iii) the rapid degradation observed in the gastrointestinal tract dramatically reduces the absorption across the epithelial tissues of protein and DNA fragments suitable for analytical detection; and (iv) although the passage of dietary DNA fragments has been suggested by several researchers, currently available PCR techniques have only allowed detection of "high copy number genes" (e.g., plant endogenous genes such as rubisco and chloroplast-specific sequences), whereas transgenes are often the result of a single insertion event.

Considering the detectable presence of GM-derived materials outside the gastrointestinal tract in livestock as an extremely rare event, the main route for the presence of transgenic material in processed meat and poultry could be an external event, such as an adventitious contamination (e.g., during slaughtering, the gastrointestinal content could come in contact with other animals' parts) or the intentional addition of GM-derived additives intended to enhance meat products properties. In particular, apart from additives produced via the use of genetically modified microorganisms (GMMs) such as antioxidants (e.g., ascorbic acid), flavor enhancer (e.g., glutamate), and enzymes (e.g., proteases to be used as tenderizer), which do not require labeling because GMMs are not directly associated with the final purified product, several additives used during meat

processing are produced from GMOs and mainly from GM soybean and maize. Soy proteins (in the form of soy flour, texturized vegetable protein [TVP], soy concentrates, and soy isolates) are by far the most commonly employed vegetal protein in the meat industry on account of their excellent water-binding properties, fat emulsification activity, and high biological value. Maize starches are often used on account of their water-binding properties, and the products obtained by their hydrolysis or thermal treatment, in the form of maltodextrin, are often used as filler or stabilizer. Soybean is also a source of lecithin and mono- and diglycerides commonly employed as emulsifiers in meat products to reduce the risk of fat and water separation, to lower cooking loss, and to improve the texture and firmness of the product.

25.2 Detection of Genetically Modified Organisms

Approved transgenes and detection methods are continuously updated, and official detection methods are validated and reported by the different national control agencies.⁶ Online databases of protein and DNA-based methods that have been validated by different research agencies are also available for consultation.⁷

25.2.1 DNA-Based Methods

GMOs currently available are the result of transformation events that provide the stable insertion of an exogenous DNA fragment into a host's genome, by means of DNA recombinant technology. The insert contains at least three elements: the gene coding for a specific desired feature and the transcriptional regulatory elements, typically a promoter and a terminator. Several additional elements could be present, depending on the transformation system employed: selection markers such as antibiotic resistance, introns, or sequences coding for signaling peptides are commonly used.⁸

A wide spectrum of analytical methods based on PCR have been developed during the past decade, and PCR-based assays are generally considered the method of choice for regulatory compliance purposes. The general procedure for performing PCR analysis includes four subsequent phases: sample collection, DNA isolation, DNA amplification, and detection of products. The latter two steps may occur simultaneously in certain PCR applications, such as real-time PCR.

Sampling, DNA extraction, and purification are crucial steps in GMO detection. Sampling plans have to be carefully designed to meet important statistical requirements involving the level of heterogeneity, the type of material (raw material, ingredients, or processed food), and the threshold limit for acceptance.⁹ DNA quality and purity are also parameters that dramatically affect the PCR efficiency.¹⁰ DNA quality is strictly dependent on degradation caused by temperature, the presence of nucleases, and low pH, and determines the minimum length of DNA-amplifiable fragments. Moreover, the presence of contaminants from the food matrix or chemicals from the method used for DNA isolation can severely affect DNA purity and could cause the inhibition of PCR reactions.

The PCR scheme involves subsequent steps at different temperatures during which: (i) the DNA is heated to separate the two complementary strands of the DNA template (denaturation, 95°C), (ii) the oligonucleotide primers anneal to their complementary sequences on the single strand target DNA (annealing step, 50–60°C), and (iii) the double-strand DNA region formed by the annealing is extended by the enzymatic activity of a thermostable DNA polymerase (extension step, 72°C). All these cycles are automatically repeated in a thermal cycler for a certain number of

cycles, and at the end of the process the original target sequence results in an exponential increase in the number of copies.

Several authors have classified PCR-based GMO assays according to a “level of specificity” criterion.^{5,11}

1. *Methods for screening purposes* are usually focused on target sequences commonly present in several GMOs. The most commonly targeted sequences pursuing this strategy are two genetic control elements, the cauliflower mosaic virus (CaMV) 35S promoter (P-35S) and the nopaline synthase gene terminator (T-NOS) from *Agrobacterium tumefaciens*.
2. *Gene-specific methods* target a portion of DNA sequence of the inserted gene. These methods amplify a gene tract directly involved in the genetic modification event, typically structural genes such as Cry 1A(b) coding for endotoxin B₁ from Bt, or the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, coding for an enzyme conferring herbicide tolerance to the GM crop.

Both the screening and the gene-specific approach are useful to investigate the presence of GMOs, but fail to reveal the GMO identity. Moreover, these methods are based on the detection of sequences naturally occurring in the environment, and this fact could lead to a significant increase of false-positive results.

3. Junction regions between two artificial construct elements such as the promoter and the functional gene are targeted by construct-specific methods; these reduce the risks of false-positive appearances and increase the chances of identifying the GM source of DNA. However, more than one GMO could share the same gene construct, preventing their unambiguous identification.
4. The highest level of specificity is obtained using event-specific methods that target the integration locus at the junction between the inserted DNA and the recipient genome.

An overview of validated PCR methods for the different strategies of GMO detection is provided in Section 25.2.1.2.

PCR assays can be followed by confirmation methods suitable to discriminate specific from unspecific amplicons. Gel electrophoresis is the simplest method to confirm the expected size of PCR products, but fails to identify the presence of unspecific amplicons having the same size of the expected PCR product. Sequencing the amplicons is the most reliable method of confirming the identity of PCR products, but it is an expensive approach and requires specific instrumentation not frequently available in control laboratories. Nested PCR is commonly used both in optimization steps and in routine analyses; it is based on a second PCR reaction in which a PCR product is reamplified using primers specifically designed for an inner region of the original target sequence. Since nested PCR consists of two PCR reactions in tandem, increased sensitivity is obtained. At the same time, however, it increases the risk of false positives by carryover or cross-contamination. Southern blot assays are another reliable confirmation method; after gel electrophoresis, DNA samples are fixed onto nitrocellulose or nylon membranes and hybridized to a specific DNA probe. Southern blot is time-consuming and quite labor-intensive, and its implementation in routine analysis is limited.

25.2.1.1 DNA Extraction Methods

Isolation of nucleic acids is one of the most crucial steps in genetic studies. The presence of a great variety of extraction and purification methods arises from the numerous parameters that

analysts have to take into account (source organism, specific matrix to be analyzed, downstream application, etc.). Regardless of the specific extraction method, the overall aim of this part of the detection process is to obtain an adequate yield of recovered DNA of high quality and purity to be used in the subsequent steps of the PCR analysis. DNA quality essentially refers to the degree of degradation of the nucleic acids recovered; the presence of DNA fragments long enough to be amplifiable is a key factor to be taken into account when designing and performing a PCR test. DNA purity mainly refers to the possible presence of PCR inhibitors in the extracted solution; the presence of proteins, bivalent cations, polyphenols, polysaccharides, and other secondary metabolites can interfere with the enzyme activity and dramatically reduce the efficiency of PCR amplification.

The extraction of nucleic acids from biological material essentially requires the following basic steps: cell lysis/sample homogenization, inactivation of nucleases, separation of the nucleic acid from other matrix components, and recovery of the purified nucleic acids.¹²

Because food matrices in general and meat samples in particular can vary greatly in their physical and chemical properties, it is difficult to devise an all-purpose extraction procedure suitable for the different matrices and meeting all the necessary criteria. For this reason, customized DNA extraction methods need to be developed or adapted from more general methods, to respond to the particular problem of the specific matrix to be analyzed and to optimize the extraction efficiency. Common extraction and purification methods for the recovery of nucleic acids reported in the literature are fundamentally based in one of the following:

- Combination of phenol and chloroform for proteins removal followed by selective precipitation of nucleic acids with isopropanol or ethanol
- Use of the ionic detergent cetyltrimethylammonium bromide (CTAB) to lyse cells and selectively insolubilize nucleic acids in a low-salt environment, followed by solubilization and precipitation with isopropanol or ethanol
- Use of detergents and chaotropic agents followed by DNA binding on silica supports (e.g., spin column or magnetic silica particles) and elution in a low-salt buffer

Several commercial methods are currently available that employ combination of the strategies mentioned earlier to perform fast and reliable extractions for specific food and feed matrices.

An overview of customized DNA extraction procedures available in the literature, clustered according to the different meat and poultry samples to be analyzed and the different processing they underwent, is reported in Table 25.1, together with the corresponding bibliographic references.

25.2.1.2 *PCR-Based Assay Formats*

25.2.1.2.1 *Qualitative PCR-Based Methods*

Conventional end-point PCR has been extensively used as a qualitative method to detect the presence of transgenic plants as raw materials and in processed foods. PCR products are usually separated and visualized using agarose gel electrophoresis in combination with DNA staining.

The main advantages of this technique are the cost effectiveness and the simplicity. Conventional PCR is carried out using instrumentation commonly available in control laboratories. The amplification and the detection steps, occurring separately, extend the analysis time, increase the risk of contamination, and reduce the automation possibilities. Despite these potential limitations, several authors have developed methods for the sensitive detection of GM crops.

Table 25.1 Customized DNA Extraction Procedures for Different Meat Samples

<i>Samples Type</i>	<i>Processing</i>	<i>Deoxyribonucleic Acid Extraction/Purification</i>	<i>Reference</i>
Beef muscle Chicken muscle Pork muscle	Unprocessed	CTAB extraction method followed by CTAB precipitation or chloroform extraction	13
Broiler muscle	Unprocessed	In-house method based on ammonium acetate extraction followed by isopropanol precipitation	14
Pork muscle	Unprocessed	In-house method based on phenol/ chloroform/isoamyl alcohol and ammonium acetate extraction followed by isopropanol precipitation	15
Beef meat Chicken meat Lamb meat Pork meat Turkey meat	Mincing Freezing Corned Steak Pie	CTAB extraction method followed by purification through a silicon spin column (Qiagen)	16
Beef meat Chicken meat Pork meat Sheep meat Turkey meat	Curing Cooking Smoking Heating Sterilization	CTAB extraction method followed by QIAquick PCR Purification Kit (Qiagen)	17
Beef meat Chicken meat Duck meat Goat meat Lamb meat Pork meat Turkey meat	Canning under different conditions (home, industrial, tropical conditions, ultra high heat)	CTAB extraction method followed by QIAquick PCR Purification Kit (Qiagen)	18
Poultry meat	Light boiling Heavy boiling Light baking Heavy baking Canning Autoclaving	Wizard DNA extraction Kit (Promega)	19
Turkey-based meat products (sausages, canned liver, ready-to-eat hamburgers)	Smoking Cooking Sterilization Frying Roasting	Wizard DNA clean-up system (Promega)	20

Conventional PCR assays have been improved, performing simultaneous amplification of several GMOs in the same reaction, and using more than one primer pair; this multiplex PCR format often requires longer optimization procedures, but results in more rapid and inexpensive assays. Several multiplex PCR methods have been developed that allow simultaneous screening of different GM events in the same reaction tube.²¹⁻²⁴

25.2.1.2.2 Quantitative PCR–Based Methods

The threshold for compulsory labeling of products containing GMOs set in many countries greatly accelerated the development of quantitative PCR-based GMO assays to comply with legislative requirements. Usually, the efficiency of quantitative methods is described using at least two fundamental parameters: the limit of detection (LOD) and the limit of quantification (LOQ). One of the main drawbacks is that these values are usually determined using standard reference material with high-quality DNA, and their value dramatically decreases when faced with complex matrices or processed products. The availability of reference material containing known amounts of GMOs is another problematic aspect in calibrating and standardizing quantitative assays, because certified reference materials (CRMs) are commercially available only for a limited number of GMOs (e.g., JRC-IRMM in Europe²⁵). To overcome problems related to CRMs, alternative strategies have been proposed, such as the use of plasmid constructs carrying the sequence to be quantified, which seems to represent a promising alternative strategy.^{26,27}

25.2.1.2.2.1 Quantitative Competitive PCR In quantitative competitive polymerase chain reaction (QC-PCR), the target amplification is coupled with coamplification of quantified internal controls that compete with target DNA for the same primers. The assay is carried out by amplifying samples with varying amounts of a previously calibrated competitor, finding the point that gives the same quantity of amplification products: the equivalence point. The end-point quantitation is then usually performed on agarose gel electrophoresis. QC-PCR methods for Roundup Ready (RR) soybean and Maximizer maize have been developed²⁸ and tested in an interlaboratory trial at the EU level.²⁹ A screening method targeting the 35S promoter and the NOS terminator has also been reported.³⁰ Even if the QC-PCR method potentially allows GMO detection with low limits of quantification, some drawbacks have limited the diffusion of this technique. The use of pipetting on a large scale increases the risk of cross-contamination and makes automation procedures difficult. Moreover, QC-PCR is time-consuming and often needs long optimization procedures.

25.2.1.2.2.2 Real-Time PCR Real-time PCR-based methods have become more and more often recognized in the past few years as the method of choice for GMO quantitation. The most distinctive feature of this technique is that the amplicon can be monitored and quantified during each cycle of the PCR reaction: the increase in amplicon amount is indirectly measured as fluorescence signal variation during amplification. Quantitation by real-time PCR relies on the setting of two parameters: (i) the threshold fluorescence signal, defined as the value statistically significant above the noise; and (ii) the threshold cycle (C_t), which is the cycle number at which the fluorescence value is above the set threshold. Quantitation can be calculated directly comparing C_t values of the GM-specific targeted gene with a reference gene. To obtain reliable measures, it is essential to perform the reactions starting with the same concentration of DNA template. Moreover, this quantitation method relies on the assumption that both amplicons are amplified with the same efficiency. As an alternative to overcome this limitation, quantitation can be done building a standard curve with a series of PCR reactions using different known initial amounts of reference material. This method allows only C_t values of the same amplicons to be compared, reducing errors in measurements.

Several chemical strategies are currently available for real-time PCR analysis. Nonspecific methods use DNA intercalating agents such as SYBR Green, and others.³¹ These assays have good sensitivity, but often require postanalysis confirmation methods to distinguish the amplicons' identity and avoid false positives. This purpose is achieved by some commercial instruments, which allow analysis of the thermal denaturation curve to define the amplicons' identity.³²

Specific methods, however, allow the simultaneous detection and confirmation of target sequences using specific probes or primers labeled with fluorescent dyes. The most widely adopted technology in real-time PCR analysis of GMOs is the TaqMan approach: a DNA oligonucleotide probe containing both a fluorophore and a quencher conjugated at each side of the molecule. During the extension step, the probe is degraded by the 5'–3' exonuclease activity of the DNA polymerase, and the quenching molecule is consequently physically separated from the fluorophore reporter, allowing the reporter to emit a detectable fluorescence that increases at each amplification cycle. A further improvement compared to TaqMan assays has been achieved through the use of minor groove binding (MGB) probes, in which a minor groove binder group increases the melting temperature of the duplex, improving the probe's selectivity and sensitivity. Alternatives, based on the same principle of physical separation between fluorophore and quencher, have been developed in scorpion primers and in molecular beacons. In these approaches a conformational change induced by the specific annealing, instead of a degradation event, drives the mechanism of fluorescence emission (a passage from a hairpin-shaped structure in solution to an unfolded conformation upon target hybridization). Other alternative technologies such as fluorescence resonance energy transfer (FRET) probes and light up probes could be promising tools also for the detection of GMOs.³¹ Comparison of the different chemistries currently available for GMO detection has been recently reported.^{33,34}

Compared to the other PCR-based methods, real-time PCR offers several advantages: (i) by performing both reaction and detection in a closed tube format, the risk of cross-contamination is greatly reduced; (ii) the high degree of automation makes real-time PCR less labor-intensive and time-consuming; and (iii) due to the possibility of setting multiplex assays and simultaneously performing several tests, the sample throughput result is increased compared with other PCR quantitation methods.

Real-time PCR has been successfully employed for quantitative analysis of genetically modified maize, soybean, rapeseed, cotton, potato, rice, tomato, and sugar beet (see Table 25.2). Several composite feed diets such as silage, commercial feed, and pellet mixed diet have been also investigated for their possible GMO content using real-time PCR.^{35,36}

25.2.1.2.2.3 PCR Enzyme-Linked Immunosorbent Assay An alternative method to perform end-point quantitation is coupling a conventional PCR with an enzymatic assay. In PCR-ELISA, a capture probe specific for the PCR amplicon is used to capture the amplicon in a well plate. PCR products, labeled during amplification, are then quantified by a conventional ELISA assay targeting the labeled amplicon. The main advantage of PCR-ELISA is that it offers a cheaper alternative to real-time PCR assays and requires less expensive instruments. Some PCR-ELISA applications have been developed for GMOs detection and quantitation.^{37,38} However, this technique does not seem to be widely adopted for accurate GMO quantitation.

25.2.1.3 Applications in Meat and Poultry Analysis

Because of the recent interest in the fate of transgenic DNA after consumption by human and animals, several studies have attempted to detect DNA fragments, related to both endogenous

Table 25.2 Validated PCR Methods for the Different Strategies of GMO Detection

<i>P</i>	Target	Primer Sequences	TaqMan Probe if Real-Time (5'-FAM 3'-TAMRA)	Reference
1	Animal mtDNA 16S rRNA gene	5'-GGTTTACGACCTCGATGT-3' 5' CCGGCTGAACTCAGATCAC-3'		39
1	Myostatin gene of mammals and poultry species	5'-TTGTGCAAAATCCTGAGACTCAT-3' 5'-ATACCAGTGCCTGGTTCAT-3'	5'-CCCATGAAAGACCGTACAAGGTACTCTG-3'	17
1	Cattle	5'-ACTCCTACCCATCATGCAGAT-3' 5'-TTTTTAAATATTCAGCTAAGAAAAAAG-3'	5'-AACATCAGGATTTTGTGCTGCAITTC-3'	18
1	Chicken	5'-TGTACCTGGGAAAGTGGTTACT-3' 5'-TTTTCGATATTTGAAATAGCAGTTACAA-3'	5'-TGAAGAAAAGAACTGAAGATGACACT GAAATTAAG-3'	18
1	Lamb	5'-ACCCGTCAAAGCAGACTTAAACG-3' 5'-TAAATATTCAGCTAAGGAAAAAAGAAAG-3'	5'-CAGGATTTTGTCCGCATTCGCTT-3'	18
1	Pig	5'-CCCCACCTCAAGTGCCT-3' 5'-CACAGACTTATTTCTCCACTGC-3'	5'-CACAGCAAGCCCTTAGCCCC-3'	18
1	Turkey	5'-TGTATTCAGTAGCACTGCTTATGACTACT-3' 5'-TTTATTAATGCTGGAAGAAATTTCCAA-3'	5'-TTATGGAGCATCGCTATCACAGAAAA-3'	18
2	Chloroplast gene for vegetal species	5'-CGAAATCGGTAGACCGCTACG-3' 5'-GGGGATAGAGGGACTTGAAC-3'		40
2	Canola	5'-GGCCAGGGTTCCGTGAT-3' 5'-CCGTGCTGTAGAACCATTGG-3'	5'-AGTCCTTATGTGCTCCACTTTCTGGTGCA-3' (5'-VIC)	41
2	Cotton	5'-AGTTTGTAGTTTTTGTATGTACATTGAG-3' 5'-GCATCTTTGAACCCCTACTG-3'	5'-AAAATAAAAATAATGGGAACAACCAT GACATGT-3'	42
2	Maize	5'-CTCCCAATCCTTTGACATCTGC-3' 5'-TCGATTTCTCTTGGTGACAGG-3'	5'-AGCAAAGTCAGAGCGCTGCAATGCA-3'	43
2	Potato	5'-GGACATGTGAAGACGGAGGC-3' 5'-CCTACCTTACCCCTCCGC-3'	5'-CTACCACCATTACCTCCGACCTCCTCA-3'	44
2	Rice	5'-TGGTGAGCGTTTTCAGTCT-3' 5'-CTGATCCACTAGCAGGAGGTCC-3'	5'-TGTGTGCTGCCAATGTGGCCTG-3'	45
2	Soybean	5'-TCCACCCCATCCACATT-3' 5'-GGCATAGAAGGTGAAGTGAAGGA-3'	5'-AACCCGGTAGCGTTGCCAGCTTCG-3'	46

2	Sugarbeet	5'-GACCTCCATATTACTGAAAGGAAG-3'	5'-CTACGAAGTTTAAAGTATGTGCCCGCTC-3'	47
2	Tomato	5'-GAGTAATTGCTCCATCCTGTTCA-3'		48
3	CaMV 35S promoter	5'-GGATCCTTAGAAGCATCTAGT-3'		49
3	Coat protein gene from potato potyvirus Y (PVY)	5'-CGTTGGTGCAATCCCTGCAATGG-3'		50
3	CP4 EPSPS	5'-CCACGCTCTCAAAGCAAGTGG-3'		15
3	CryIA(b)	5'-TCCTCTCCAAATGAAATGAACCTTCC-3'		46
3	Figwort mosaic virus (P-FMV) promoter	5'-GAATCAAGGCTATCACGTCC-3'		51
3	Hygromycin phosphotransferase (hph) gene	5'-CATCCGCACTGCCCTCATAACC-3'		52
3	Neomycin phosphotransferase II (nptII) gene	5'-GCGTGGCCGATGAAGGTGCTGTC-3'		53
3	Nopaline synthase (NOS) terminator	5'-CGGTCCCTCATGTTCCGGGGTCTC-3'		49
4	Canola GT73	5'-CCGACCCCTGAGCCAGC-3'		54
4	Canola Ms8	5'-GGTGCCACCGTTGTTCTGA-3'	5'-TTCCCGGACATGAAGATCATCCTCCTT-3'	55
4	Canola Rf3	5'-GCCAAAAAGCTACAGGAGATCAATG-3'	5'-AATATAATCGACGGATCCCCGGGAATTC-3'	56
		5'-GCTGCTCGATGTGACAAAGATTAC-3'	5'-CGCACCGCTTATCGACCATAAGCCCCA-3'	
		5'-CGCCGATGGTTTCTACAA-3'		
		5'-GGCGTCCGGTTTCCACTAT-3'		
		5'-GGATCTCCTGTCATCT-3'		
		5'-GATCATCCTGATCGAC-3'		
		5'-GCATGACGTTATTATGAGATGGG-3'		
		5'-GACACCGCGCGGATAATTATCC-3'		
		5'-CCATTTGACCATCACTACTATTGCT-3'		
		5'-GCTTATACGAAGGCAAGAAAAGA-3'		
		5'-GTTAGAAAAGTAAACAATTAATATAGCCCGG-3'		
		5'-GGAGGGTGTITGGTTATC-3'		
		5'-AGCATTTAGCATGTACCATCAGACA-3'		
		3'-CATAAAGGAAGATGGAGACTTGAG-3'		

(Continued)

Table 25.2 (Continued)

<i>P</i>	Target	Primer Sequences	TaqMan Probe if Real-Time (5'-FAM 3'-TAMRA)	Reference
4	Canola T45 (HCN28)	5'-CAATGGACACATGAATTATGC-3' 5'-GACTCTGTATGAAGCTGTTCCG-3'	5'-TAGAGGACCCTAACAGAACTCGCCGT-3'	41
4	Cotton MON 1445	5'-GGAGTAAAGACGATTCAGATCAAAACAC-3' 5'-ATCGACCTGCAGCCCAAGCT-3'	5'-ATCAGATTGCTGTTCCCGCCCTCAGTTT-3'	57
4	Cotton 281-24-236	5'-CTCATTTGCTGATCCATGTAGATTTC-3' 5'-GGACAATGCTGGGCTTTGTG-3'	5'-TTGGGTAAATAAAGTCAGATTAGAGGG AGACAA-3'	42
4	Cotton 3006-210-23	5'-AAATATTAACAATGCATGAGTATGATG-3' 5'-ACTCTTCTTTTCTCCATAITGACC-3'	5'-FACTCATTTGCTGATCCATGTAGATTTCGCCG-3'	42
4	Cotton MON 531	5'-TCCCATTCCGAGTTTTCACGT-3' 5'-AACCAATGCCACCCACTGA-3'	5'-TTGTCCCTCCACTTCTTCTC-3'	58
4	Cotton LLCotton25	5'-CAGATTTTGTGGGATGGAAATTC-3' 5'-CAAGGAACATTAACACTGAG-3'	5'-CTTAACAGTACTGGGCCGTCGACCCGC-3'	59
4	Maize Bt10	5'-CACACAGGAGATTATTATAGGG-3' 5'-GGGAATAAGGGCGCACCCG-3'	60	
4	Maize Bt11	5'-AAAAGACCACAACAAGCCCG-3' 5'-CAATGCGTCTCCACCAAGTACT-3'	5'-CGACCATGGACAACAACCCAAACATCA-3'	43
4	Maize CBH-351	5'-CCTTCGCAAGACCCCTCCTCTATA-3' 5'-GTAGCTGTCCGGTGTAGTCTCCTCGT-3'	21	
4	Maize DAS-59122-7	5'-GGGATAAGCAAGTAAAGCGCTC-3' 5'-CCTTAATTCCTCGCTCATGATCAG-3'	5'-TTTAAACTGAAGCGCGGAAACCGACAA-3'	61
4	Maize event 176	5'-TGTTCCACCAGCAGCAACCAG-3' 5'-ACTCCACTTTGTGCAGAACAGATCT-3'	5'-CCGACGTGACCGACTACCACATCGA-3'	43
4	Maize GA21	5'-GAAGCCTCGGCAACGTCA-3' 5'-ATCCGGTTGGAAGCGACTT-3'	5'-AAGGATCCGGTGCATGGCCCG-3'	43
4	Maize MIR604	5'-GCCGACCCCAATCAACAG-3' 5'-GGTCATAACGTGACTCCCTTAATTC-3'	5'-AGCGGGAAACCGACAATCTGATCATG-3'	62
4	Maize MON 810	5'-GATGCCCTTCTCCCTAGTGTGA-3' 5'-GGATGCACCTCGTTGATGTTG-3'	5'-AGATACCAAGCGGCCATGGACAACAA-3'	43

4	Maize MON 863	5'-GTAGGATCGGAAAAGCTTGGTAC-3' 5'-TGTTACCGCCCTAAATGCTGAAC-3'	5'-TCAACACCCATCCGAACAAGTAGGGTCA-3'	63
4	Maize NK603	5'-ATGAATGACCTCGAGTAAGCTTGTTAA-3' 5'-AGAGATAACAGGATCCACTCAAACACT-3'	5'-TGGTACCA CGGCACACACTTCCACTC-3'	64
4	Maize T25	5'-GCCAGTTAGGCCAGTTACCCA-3' 5'-TGAGCGAAAACCCATAAGAACCCCT-3'	5'-TGCAGGCATGCCCCGCTGAAATC-3'	43
4	Maize TC1507	5'-JAGTCTTCGGCCAGATGG-3' 5'-CTTTGCCAAGATCAAGCG-3'	5'-TAACTCAAGGCCCTCACTCCCG-3'	65
4	Potato EH92-527-1	5'-GTGTCAAAACACAATTTACAGCA-3' 5'-TCCCCTTAATTCCTCCGCTCATGA-3'	5'-AGATTGTCGTTTCCCGCCTTCAGTT-3'	44
4	Rice LLRICE601	5'-TCTAGGATCCGAAGCAGATCGT-3' 5'-GGAGGGCCGGAGTGT-3'	5'-CCACCTCCCAACAATAAAAGCGCCTG-3'	66
4	Rice LLRICE62	5'-AGCTGGCGTAATAGCCGAAGAGG-3' 5'-TGCTAACGGGTGCATCGTCTA-3'	5'-CGCACCCGATTATTTAFACTTTIAGTCCACCCT-3'	45
4	Soybean A2704-12	5'-GCAAAAAGCGGTTAGCTCCT-3' 5'-ATTCAGGCTGCGCAACTGT-3'	5'-CGGTCTCCCGATCGCCCTTCC-3'	67
4	Soybean GTS 40-3-2	5'-CCGGAAGGCCAGAGGAT-3' 5'-GGATTCAGCATCAGTGGCTACA-3'	5'-CCGGCTGCTTGACCCGTGAAG-3'	68
4	Sugarbeet H7-1	5'-JGGGATCTGGTGGCTTAACT-3' 5'-AATGCTGCTAAATCCTGAG-3'	5'-AAGGGGGAAAACGACAATCT-3'	47
4	Tomato Nema 282F	5'-GGATCCTTAGAAGCATCTAGT-3' 5'-CATCGCAAGACCGGCAACAG-3'		48

Note: P, purpose of the analysis; 1, presence of animal amplifiable material/identification of animal species; 2, presence of vegetal amplifiable material/identification of vegetal species; 3, identification of transgenic constructs; 4, identification of transgenic events.

genes and transgenes, using PCR-based technologies, in livestock and in the processed meat and poultry obtained.

The fate of chloroplast-specific gene fragments of different lengths (199 and 532 bp) and a Bt176-specific fragment has been evaluated in cattle and chicken fed a diet containing conventional or GM maize.⁶⁹ Only the short DNA amplicon from chloroplast was detected in blood lymphocytes of cows, but no plant DNA was detectable in muscle, liver, spleen, or kidney. In contrast, in all chicken tissues (muscle, liver, spleen, and kidney) the short maize chloroplast gene fragment was amplified. However, a Cry 1A(b)-specific sequence was not detectable in any of the analyzed sample.

An optimized DNA extraction protocol combined with PCR has been used to detect feed-derived plant DNA in muscle meat from chickens, swine, and beef steers fed MON 810 maize.¹³ Short fragments (173 bp) amplified from the high copy number chloroplast-encoded maize rubisco gene (*rbcl*) were detected in 5, 15, and 53% of the muscle samples from beef steers, broiler chickens, and swine, respectively. Only one pork sample out of 118 tested positive for the screening of P-35S; however, further analysis performed with a specific MON 810 PCR method generated indeterminate results, suggesting that the number of target copies in the sample, where present, were below the detection limit of the method.

PCR has also been used to investigate the fate of feed-ingested foreign DNA in pigs fed Bt maize.⁷⁰ Fragments of transgenic DNA were detected in the gastrointestinal tract of pigs up to 48 h after the last feeding with transgenic maize. Chloroplast DNA was detected in blood, liver, spleen, kidney, lymphatic glands, ovary, *musculus longissimus dorsi*, *musculus trapezius*, and *gluteus maximus*. In contrast, the Bt maize Cry 1A(b) gene was never detected in tissue samples.

The persistence of plant-derived recombinant DNA in sheep and pigs fed genetically modified (RR) canola has been assessed by PCR and Southern hybridization analysis of DNA extracted from digesta, gastrointestinal tract tissues, and visceral organs.⁷¹ The study confirmed that feed-ingested DNA fragments (endogenous and transgenic) do survive to the terminal gastrointestinal tract, and that uptake into gut epithelial tissues does occur; furthermore, a very low frequency of transmittance to visceral tissue was confirmed in pigs, but not in sheep.

A study was performed to assess whether processing and thermal treatments influence the detection of genetically modified DNA in different kinds of processed meat products (sausages, canned liver, ready-to-eat hamburgers) prepared with soybean meal spiked with a known amount of RR soybean.²⁰ The products were tested for the presence of specific 35S promoter and NOS terminator sequences, at different stages of processing, by PCR. The lowest contamination level (0.5%) was successfully detected in all raw and processed meat products at the different degrees of processing evaluated.

In a recent work, the detection of transgenic soybean was performed using a nested PCR protocol applied to several meat additives (blends, spices, taste enhancers), soy protein-based ingredients for meat products (soy protein and texturized soy protein), and processed meat samples (chicken mortadella, hot dog, cooked ham, hamburger, chicken-fried steaks) present on the Brazilian market.⁷² The reported results indicated that RR soybean was detectable in 3 out of 18 of the meat additives, 12 out of 14 of the soy protein ingredients, and 3 out of 8 processed meats tested.

25.2.2 Protein-Based Methods

Apart from transformation events bearing an antisense sequence, GM plants usually undergo the insertion of transgenes coding for novel proteins. These proteins represent in most cases suitable

targets for GMO detection. A wide spectrum of immunoassay-based technologies has been developed in the past decades, covering an enormous range of purposes and scientific disciplines.

25.2.2.1 *Antibody-Based Assay Formats*

25.2.2.1.1 Enzyme-Linked Immunosorbent Assay

ELISA is the most commonly employed technique among immunoassay strategies. ELISA assays allow the detection, and often the quantitation, of several classes of molecules such as proteins, peptides, antibodies, hormones, and other small molecules able to elicit immune response (hap- tens). A standard 96-well (or 384-well) polystyrene plate is the most common format used to perform ELISAs. The first step of the assay usually involves the target protein (antigen) absorption to a solid surface (direct ELISA) or the bounding of the antigen to a specific antibody, fixed at the bottom of a plate well (sandwich ELISA). The antigen is then bound by an antibody coupled with an enzyme (typically horseradish peroxidase [HRP] or alkaline phosphatase [AP]). After the formation of the complex, a substrate that produces a detectable product is added. Several substrates and instruments (luminometers, spectrophotometers, fluorometers) are available to meet the different technical needs.

Variants of ELISA assay with improved sensitivity have been developed using signal amplification strategies. The most common approach is based on the addition of a secondary enzyme-labeled antibody that binds a primary antibody specifically linked to the antigen. The binding of several secondary antibodies to a single primary immunoglobulin results in a strong signal enhancement. Another strategy consists of forming a biotin/streptavidin–derived complex linking more copy numbers of the enzyme to the same antibody.

Competitive ELISA formats have also been developed. These assays are particularly suitable for molecules that have only one epitope or when only one specific antibody is available. Several applications of this format are available. One of the most common uses an enzyme-conjugated antigen as standard: unlabeled antigen (from sample) competes with known amounts of labeled antigens for a limited number of specific binding sites of a capture antibody fixed on the well plate.

The main advantages of ELISA assay are that it provides quantitative information using an economical, high-throughput, and non-labor-intensive approach.

25.2.2.1.2 Lateral Flow Assays

Lateral flow assay technology commonly consists of a nitrocellulose strip containing specific antibodies conjugated to a color reactant. One end of the strip is placed in a tube containing the protein extract, which then starts to flow to the other end of the strip. When the target protein is present, a complex with color reagent–conjugated antibodies is formed and passes through two capture zones containing respectively a second antigen-specific antibody (test line) and an antibody for the labeled immunoglobulin excess (control line). When both lines give a positive signal, the test indicates a positive sample. When only the control line is positive, the test gives a negative sample. Lateral flow strip tests are very inexpensive, take a short time to analyze, and do not require a high degree of technical skills to be performed. All these reasons make this assay particularly suitable for field tests.

Several drawbacks have so far limited the application of antibody-based assay formats in GMO detection: (i) the presence of other substances in complex matrices (other proteins, phenolic

compounds, surfactants, fatty acids) can interfere with the assay; (ii) GM protein can be expressed in a very low amount, and the amount of the target protein expressed could be highly variable in different plant tissues or development stages; and (iii) matrices that undergo industrial processing, e.g., heating, could change the conformational structure of active epitopes, resulting in nonre-active proteins. This problem should be carefully evaluated for each sample when choosing the appropriate assay format.

Although protein-based methods have not found wide application in GMO detection if compared to PCR, several works report their use in this field, and innovative applications have also been developed and tested.^{9,73,74}

25.2.2.2 *Applications in Meat and Poultry Analysis*

The potential presence in food products of novel proteins as a consequence of GMOs entering the food chain has become, in the last few years, a relevant issue at national and international policy levels, also raising concern among citizens. On account of this, several attempts to investigate the fate of transgenic proteins have been performed on livestock and derived productions.

The possible transfer of the Cry 9C protein to blood, liver, and muscle in broiler chicks fed with StarLink corn has been investigated.⁷⁵ The determination of Cry 9C protein in the analytical materials was performed using a commercial GMO Bt9 maize test kit, and no positive samples were detected in the examined tissues.

A study was conducted to determine the content of GM protein from RR soybeans in tissues and eggs of laying hens.⁷⁶ A commercial double antibody sandwich incorporated in a lateral flow strip format, specific for the CP4 EPSPS protein, has been used. Whole egg, egg albumen, liver, and feces were all negative for GM protein.

The attempt to detect the Cry 1A(b) protein in chicken breast muscle samples from animals fed YieldGard Corn Borer Corn event MON 810 has been published.¹⁴ Analyses were performed using an in-house developed competitive ELISA with an LOD of approximately 60 ng of protein per gram of chicken muscle. Neither the Cry 1A(b) protein nor the immunoreactive peptide fragments were detectable in the breast muscle samples.

Using a similar strategy, the same author also investigated the presence of CP4 EPSPS protein in the muscle of pigs fed a diet containing RR soybean.¹⁵ A competitive immunoassay, with an LOD of approximately 94 ng of CP4 EPSPS protein per gram of pork muscle, was developed by the authors and used to test samples; neither the CP4 EPSPS protein nor immunoreactive peptide fragments were detected in any samples.

In another work, three different assays to detect Cry 1A(b) protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11 were employed.⁷⁷ Two commercial kits (a conventional microplate-format ELISA and a test strip format immunochromatographic assay) and immunoblotting were used to test pig samples. The Cry 1A(b) protein was detected in the contents of stomach, duodenum, ileum, cecum, and rectum.

25.2.3 *Alternative Techniques for GMO Detection*

With the number of GMOs developed by biotech companies constantly increasing and expected to have an even higher impact on worldwide cultivations and markets in the coming years,² new technologies and instruments will be needed to face the challenges of high throughput and

affordable detection of an increasing number of transgenes. For both qualitative and quantitative analysis, routine procedures such as PCR and immunodetection methods appear to be inadequate when confronted with the future demand to screen very large numbers of different GMOs. Several analytical approaches have been used to develop new detection systems able to implement the currently available methodologies in terms of sensitivity, specificity, robustness, and sample throughput.

Although most of the work on the development of new detection methods cited in the literature mainly focuses on analytical systems for the detection of GMOs in grains or plant products, several approaches also seem to be suitable for performing analysis on more complex matrices, such as meat products.

NIR spectroscopy, usually employed for the nondestructive analysis of grains for the prediction of moisture, protein, oil, fiber, and starch, has been described as a tool to discriminate between sample sets of RR soybean and nontransgenic soybeans.⁷⁸ More recently, visible/NIR (vis/NIR) spectroscopy combined with multivariate analysis was used to analyze tomato leaves and successfully discriminate between genetically modified and conventional tomatoes.⁷⁹ Although NIR techniques combine rapidity, ease of use, and cost effectiveness, their ability to resolve small quantities of GM varieties is assumed to be low: in fact the technique discriminates according to structural changes that are larger than those produced by single gene modifications. Further advancement in the development of the technique still needs to be accomplished before it could be evaluated for use in complex matrices.

Some authors have proposed chromatographic techniques for the detection of GMOs. Conventional chromatographic methods combined with efficient detection systems such as mass spectrometry could be applicable when significant changes occur in the composition of GM plants or derived products. This approach has been used to investigate the triglyceride patterns of oil derived from GM canola, showing that increased triacylglycerol content characterizes the transgenic canola variety.⁸⁰ Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and nanoelectrospray ionization quadrupole time-of-flight (nano ESI-QTOF) were successfully applied to the detection of the transgenic protein CP4 EPSPS in 0.9% GM soybean after fractionation by gel filtration, anion-exchange chromatography, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).⁸¹

Again these methodologies, although very sensitive, appear at present only to be suitable for differentiating between GM and conventional varieties, but they lack the specificity needed for detection in composite food matrices.

A recent application has been described that uses anion exchange liquid chromatography coupled with a fluorescent detector in combination with peptide nucleic acid (PNA) probes to detect and univocally identify PCR amplicons of RR soybean or Bt176 maize both on CRM and in commercial samples.⁸²

25.2.3.1 DNA Microarray Technology

With the number of genetic targets to be monitored constantly increasing, the detection of GMOs in the near future appears to be moving toward the need for higher throughput analysis that can simultaneously detect a high number of targets of interest and lower the cost of detecting an increased variety of genetic targets. In this context, one of the more promising technologies available appears to be microarray systems. In their general form, microarray systems are oligonucleotide probe-based platforms on which a high number of nucleic acid targets can be

simultaneously detected with high specificity. This would imply, in the case of GMO detection, the potential for rapid and efficient screening of a large number of control, gene-specific, and transgene-specific nucleic acid targets.

The main advantages of DNA microarray technology are miniaturization, high sensitivity, and screening throughput. Its main limitation is at present the strict dependence on PCR or other amplification techniques to amplify and label DNA or mRNA target sequences before performing the microarray analysis of a sample. The presence of this PCR step, at present still not likely to be overcome, imposes on this technology all the limitations discussed in the previous PCR section. Moreover, the possibility of quantifying GMO content in the sample is lost, because amplification and labeling are performed using end-point PCR, which is strictly qualitative. Different DNA microarray approaches, at both the research and the commercial stage, have been described for the detection of GMOs in food and feed systems, and their approach could be valuable also for the specific analysis of meat products.

A recent paper describes the development of a method for screening GMOs using multiplex-PCR coupled with oligonucleotide microarray.⁸³ The authors developed an array of 20 oligonucleotide probes for the detection of the majority of the genetic construct, covering 95% of commercially available transgenes (soybean, maize, cotton, and canola), with a detection limit of 0.5 and 1.0% for transgenic soybean and maize, respectively.

A multiplex DNA microarray chip was developed for simultaneous identification of nine GMOs, five plant species, and three GMO screening elements.⁸⁴ The targets were labeled with biotin during amplification, and the arrays could be detected using a colorimetric analysis with a detection limit below 0.3%.

A commercial microarray system for the qualitative detection of EU-approved GMOs has been recently commercialized in Europe.⁸⁵ The system combines the identification of GMOs by characterization of their genetic elements with a colorimetric detection based on silver.

A multiplex quantitative DNA array-based PCR (MQDA-PCR) method has been described for the quantification of seven different transgenic maize types in food and feed samples.⁸⁶ The authors were able to correctly characterize the presence of transgenic maize in the range 0.1–2.0% using a two-step PCR, which used opportunely labeled primers, and a DNA array spotted on a nylon membrane.

Ligation detection reaction (LDR), in combination with multiplex PCR and a universal array, has been described as a sensitive tool for GMO detection.⁸⁷ The authors were able to detect trace amounts of five transgenic events (maize and soybean) in heterogeneous samples both in reference materials and in commercial samples.

A class of synthetic oligonucleotide analogs with increased hybridization sensitivity and specificity has been described in a recent paper,⁸⁸ in which the authors used PNAs as capture probes for the detection of five GM maize and soybean products amplified by a multiplex PCR with a LOD of 0.25%.

25.2.3.2 *Biosensors*

Although only at research stage, several biosensor-based methods have been developed and tested for the detection of GMOs. Their main advantage is the fact that detection is based on physical principles, resulting in the possibility of performing the analysis in a faster and more economical way than conventional techniques. Their major drawback is that, as do the previously described techniques, they rely on PCR, because their sensitivity is not high enough for standalone analysis. As research on biosensors has continuously improved over the past few years,

innovative techniques and detection systems are likely to be developed, which could in the near future adequately fulfill the requirements of GMO detection.

A biosensor based on quartz crystal microbalance (QCM) has been described for the detection of sequences of the 35S promoter and NOS terminator.⁸⁹ PCR products obtained from CRM and real samples were correctly identified in a label-free hybridization reaction showing how this approach could be a sensitive and specific method for the detection of GMOs in food samples.

An electrochemical biosensor based on disposable screen-printed gold electrodes has been recently described for the detection of characteristic sequences of soybean and the 35S promoter.⁹⁰ The applied detection scheme, based on the enzymatic amplification of hybridization signals by a streptavidin-AP conjugate, led to a highly sensitive detection of the target sequences without the need for chemical or physical treatment of the electrode surfaces.

A biosensor based on surface plasmon resonance (SPR) has been reported to allow for the discrimination between samples containing 0.5 and 2.0% Bt176 maize reference material.⁹¹ The PCR products amplified by multiplex PCR were immobilized on the surface of the sensor, and oligonucleotide probes were flowed through the cell and hybridized to their specific target, generating a quantifiable signal.

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Chapter 26

Detection of Adulterations: Addition of Foreign Proteins

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26.1 Reasons for the Addition of Foreign Proteins in Processed Meats

The addition of foreign proteins to processed meats is a very common practice. The main aims of such addition are to assist in the management and production of these products, especially to improve the water-binding capacity of meat, resulting in less water exudation upon sterilization, and, in the case of comminuted meats, to assist in the emulsion of fat particles. Other reasons are to obtain less-fatty meat products, to exploit low-quality meat pieces, and, in the case of soybean proteins, to obtain health benefits.

26.1.1 Stabilization and Sensory Improvement of Processed Meats

Comminuted meat products are complex food systems in which water absorption, gelation, and emulsion formation influence stability and sensory characteristics of the cooked product. During comminution of fine sausage emulsions, a relatively large amount of small fat or oil droplets are liberated from the fat cells. All this fat needs sufficient protein coating to prevent it from flowing back together during heating. This task is performed by the soluble myofibrillar proteins present in the meat, which also act to bind meat water. Nevertheless, frequently the meat protein content in processed meats is insufficient to support an emulsion, and foreign proteins are usually added to stabilize it. Different sources of foreign proteins have been added to meat emulsions and numerous studies have reported the benefits of these additions.¹⁻⁴

Foreign proteins are also added for the improvement of organoleptic characteristics such as texture,⁵⁻⁹ color,^{4,10} flavor,¹¹ and, in general, the quality of the final product.^{12,13} Fermented sausages are another kind of processed meats (not heat treated) to which the addition of foreign proteins is standard. The reason for such addition is to improve water-binding and textural properties that are damaged during vacuum packaging. For example, the addition of 2.5% of soybean protein isolate (SPI) prevents drip loss without introducing any change in the flavor, aroma, or juiciness characteristics of the product.^{14,15}

26.1.2 Reduction of Meat Fat Content

Processed meats normally contain higher fat content than whole-muscle products. Fat provides flavor, texture, juiciness, and water entrapment. Therefore, lowering the fat content in emulsified products has been reported to increase toughness and significantly alter the texture, flavor, and color of the resulting low-fat product.^{16,17}

The replacement of fat by water is an alternative, but resulting products have been reported to increase cooking and purge losses. Another challenge is the formulation of low-salt meat products, since the use of low sodium chloride content affects the water-holding capacity and emulsifying properties of meat. The addition of foreign proteins, especially soybean and milk proteins, to comminuted meats can balance these negative effects.¹⁸⁻²³ In fact, added proteins are capable of forming gels upon heating entrapping liquid and moisture. This gelling action in a low-fat/high-added-water

formulation has the potential to return some of the texture often lost when levels of water addition are high.²⁴

Another approach to the reduction of fat content is the direct addition of foreign proteins as fat replacers (protein-based fat replacers or substitutes).^{17,25–27}

26.1.3 Exploitation of Low-Quality Meats

The meat industry is constantly looking for ways to enable the efficient utilization of meat from spent or aged animals. Spent animal meat is tougher and less juicy due to high collagen content and a high degree of crosslinkages. Quality attributes of spent animal meat can be improved by the addition of foreign proteins, especially milk proteins.^{13,28–31}

Another approach to the exploitation of low-quality meat is the manufacture of *restructured meats*. Restructuration of meat uses less-valuable meat pieces to produce palatable meat products at reduced cost. Binding of these meat pieces and texture of the final product are the main characteristics that influence the acceptability of these products. Cohesion among meat pieces in structured meat products is accomplished by the formation of a protein matrix after extraction of muscle proteins, which requires the addition of salts and tumbling. The process brings salt-soluble meat proteins to the meat surface, forming a tacky exudate that coagulates upon cooking to bond the meat pieces into a continuous body. Nevertheless, due to damage to muscle texture produced during the tumbling and to the increasing concern of consumers over the sodium content of food, nonmeat proteins have been in demand as binders in restructured meats.^{32–38}

26.1.4 Health Benefits

The consumption of soybean protein is related to health benefits. New food-based recommendations issued by the American Heart Association with the objective of reducing risk for cardiovascular disease promoted the inclusion in the diet of specific foods with cardioprotective effects, including soybean. The available evidence indicates that the daily consumption of 25 g of soybean protein could decrease total and low-density lipoprotein (LDL)-cholesterol levels in hypercholesterolemic individuals.^{39–41}

26.2 Kinds of Foreign Proteins Added to Processed Meats

The foreign proteins most frequently added to processed meats are soybean proteins, wheat gluten, and milk proteins. Other proteins used to a lesser extent are corn gluten, blood plasma, pea proteins, and egg proteins.²⁴

Soybean proteins can be added to meat products as textured soybean (50% protein), soybean protein concentrate (70% proteins), or SPI (90% proteins).⁴² Water solubility of soybean proteins significantly contributes to improve functional properties of soybean-containing products, including water-holding capacity, foaming properties, appearance, and texture. Moreover, modification of soybean proteins by ultracentrifugation, low-dose irradiation, or treatment with various chemicals (e.g., proteolytic enzymes) contributes to the improvement of soybean protein functionality.^{43,44} An even greater improvement of soybean protein functionality can be achieved by the heating of these proteins before their addition to meat. In fact, the high denaturation temperatures of the major soybean proteins (75–90°C) prevent the protein from undergoing sufficient structural

changes under common meat heating conditions (65–73°C), thereby limiting their interaction with meat proteins and not contributing to meat gelling properties.⁴⁵

Other vegetable proteins have also been added to processed meats (especially sausages), but their use is far less common than the industry applications of soybean proteins. For example, the addition of wheat gluten is advantageous due to its functionality and low cost but is limited due to its poor solubility. Chemical (acid deamidation), enzymatical, or physical modification of wheat gluten can result in a product with enhanced functional properties.^{46,47} The case of corn gluten meal is similar, since it is not suitable for use in the food industry due to its low functionality, poor solubility, etc. Nevertheless, a simple hydrolysis of native corn gluten meal or increasing the pH of the native corn gluten meal results in an improvement of functional properties.⁴⁸

Various milk products (nonfat dry milk, whey proteins, sodium caseinate, etc.) have been added to meat products. Skim milk powder (35% protein), which is widely used as filler in comminuted meat products, has good water-binding properties, but lactose may cause discoloration of meat products because of Maillard reactions. Whey proteins act as binders and extenders, gelling when they are heated. Sodium caseinate (90% protein) is completely soluble in water and in solutions with pH lower than 9, emulsifying up to 188 mL of oil/g of protein.⁴⁹ Nevertheless, in comparison with SPI, the incorporation of sodium caseinate results in high moisture loss.⁵⁰

26.3 Methods Used for the Detection of Foreign Proteins in Processed Meats

There is an extensive literature dealing with the detection of foreign proteins, especially soybean proteins, in processed meat products. Methods can be divided in two groups—methods determining soybean proteins based on the presence of substances accompanying these proteins and methods based on the determination of proteins themselves.

Chemical methods have been employed for the determination of certain compounds or tracers that could reveal the presence of certain foreign proteins. The compounds analyzed were oligosaccharides, amino acids, phytate or phytic acid, metals, etc. The main drawback of these methods is their low specificity.⁵¹ Microscopic methods enable the visualization of characteristic structural forms of the soybean such as palisade and hourglass cells present in the bean hull and calcium oxalate crystals from the cotyledon cells. In the case of soybeans, histological methods based on the selective stain of certain compounds present in the bean, normally carbohydrate-containing cells, have also been employed. These methods proved useful when soybean flour and textured soybean were added, but their application was limited when soybean protein concentrates or isolates were employed.^{52–54}

Currently, the most common methods employed for the determination of foreign proteins in meat products are based on electrophoresis, immunological reactions, and chromatography.

26.4 Electrophoretic Methods for the Detection of Foreign Proteins in Processed Meats

The use of electrophoretic techniques for the determination of foreign proteins in meat products requires the prior solubilization of these proteins. Protein solubilization is more difficult, with the most severely heated samples necessitating the use of detergents or concentrated solutions of urea

containing mercaptoethanol to disrupt disulfide crosslinks. Regarding the support material, most electrophoretic methods use polyacrylamide gels (polyacrylamide gel electrophoresis [PAGE]), although starch gels and cellulose acetate membranes have also been employed. Most PAGE methods employ sodium dodecyl sulfate (SDS). SDS not only solubilizes the proteins but also confers a negative charge in proportion to their mass. Since the mass-to-charge ratio is uniform for most proteins, all proteins migrating to the cathode will cross the gel matrix and will separate as a function of their molecular weights. Table 26.1 groups the electrophoretic methods developed for the determination of foreign proteins in processed meats, most of them devoted to soybean proteins.

Olsman⁵⁵ and Thorson et al.⁵⁶ reported the first methods using electrophoresis for the detection of soybean proteins and caseins in heated meats. In both cases, urea was employed for the solubilization of foreign proteins, although Olsman mixed it with mercaptoethanol. The main difference between them was the supporting material and the electrophoretic mode employed, a starch gel in slab in the case of Olsman and a polyacrylamide gel in tube in the case of Thorson and coworkers. Olsman obtained significantly better detection limits than Thorson and coworkers, who, in addition, had difficulties in the detection of soybean proteins due to co-elution of meat bands with the main soybean protein bands. Nevertheless, Olsman's method was not adequate for routine analysis due to the lengthy time required for a single analysis (24 h). Detection of soybean proteins by PAGE was improved by Freimuth and Krause⁵⁷ (in the slab mode) and by Fischer and Belitz⁵⁸ (in tube). While Freimuth and Krause extracted soybean proteins with a urea-lactate buffer and separated them at pH 3.1, Fischer and Belitz employed a tris-glycine buffer and the separation was carried out at basic pH. Fischer and Belitz's method was valid for highly cooked sausages, yielding results within 12 h.

Hofmann and Penny^{59,60} developed another approach based on the use of SDS-PAGE in slab and a tris-boric acid buffer for the extraction of proteins. The method enabled the detection of soybean proteins in meat products heated up to 100°C, whereas those heated to higher temperatures (121°C) showed less clearly defined bands. Hofmann^{61,62} also applied the method to the identification of foreign proteins other than soybean (egg white, egg yolk, milk, and wheat proteins) in meat products. Every protein showed a characteristic pattern that enabled its identification, with the exceptions of egg yolk proteins and wheat proteins, which could not be identified because their protein pattern was very complex (in the case of egg yolk) or was not stained properly (in the case of wheat proteins).^{61,62} Other authors tried to improve Hofmann and Penny's method. Matthey⁸⁵ and Smith⁸⁶ used a 6% acrylamide gel and Bergen and Bosch⁸⁷ employed 10% instead of the 8% used by Hofmann and Penny. Moreover, Smith⁸⁶ and Endean⁸⁸ also cooled the front of the gel to avoid band distortions. Parsons and Lawrie⁶³ also applied an electrophoretic method similar to Hofmann and Penny's. In this case, proteins were extracted with a buffered solution containing 10 M urea and the acrylamide concentration was varied from 3 to 8%. The method enabled the quantification of soybean proteins in meat products heated up to 100°C, while at sterility temperatures (127°C for 24 min) only qualitative identification was possible, with no interference observed from field beans or egg albumin.⁶³ A further investigation on the reliability of this method was performed by Tateo.⁸⁹

Spell⁶⁴ and Frouin et al.⁶⁵ focused their efforts on the improvement of sensitivity in the determination of soybean and milk proteins by PAGE in sterilized meats. Frouin et al.⁶⁵ proposed a first fractionation of proteins to eliminate those high molecular-weight interfering proteins. Detection limits obtained by this method were better than those yielded by the PAGE method of Spell.⁶⁴ Lee et al.⁶⁶ proposed the use of a preconcentration technique based on SDS-PAGE to detect soybean proteins in cooked meat-soybean blends. This preconcentration step yielded

Table 26.1 Electrophoretic Methods for the Determination of Foreign Proteins in Processed Meats

Sample	Foreign Proteins	Technique	Detection Limit	Reference
Luncheon meat heated at 115°C and liver paste heated at 105°C	Soybean proteins and caseins	Urea-starch gel electrophoresis	0.50% for soybean proteins and 0.25% for caseins	55
Heated meat products (110°C)	Soybean proteins and caseins	PAGE (in tube)	3% for caseins	56
Cooked sausages	Soybean proteins	PAGE	—	57
Sausages (116°C)	Soybean proteins	PAGE (in tube)	—	58
Heated meats (pork and beef) (100°C) and sausages	Soybean, egg, milk, and wheat proteins	SDS-PAGE	5% for soybean proteins	59–62
Heated meats (sausages, pies, and beefburgers) (100°C)	Soybean proteins, field bean proteins, and egg albumin	SDS-PAGE	—	63
Heated meats (sausages) (120°C)	Soybean and milk proteins	PAGE	2% for soybean and milk proteins	64
Meat products (pate, ham, and sausages) sterilized at 117°C for 1 h 15 min	Soybean and milk proteins	SDS-PAGE (in tube)	1% for soybean and milk proteins	65
Cooked model meats	Soybean, milk, and egg white proteins	Stacking SDS-PAGE	—	66
Beef burgers, sausages, pies, and canned meat autoclaved at 110–115°C	Soybean proteins	PAGE	—	67,68
Cooked meats (paté, corned beef, bolognaise sauce, ravioli, and sausages)	Soybean proteins, wheat gluten, and milk proteins	Urea-PAGE (in tube)	1% for soybean proteins	69
Sausages and beefburgers	Soybean proteins	Isoelectric-focusing PAGE	—	70–72

Heated pork and beef (74°C for 150 min)	Soybean proteins and caseins	PAGE (in tube)	1% for soybean proteins and caseins	73
Cooked sausages	Soybean proteins and caseins	PAGE (in tube)	—	74
Pasteurized meats (sausages and ham; 70°C)	Soybean proteins	SDS-PAGE	0.5% for soybean proteins	75
Model products consisting of roe and deer meats heated up to 70°C	Soybean proteins, caseins, and egg white proteins	PAGE (in tube)	—	76
Model cooked beef and pork meats	Soybean proteins	SDS-PAGE	—	77
Frankfurters	Soybean proteins	PoroPAGE	—	78
Frankfurters	Soybean proteins	SDS-PAGE	3% for soybean proteins	79
Model pork and beef meats autoclaved to 118°C for 20 min	Soybean proteins, sunflower proteins, and field bean proteins	SDS-PAGE	—	80
Model beef frankfurters	Soybean proteins	SDS-PAGE	<1% for soybean proteins	81
Cooked pork meat products (ham)	Soybean proteins, whey proteins, and caseins	SDS-PAGE	0.5% for soybean proteins and caseins and 1% for whey proteins	82
Model meat samples cooked to 100°C for 15 min, hamburgers, and sausages	Soybean proteins, wheat gluten, milk proteins, and egg proteins	SDS-PAGE	—	83,84

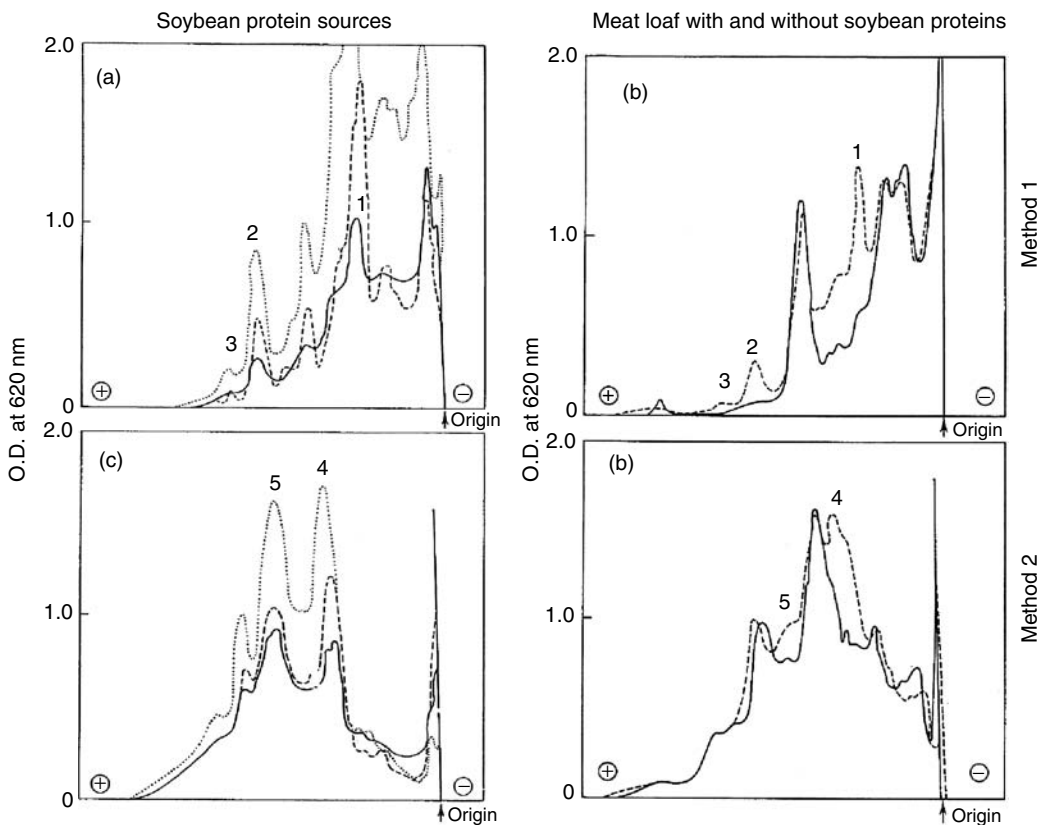


Figure 26.1 Densitograms corresponding to the SDS-PAGE separation of different soybean protein sources (textured soybean [—], SPI [---], and soybean flour [...]) and a meat loaf with (- - -) and without (—) SPI by method 1 (based on Olsman's⁵⁵ approach) (a,b) and method 2 (based on Parsons and Lawrie's⁶⁷ approach) (c,d). Labeled bands 1, 2, 3, 4, and 5 were from soybean proteins. (From Guy, R.C.E. et al., *J. Sci. Food. Agric.*, 24, 1551, 1973. With permission.)

high-resolution separations and accurate determinations of soybean proteins in the presence of milk proteins and egg white proteins.

A comparative study of two different methods (extraction of proteins with a solution containing 8 M urea and 1% 2-mercaptoethanol at 18–20°C for 16 h (method 1, based on Olsman's⁵⁵ approach) and extraction of proteins with 10 M urea and 4% 2-mercaptoethanol at 100°C for 30 min (method 2, based on Parsons and Lawrie's⁶³ approach)) for the extraction of soybean proteins in meat products) was published by Guy et al.⁶⁷ Figure 26.1 shows the densitograms obtained for different soybean protein sources and for meatloaf with and without 5% SPI using both methods. The three soybean protein sources showed characteristic peaks that could be observed in the pattern corresponding to the meatloaf containing soybean proteins (peaks 1, 2, and 3 by method 1 and peaks 4 and 5 by method 2). From these results, the authors concluded that method 1 provided a better separation of soybean proteins in cooked meats than method 2. Moreover, this method was reproducible and free from interference from other nonmeat proteins (milk proteins, egg proteins, and wheat gluten).⁶⁸

Homayounfar⁶⁹ took up again Freimuth and Krause's⁵⁷ idea of developing the protein separation in an acid environment instead of the neutral or slightly alkaline conditions normally used. Four bands pertaining to soybean proteins were observed when the concentration of soybean proteins in the meat was higher than 5%, two of them disappearing at lower proportions. This method was used by Baylac et al. for the determination of various foreign proteins (soybean proteins, caseins, whey proteins, egg white proteins, wheat gluten, and blood plasma) in model fresh, pasteurized, or canned meat products.⁹⁰

Isoelectric focusing in polyacrylamide gels enables higher resolution than conventional electrophoresis and has been applied to the determination of soybean proteins in cooked meats.^{70–72} Although patterns observed by isoelectric focusing were more complex than those obtained by conventional electrophoresis, they could be simplified since meat bands disappeared when applying limited heating. The technique proved adequate for raw meats but failed with severely heated meat products since denaturation of soybean proteins made them insoluble in the extracting solution (urea-mercaptoethanol). A similar conclusion was drawn by Vállas-Gellei,⁷³ who observed that samples heated to 74°C for 150 min yielded weaker meat bands due to the high sensitivity of meat proteins to thermal denaturation, while soybean protein and casein bands remained unchanged or even stronger.

Other approaches have been developed to improve different aspects of the application of electrophoresis to the determination of foreign proteins in processed meats. Richardson⁷⁴ reduced the whole analysis time from the 2–3 days usually required in the slab mode to a single working day. Armstrong et al.⁷⁵ proposed the use of an internal standard protein (hemocyanin) to compensate for variations in the meat pattern and obtained accurate determination of soybean proteins in meats. Ring et al.⁷⁶ developed a unique separation method enabling the simultaneous differentiation between closely related meat species and the identification of added nonmeat proteins (caseins, egg white albumin, and soybean proteins) in cooked meat products. Molander⁷⁷ compared standard curves obtained by SDS-PAGE for the determination of soybean proteins in meat products subjected to different degrees of heat treatment. Although the method was accurate for raw or slightly heated meats, it failed with severely heated meats. In any case, the presence of other ingredients (milk powder, potato flour, bread-crumbs, caseins, and whole blood) did not seem to affect the determination of soybean proteins. Heinert and Baumann⁷⁸ proposed the use of a porosity gradient in PAGE in the presence of SDS and urea to obtain two soybean protein bands separated from those of meat proteins, which proved adequate for the detection of soybean proteins in sausages. Feigl⁷⁹ proposed an SDS-PAGE method using commercially available gel plates for its application as a routine procedure for the determination of soybean proteins in meat products.

Lacourt et al.,⁸⁰ Woychik et al.,⁸¹ and López et al.⁸² applied essentially the Laemmli⁹¹ SDS-PAGE procedure using a tris-glycine buffer for the detection of soybean proteins in heated meats. This stacked buffer system provided a resolution above that obtained without stacking. Lacourt et al.⁸⁰ studied model beef and pork meats sterilized at 118°C for 20 min that contained soybean, sunflower, or field bean proteins. Despite the high resolution power of the method, they observed that, especially at low concentrations, differentiation among these three foreign proteins was not feasible. Woychik et al.⁸¹ applied the Laemmli procedure to quantitate soybean proteins in pasteurized frankfurters based on the α -conglycinin/actin peak height ratios. López et al.⁸² applied the method to the determination of soybean proteins in cooked ham, and was able to quantitate down to 0.5% of soybean proteins and caseins and 1% of whey proteins.

Olivera Carrión and Valencia⁸³ developed a PAGE method in the slab mode enabling the identification of soybean proteins in various model and commercial processed meats heated to 100°C. Quantification was performed from the area ratio corresponding to the bands appearing

at 19,500 and 52,000 Da. No interferences from meat proteins or other extrinsic proteins (egg proteins, wheat gluten, milk casein, and whey) were observed.⁸⁴

26.5 Immunological Methods for the Detection of Foreign Proteins in Processed Meats

Many immunological methods have been developed for the determination of foreign proteins in processed meat, soybean proteins being those most extensively determined. The determination of soybean proteins, and foreign proteins in general, in meats is limited by the low extraction efficiency observed, a result of the mild extracting conditions used to avoid the loss of protein antigenicity. In fact, the use of extracting solutions containing urea or SDS, despite being very efficient, could destroy the immunogenic properties of proteins. In this respect, Hyslop⁹² suggested the possibility of using a 2% SDS solution for the extraction of soybean proteins with the posterior removal of SDS to regain protein immunogenicity. Moreover, in the case of processed meats there is an additional limitation related to the structural changes occurring in foreign proteins due to the processing. The subjection of soybean proteins to heat treatment improves their nutritional value by denaturing various antinutritional factors. Nevertheless, the susceptibility of the major soybean proteins to heat processing has been well documented.⁹³ Moreover, in the case of soybean proteins, their antigenic properties depend on the source of the added soybean protein (soybean flour, textured soybean, soybean protein concentrate, or SPI).^{51,52}

Table 26.2 groups the immunological methods that have been developed and applied to the analysis of foreign proteins in processed meat products. Immunological methods have been grouped in five categories: serology, immunodiffusion, indirect hemagglutination, methods involving an electrophoretic separation and an immunological reaction, and immunoassays.

26.5.1 Serology

Early immunological methods consisted of serological reactions applied to the determination of soybean proteins. Serological methods are based on the specific interaction between an antigen and an antibody. Major limitations were observed in their application to meats heated to extremes.¹⁴² In 1939, Glynn published a serological method enabling the detection of soybean flour in sausages.⁹⁴ Other research refined this method (by the optimization of the time and temperature of incubation of the serum with the soybean proteins) for application in quantitative analysis.^{95,96} Degenkolb and Hingerle^{97,98} developed a screening method for the detection of foreign proteins in meats. Samples yielding a positive precipitation reaction were later subjected to a volumetric assay. This assay proved useful with products heated up to 110–115°C, using antibodies different from those employed with products heated up to 70°C. Krüger and Grossklaus,⁹⁹ using this method for the determination of soybean proteins in canned meats heated at 100°C, obtained a detection limit of 0.2%. Moreover, quantitative determination of added soybean proteins was possible in scalded meat products (heated to 75°C).

26.5.2 Immunodiffusion

In immunodiffusion, antigen–antibody reactions take place in an agar or agarose gel medium. Single immunodiffusion involves the antigen diffusing into a gel containing the corresponding

Table 26.2 Immunological Methods for the Determination of Foreign Proteins in Processed Meats

Sample	Foreign Proteins	Detection Limit	Reference
Serology			
Sausages	Soybean proteins	—	94–96
Sausages and canned meats heated to 120°C	Soybean proteins	0.2%	97–99
Immunodiffusion			
Canned meat	Soybean proteins	—	100
Model sausages	Soybean proteins	—	101
Sausages	Soybean proteins, hydrolyzed milk proteins, and ovalbumin	0.3 mg/mL for soybean proteins and 0.5 mg/mL for hydrolyzed milk proteins and ovalbumin	102, 103
Heated meats	Soybean proteins	—	104
Heated and unheated model meats (60 min at 121°C) and commercial beefburgers, meat balls, sausages, and canned stewed steak	Soybean proteins	1%	105
Canned meat heated to 120°C for 50 min	Soybean proteins	—	106
Indirect Hemagglutination			
Sausages	Soybean proteins, milk proteins, and ovalbumin	—	107, 108
Model frankfurters (75–120°C)	Soybean proteins	—	109
Sausages	Soybean proteins, hydrolyzed milk proteins, and ovalbumin	1.0 mg/mL for soybean and hydrolyzed milk proteins and 5.0 mg/mL for ovalbumin	102
Heated meats (121°C)	Soybean proteins	—	110
Electrophoresis + Immunological Methods			
Heated meats	Milk proteins	—	111
Luncheon meat	Soybean proteins and caseins	—	112
Model heated meats (65–125°C)	Soybean proteins	—	113
Model frankfurters (75–120°C)	Soybean proteins	—	109
Model cooked sausages (78 and 114°C) and commercial meat products (sausages, luncheon meat, meatballs, ham, and roast turkey)	Caseins	—	114, 115

(Continued)

Table 26.2 (Continued)

Sample	Foreign Proteins	Detection Limit	Reference
Model sausages	Soybean and mustard proteins	—	116
Model cooked meats (71°C)	Soybean proteins	—	117
Model cooked meats (60–125°C)	Soybean proteins	2.5%	118
Model heated sausages (121°C for 45 min)	Soybean proteins	0.1%	119
Model heated meats (100°C)	Soybean proteins	0.02%	120
Model heated meats (100°C)	Soybean proteins, caseins, whey proteins, ovalbumin, and wheat gluten (modified and nonmodified)	0.1% for each protein	121,122
Model heated meats (60–100°C)	Soybean proteins	0.5%	123
Sausages	Egg proteins	—	124
Sausages	Milk proteins	—	125
Immunoassays			
Model heated meats	Soybean proteins	—	126,127
Pasteurized hamburger and canned luncheon meats sterilized at 120°C for 30 min	Soybean proteins	0.1%	128
Meat balls, beef croquettes, fried chicken, and hamburger	Soybean proteins	—	129
Commercial hamburger	Soybean proteins	2 ppm (0.0002%)	130
Commercial hamburger	Soybean proteins	—	131
Autoclaved model meats (121°C for 20 min), sausages, ham, paté, and hamburger	Soybean proteins	—	132,133
Model and commercial sausages	Soybean proteins	—	134
Model pork sausages (80°C for 20 min)	Soybean proteins	—	135
Fermented sausage (chorizo)	Soybean proteins	1%	136
Model heated meats	Wheat gluten	—	137,138
Model heated meats (100°C for 5 min) and commercial sausages	Wheat gluten	0.2%	139,140
Sausages	Soybean proteins, pea proteins, and wheat gluten	0.05–0.1% for soybean and pea proteins and 0.025–0.5% for wheat gluten	141

antibodies. Peter¹⁰⁰ found this technique to be adequate for the screening of soybean proteins in meats. In this respect, Hauser et al.¹⁰¹ prepared ready-to-use agar layers for the routine application of this technique to the determination of soybean proteins in meats. They concluded that the successful application of this technique required knowing the soybean protein source added.

Double immunodiffusion, or Ouchterlony immunodiffusion, involves both antigen and antiserum to diffuse from different wells in an agarose or agar gel. This technique has been applied for the screening of various foreign proteins (soybean proteins, hydrolyzed milk proteins, and ovalbumin) in sausages. After 2 h incubation the method enabled the detection of up to 0.3 mg/mL of soybean proteins and 0.5 mg/mL of hydrolyzed milk proteins and ovalbumin.¹⁰² Appelqvist et al.¹⁰³ also applied this method to the determination of soybean and milk proteins in meat products. Günther and Baudner¹⁰⁴ found that the use of cellulose acetate membranes was also suitable for the qualitative detection of soybean proteins in processed meats, though agar gels were more adequate for quantitation.

Several approaches have been developed to improve the antisera performance. The use of a commercial soybean protein antiserum proved useful with raw meats but did not solve the problem of decreasing sensitivity observed when meats are severely heated.^{52,143} Hammond et al.¹⁰⁵ prepared an antiserum against both heated (121°C) and unheated SPI. Nevertheless, the lack of specificity due to cross-reactivity with certain spices, onion, and hydrolyzed vegetable proteins, combined with the inability of the method to respond to severely processed products, limited its application. Another proposal was suggested by Baudner et al.,¹⁰⁶ who proved the suitability of an antiserum against a soybean protein fragment stable at 120°C and conjugated with a carrier for the detection of soybean proteins in meats.

The double immunodiffusion method proposed by Ouchterlony and the starch gel electrophoretic method proposed by Olsman⁵⁵ were evaluated in a collaborative study for the detection of caseins and soybean proteins in meat products. In general, results were more successful by immunodiffusion, since electrophoretic patterns were difficult to interpret. Nevertheless, and as expected, soybean proteins could not be detected in meats heated to temperatures higher than 100°C. In the case of caseins, false positives were obtained due to the presence of undenatured bovine blood proteins with similar immunogenic properties.¹⁴⁴

26.5.3 Indirect Hemagglutination

Indirect hemagglutination uses erythrocytes coated with antigenic molecules. When these aggregates are added to a solution containing the corresponding antibodies, the cells agglutinate and, due to their large size, their detection is possible even in low concentrations. Kotter et al.^{107,108} applied this technique to the determination of different foreign proteins in meats, concluding that the high labor intensity and time requirements limited its application. Regarding feasibility and reliability, conclusions published by various authors have been contradictory.^{97,98,145} Krüger and Grossklaus¹⁰⁹ obtained quantitative results for products heated at 75°C, but the technique failed with more severely heated products, even when using antiserum against soybean proteins heated at 110°C. Kraack¹⁰² used this technique for the confirmation of results obtained by a screening serological test. He observed detection limits much higher than that obtained by immunodiffusion. Herrmann and Wagenstaller¹¹⁰ could quantify soybean proteins in meat products heated up to 115°C, and found it possible to detect soybean proteins in products heated up to 121°C.

26.5.4 Immunological Methods Comprising Electrophoretical Separations

In this section, all immunological methods consisting of a first electrophoretical separation have been grouped. Among these methods are immunoelectrophoresis and Western blot (immunoblotting).

Immunoelectrophoretical methods combine electrophoresis and immunodiffusion. Proteins separated by electrophoresis are transferred onto a membrane and detected by radio- or enzyme-labeled antibodies.¹⁴⁶ The development of electroimmunodiffusion, also known as Laurell immunoelectrophoresis, constituted a significant advance. Since the electrophoretic separation takes place on a gel containing a uniform concentration of the antiserum, no transference of proteins is required. The antigenic proteins present in the sample form complexes with antibodies, which migrate as well, resulting in rocket-shaped precipitation lines (rocket electrophoresis). The length of these lines is proportional to the concentration of antigen in the sample. Laurell immunoelectrophoresis is rapid compared to immunodiffusion methods and can be applied for quantitative analysis.

Early applications of immunoelectrophoresis were devoted to the qualitative analysis of soybean and milk proteins in meat products.^{111,112} Kamm¹¹³ was the first to propose the immunochemical quantitation of soybean proteins in cooked meats by immunoelectrophoresis. He prepared an antiserum against crude soybean globulin that contained three antigenic species. One of these species disappeared after heating at 65°C, others after heating at 100°C, and the most stable one was removed at commercial sterility temperature (125°C for 25–30 min), meaning the method was not adequate for severely cooked products. Krüger and Grossklaus¹⁰⁹ studied the effect of temperature on the immunoelectrophoretic signal. They applied the method to model canned frankfurters heated to temperatures ranging from 75 to 120°C and containing from 0.1 to 0.4% of soybean proteins. The method yielded quantitative results for products subjected to scalding temperatures (75°C), but inadequate when products were subjected to higher temperatures. Sinell and Mentz^{114,115} used Laurell's technique to quantitate milk proteins in sausages with antibodies against α - and β -caseins. The quantitative determination of this part of milk proteins enabled the measurement of the whole.

Various efforts have been made for the improvement of these results. Merkl¹¹⁶ avoided cross-reactivity in the determination of soybean proteins in meat products containing mustard by pH adjustment of the agarose gel. Koh¹¹⁷ prepared antibodies against renatured soybean proteins by extracting soybean proteins under denaturing conditions with urea and mercaptoethanol and removing them by dialyzing. The renatured proteins surprisingly kept their antigenic properties, making the method suitable for the identification and quantification of soybean proteins in heated (71°C) beef mixtures. Poli et al.¹¹⁸ developed a rapid and sensitive method combining electrophoretic separation with an indirect immunofluorescence detection. The method enabled the detection down to 2.5% of soybean proteins in meat products, even when they were sterilized. A further reduction of detection limits (0.1% of soybean proteins) was obtained by Heitmann,¹¹⁹ who also used immunofluorescence detection. Janssen et al.¹²⁰ proposed the use of a Western blot method for the sensitive determination of soybean proteins in processed meats. In this case, proteins separated by SDS-PAGE are transferred to a nitrocellulose membrane and immunostained with peroxidase. Under these conditions meat proteins did not stain and soybean proteins were detected at a level of 0.02%. The method was also valid for the detection of other nonmeat proteins (ovalbumin, wheat gluten, caseins, and whey proteins) added at a level down to 0.1% in meats heated up to 100°C.¹²¹ Moreover, the elimination of the separation step enabled the rapid screening of samples by a dot blot procedure.¹²⁷ This rapid method using

immunoperoxidase staining was compared with an immunogold-silver staining method. Though the immunogold-silver procedure proved to be more sensitive, it was much more expensive than the immunoperoxidase method.¹⁴⁷ In any case, it was recommended that positive samples be re-examined using the whole procedure, including the electrophoretic separation. Körs¹²³ could improve the proposed method by the substitution of SDS by a less denaturing detergent (CTAB, *N*-cetyl-*N,N,N*-trimethylammonium bromide). He concluded that the intensity of the soybean protein bands depended only on the heating temperature at low additions (0.5–1%), independent of temperature at higher proportions.

Although the use of wheat gluten as meat extender has not been as extensive as the use of soybean proteins, the modification of wheat gluten to obtain a more readily soluble product has opened new possibilities for its application in the meat industry. Janssen et al.¹²² proved that their proposed Western blot method¹²¹ was capable of detecting this modified gluten and could also discriminate between modified and nonmodified wheat gluten.

Brehmer et al. focused their efforts on the determination of foreign proteins present in cooked meats other than soybean proteins and wheat gluten. They developed immunoelectrophoretical methods sensitive to egg proteins¹²⁴ and milk proteins (based on the detection of the α -casein fraction)¹²⁵ in cooked meats.

26.5.5 Immunoassays

A number of immunoassays have been developed for the detection of foreign proteins, especially soybean proteins, in cooked meats. The most commonly used immunoassay, the enzyme-linked immunosorbent assay (ELISA), has shown certain advantages compared to previous immunological techniques, such as their suitability for routine analysis and easy semi-automation. Unlike the classical immunochemical methods, ELISA does not rely on the precipitation of the antigen–antibody complex since the presence of the complex is monitored by colorimetric measurement of an enzyme linked to it.

Based on the idea of Koh¹¹⁷ for the extraction of proteins, Hitchcock et al.¹²⁶ developed an ELISA method working with sterilized meat products for the detection of soybean proteins. The sample extract, prepared in a hot concentrated solution of urea, was cooled, diluted for the renaturation of soybean proteins, and treated with a known excess of soybean protein antiserum. The soybean protein in the sample (the antigen) interacted with the antibody while the unreacted antibody was trapped on an immunosorbent that contained an immobilized standard of soybean protein antigen. The captured antibody was determined after adding a second antibody to which an enzyme had been covalently attached (conjugate). The captured enzyme (alkaline phosphatase) was determined by adding *p*-nitrophenyl phosphate as a chromogenic substrate. Finally, the optical density after incubation was measured at 405–410 nm. Olsman et al.¹²⁷ organized a collaborative trial in which various meat products heated at 80°C, containing soybean proteins from different sources, were analyzed using an SDS-PAGE method⁷⁶ and the ELISA method of Hitchcock et al.¹²⁶ Both methods were suitable for qualitative purposes, with SDS-PAGE being more precise and ELISA more accurate. In 1985 this method was adopted as the AOAC official first action.

Although this method was considered one of the best methods for high specificity and sensitivity, reliable quantitative analysis could be obtained only if the source of soybean proteins was known and when meats were not subjected to severe heating processing. Moreover,

the long time needed for completion of an analysis (several days were needed to prepare samples) also limited its routine application. Several approaches have been developed to overcome these limitations:

1. *Improvement of antibody performance:* Menzel and Hagemeister¹⁴⁸ reported that antibodies against formaldehyde-treated soybean proteins reacted with both native and heated soybean proteins (125°C). The author suggested the applicability of these antibodies for soybean protein determination in processed meats, but no corroborative data demonstrated it. Ravestein and Driedonks¹²⁸ prepared antibodies against soybean proteins denatured with SDS instead of the urea used by Hitchcock. This modification made the method feasible for heated meats, independent of the soybean variety and soybean protein source. Moreover, this method was demonstrated to have no interference from meat proteins and other non-soybean vegetable proteins, making possible the quantitation and detection down to 0.5% and 0.1%, respectively, of soybean proteins. Monoclonal and polyclonal antibodies against different fractions of soybean proteins, rather than against all soybean proteins, have been proposed and used in ELISA systems for the detection of soybean proteins in processed meat products. Tsuji et al.¹²⁹ prepared two monoclonal antibodies against the major soybean allergen (Gly m Bd 30K) and used them in an ELISA method for the measurement of this allergen in different meat products. Yeung and Collins¹³⁰ developed polyclonal antibodies specific to soybean proteins with no demonstrated cross-reactivity with any nuts, legumes, or other ingredients in hamburgers. Macedo-Silva et al.¹³¹ proposed the use of the 7S fraction of soybean proteins to prepare a polyclonal antibody since it yielded higher immunogenicity than the 11S fraction.
2. *Reduction of analysis time:* Griffiths et al.¹⁴⁹ modified the ELISA method of Hitchcock, using commercial immunoreagents (antisera and labeled antiglobulin) and commercial microtiter plates. This method was subjected to a collaborative trial involving 23 U.K. laboratories.¹⁵⁰ Rittenburg et al.¹³² developed a ready-to-use kit containing standardized reagents that enabled the complete analysis of a meat sample in a working day. The performance of this kit was evaluated in another collaborative trial, which concluded that using a single arbitrary soybean standard as a reference enabled the reliable estimation of the level of soybean proteins in a pasteurized meat product of entirely unknown composition. Moreover, suitable repeatability and reproducibility (RSD values of 1 and 2%, respectively) and recoveries ranging from 80 to 100% were obtained.¹³³ Another improvement was introduced by Medina,¹³⁴ who reduced the analysis time and complexity of the ELISA procedure by the use of a simple and rapid sample preparation based on the direct extraction of soybean proteins in a carbonate buffer. Results reported for the analysis of various model and commercial sausages demonstrated the validity of the proposed method. On the other hand, Koppelman¹⁵¹ demonstrated that the use of an extremely high pH (pH 12) for the extraction of soybean proteins yielded higher recoveries than those observed in other (native) conditions (Tris buffer, pH 8.2) or commercially available test conditions (urea and dithiothreitol); it was possible to detect down to 1 ppm of soybean proteins. Although this extraction procedure was suggested as a solid alternative to other preparation procedures used for the determination of soybean proteins by ELISA in meats, no corroborative data in meats was shown.
3. *Improvement of sensitivity and accuracy:* The denaturation of soybean proteins by heating made their determination by immunological methods limited in sensitivity and accuracy. Since protein denaturation rarely affects its primary structure, Yasumoto et al.¹³⁵ proposed the detection of the presence of soybean proteins by the identification of characteristic peptides.

For that purpose, they prepared antibodies against a peptide fragment of the 11S soybean globulin, the major soybean protein exhibiting the most heat-stable antigenicity. Quantitative results obtained in model sausages demonstrated agreement between the added and the determined soybean protein content.

The application of the ELISA procedure has extended to the determination of soybean proteins in meat products with processing other than heating. González-Córdova et al.¹³⁶ developed an ELISA method for use in the determination of soybean proteins in fermented sausages (chorizo). The method proved specific and accurate, and the total time needed for the completion of an analysis was just 4 hours.

Although most methods were focused on the analysis of soybean proteins, there are some examples in which other nonmeat proteins have been analyzed in processed meats. Skerritt and Hill¹³⁷ developed an immunological method based on the detection of ω -gliadins for the determination of wheat gluten. Since ω -gliadins are heat-stable proteins, this test seemed suitable for the detection of wheat gluten in heat-processed meats. The main limitation of this test was the dependence of the response on the wheat gluten standard used. This method was subjected to a collaborative study in 15 laboratories. In the case of processed meats, the method proved semi-quantitative.¹³⁸ The use of antibodies allowing the recognition of total gliadins instead of only a part of them yielded more accurate determinations they were more affected by heating.¹⁵² Marcin et al.^{139,140} proposed a dot EIA (enzyme immunoassay) test that enabled the detection despite down to 0.2% of wheat gluten in sausages.

Finally, Brehmer et al.¹⁴¹ have applied the ELISA method to quantitate various foreign proteins (soybean proteins, pea proteins, and wheat gluten) in sausages, observing very low detection limits.

26.6 Chromatographic Methods for the Detection of Foreign Proteins in Processed Meats

The analysis of amino acids, peptides, or whole proteins by chromatography has been an alternative to electrophoretic and immunological methods for the detection of foreign proteins in processed meats. This section is devoted to a discussion of chromatographic methods, grouped in Table 26.3, applied to the determination of foreign proteins in processed meats.

26.6.1 Analysis of Amino Acids

The chromatographic analysis of amino acids consists of three steps: hydrolysis of the sample, chromatographic analysis of the hydrolyzed sample, and comparison of the amino acid pattern with a collection of amino acid patterns from different proteins. This comparison is assisted by a computer program based on a regression method, which can determine the types of proteins present in a sample. The main advantage is that this strategy works equally well for mixtures of native or denatured proteins since the amino acids are less prone to undergoing changes during processing than are proteins. The principal difficulties observed are due to the fact that all proteins contain all the major 17 amino acids, though in varying amounts. An additional problem in the case of soybean and meat proteins is that soybean and muscle proteins present a similar amino acid composition.⁵¹

Table 26.3 Chromatographic Methods for the Determination of Foreign Proteins in Processed Meats

<i>Sample</i>	<i>Foreign Proteins</i>	<i>Chromatographic Mode</i>	<i>Detection Limit</i>	<i>Reference</i>
Analysis of Amino Acids				
Pasteurized meat samples	Soybean proteins, egg white proteins, wheat proteins, caseins, potato proteins, and sinew proteins	Ion exchange	—	51
Model heated meats	Soybean proteins, caseins, and whey proteins	RP	—	153
Model heated meats	Soybean and wheat proteins	RP	—	154
Analysis of Peptides				
Model heated meats (120°C for 3 h)	Soybean proteins	Ion exchange	5–10%	155–157
Model heated meats (100°C for 30 min)	Soybean proteins	Ion exchange	—	158
Model heated meats (120°C for 3 h)	Soybean proteins	Ion exchange	2%	72,159
Analysis of Whole Proteins				
Commercial loaf meats	Soybean proteins	RP	0.19%	160
Model heated meats (pork, turkey, chicken, and beef), sausages, and meatloaf	Soybean proteins, caseins, and whey proteins	RP (perfusion)	0.07% for soybean proteins	161–163
Commercial cured meats (dry-fermented (Spanish chorizo) and to spread)	Soybean proteins	RP (perfusion)	0.04%	164

Lindqvist et al.¹⁶⁵ published the first application of this mathematical approach to the determination of proteins in mixtures. They used a stepwise multiregression analysis adapted to perform the comparison of the amino acid pattern corresponding to a composite sample with those of simple substances arranged in a data bank. This program selected from the bank those proteins whose amino acid patterns best matched that of the sample and calculated the proportion of every protein in the mixture. They applied the method to two model mixtures containing soybean and milk proteins but in no case used meat proteins.

Olsman⁵¹ applied, for the first time, a similar multiregression procedure to identify foreign proteins (soybean proteins, egg white protein, wheat proteins, caseins, potato proteins, and sinew proteins) in pasteurized meat products. Lindberg et al.¹⁵³ applied partial least-squares regression

analysis to determine various proteins in model heated meat products containing ground beef mixed with some common meat extenders (collagen, soybean proteins, and milk proteins). Samples were totally hydrolyzed, derivatized with dansyl chloride, and analyzed by reversed-phase (RP-) high-performance liquid chromatography (HPLC). Separation was carried out with a binary gradient acetonitrile-phosphate buffer water in 25 min. The method seemed to be very little affected by heating, with observing accuracies of 94% for heated meats. Zhi-Ling et al.¹⁵⁴ employed a similar procedure to determine muscle, collagen, shrimp, wheat, and soybean proteins in heated simulated mixtures. Chromatographic separation was performed in a column similar to that used previously, with an analysis time of 12 min, using a binary gradient acetonitrile-acetate buffer water. The accuracy in the determination of soybean proteins was not as good as that observed for wheat gluten and collagen since soybean proteins presented a similar amino acid profile to shrimp and muscle proteins.

26.6.2 Analysis of Peptides

Another approach to the determination of foreign proteins in meat samples has been the determination of characteristic peptides of the searched proteins. This proposal involves the partial hydrolysis, normally by enzymatic digestion, of proteins and the separation of soluble peptides by HPLC. Special care is needed in the case of heated samples in order to avoid aggregation of individual proteins, which could be difficult to dissolve. The studies published using this idea were focused on the analysis of soybean proteins and used ion-exchange chromatography for the separation of characteristic peptides.

Bailey et al. applied this approach for the first time to the determination of soybean proteins in heated meats.^{155–157} They isolated a characteristic peptide from soybean proteins (*Ser-Gln-Gln-Ala-Arg* from 11S globulin) by ion-exchange chromatography of the extracts obtained by trypsin digestion. The method was valid for heated samples but was not as sensitive as other methods. Moreover, the analysis time was extremely long (180 min), and this characteristic peak was badly resolved from meat. Llewellyn et al.¹⁵⁸ improved Bailey's method by the introduction of a filtration step before separation, the use of a larger column, and the reduction of flow rate by half.¹⁵⁸ Two characteristic peptides from soybean were selected for the determination of soybean proteins. Despite these efforts, the method continued to be inaccurate since these two soybean peptides proved to overlap with some minor peaks from meat. A further development of the method using an even longer column could improve the resolution of the target peaks and yield lower detection limits for soybean proteins. Nevertheless, the method presented limitations for quantitative purposes and it was not adequate for routine analysis since the total time required for a single analysis was 5–6 days.^{72,159}

26.6.3 Analysis of Whole Proteins

The determination of whole soybean proteins in cooked meats has also been approached by HPLC. Various methods enabling the determination of soybean proteins in raw meats have appeared, the group of Marina et al. being the first to focus its efforts on the determination of soybean proteins in heat-processed meats by the analysis of whole proteins by HPLC. They developed conventional and perfusion HPLC methods in the RP mode, applying them to the

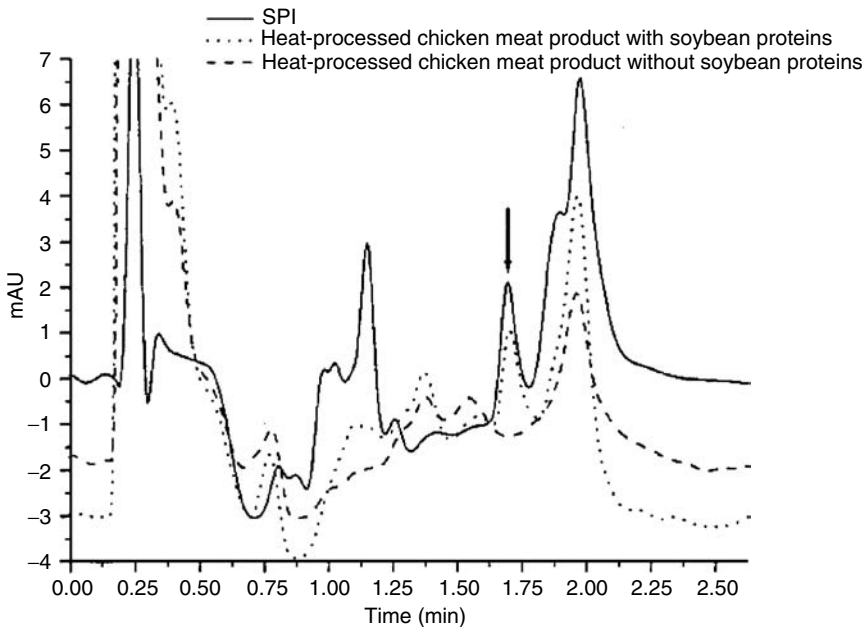


Figure 26.2 Chromatograms obtained by perfusion HPLC from a heat-processed chicken meat with and without SPI and an SPI. The arrow shows the selected soybean protein peak. (From Castro, F. et al., *Food Chem.*, 100, 468, 2007. With permission.)

determination of soybean proteins in commercial heated meats. Moreover, they could also identify additions of caseins and whey proteins in the meats by perfusion HPLC. As examples, Figure 26.2 shows the chromatograms corresponding to a heat-processed chicken product with and without soybean proteins and an SPI using the perfusion method; the separations obtained by conventional HPLC for a commercial heat-processed meat (containing turkey and pork) and an SPI are presented in Figure 26.3. As expected, perfusion chromatography enabled a much shorter separation than conventional HPLC. Nevertheless, in both cases it was possible to obtain a soybean protein peak totally isolated from meat bands, which was used for quantitation. Both methods enabled detection limits significantly lower than those obtained with any previous technique. Quantitative results obtained by both methods were very similar, with the soybean protein content in commercial meats between 0.60 and 1.54%. Moreover, the results obtained by perfusion HPLC were compared with those observed applying the official ELISA method, with the conclusion that the proposed method could be a serious alternative to the official ELISA method, enabling a significant reduction of analysis time, price, and the complexity of the method itself.^{160–163}

The same group has also extended its interest to the analysis of other processed meats, such as cured meat products also containing soybean proteins. They have proposed a new perfusion HPLC method that enabled the isolation of a soybean protein peak that proved adequate for the detection and determination of soybean proteins. Figure 26.4 shows the separations obtained for an SPI and for various cured meat products with and without soybean proteins.¹⁶⁴

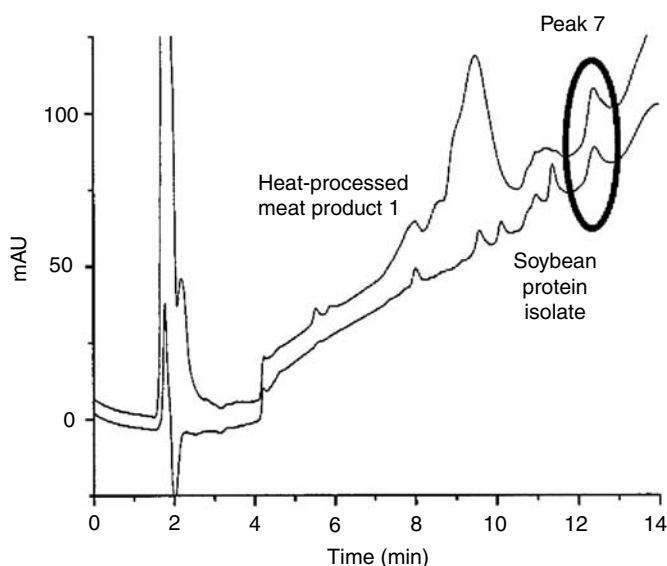


Figure 26.3 Chromatograms obtained from a heat-processed meat product and an SPI by conventional HPLC. (From García, M.C. et al., *Anal. Chim. Acta*, 559, 215, 2006. With permission.)

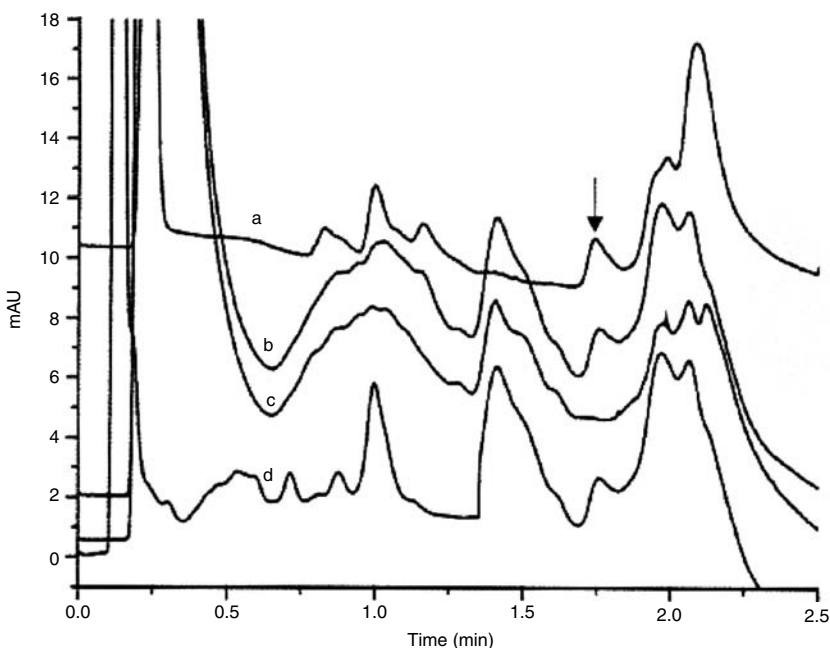


Figure 26.4 Separations corresponding to an SPI (a), a cured meat product to spread spiked with SPI (b), a cured meat product to spread without soybean proteins (c), and a cured meat product (dry-fermented sausage) with soybean proteins (d) in its composition obtained by perfusion HPLC. The arrow shows the selected soybean protein peak. (From Criado, M. et al., *J. Sep. Sci.*, 28, 987, 2005. With permission.)

26.7 Other Methods for the Detection of Foreign Proteins in Processed Meats

Deoxyribonucleic acid (DNA) analysis has also been applied to the detection of foreign food constituents. The stability of DNA made these methods appropriate for the analysis of heated products where antibody based methods fail. Moreover, the unique specificity of the target in these methods ensures the discrimination and avoids cross-reactivity. The main disadvantage of these methods is that they are qualitative or semiquantitative (by the incorporation of internal standards). Superior quantification could be achieved by using real-time polymerase-chain reaction (PCR) or a PCR-ELISA.^{115,166} Meyer et al.¹⁶⁷ designed a PCR protocol for the amplification of 414 and 118 bp fragments of the Lectin gene *Lel* and compared its performance with the commercial ELISA test (based on polyclonal antibodies against renatured soybean proteins) for the detection of soybean proteins in both fresh and processed meats (hamburgers, frankfurters, and heat processed mixtures of soybean and beef meat). The ELISA kit yielded higher recoveries and could quantify soybean proteins in meat products. However, sample preparation using a denaturation–renaturation step was very time consuming. In contrast, the oligonucleotides used in PCR were synthesized rapidly and could be stored for several years. They concluded that PCR could be an interesting method to confirm ELISA results.

Boutten et al.¹⁶⁸ combined immunohistochemistry and video image analysis and applied the method to the detection of soybean proteins in processed meats. They used the visual images provided by histochemical techniques and the specificity of antibodies. Polyclonal antibodies against both raw and heated SPI and soybean protein concentrate were employed. No interference was observed when other proteins were added. Moreover, the labeled soybean surface was proportional to the percentage of soybean proteins added, making this method adequate for the estimation of soybean proteins.

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Chapter 27

Detection of Adulterations: Identification of Animal Species

Johannes Arjen Lenstra

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27.1 Introduction

Species substitution during food production results from economic fraud or negligence. It may not only lead to unwanted disrespect of religious rules, but can also have harmful health effects. For these reasons several methods have been developed for the identification of the species origin of meat samples. In addition, the same methodology can be applied to the control of poaching and illegal trade in animal products.

Earlier reviews described the state of the art in species identification in 2001 [1] and 2003 [2], more general aspects of food forensics [3,4], or traceability at the level of the subspecies or breed [5]. In this chapter we review the considerable progress during recent years. The almost complete dominance of deoxyribonucleic acid (DNA)-based methods has not led to the abandonment of other techniques. However, most reports describe wider applications or refinement of polymerase chain reaction (PCR)-based species identification. There is now a growing emphasis on convenient real-time PCR assays, which allow a quantitative interpretation of the results.

In addition to the published work, the Web site www.molspec.org offers a detailed description of the detection of several food species.

27.2 Alternatives to Polymerase Chain Reaction

Immunochemical methods require no expensive equipment or elaborate protocols and are still in use. Species-specific proteins, or epitopes, have been developed for most animals used for meat production, including pig, cattle, sheep, and poultry, but threshold values have yet to be determined empirically [6]. Although heating decreases the sensitivity and specificity of the antisera, adequate performance of a species-specific enzyme-linked immunosorbent assay (ELISA) with commercial antisera [6] and of a pork-specific indirect ELISA [7] has been reported. However, ELISA procedures are not yet adequate for a sensitive detection of ruminant material in feed [8].

Capillary electrophoresis has been described as a flexible tool for the analysis of species-specific proteins in unheated meat product [9].

27.3 Deoxyribonucleic Acid Methods

27.3.1 *Deoxyribonucleic Acid Extraction*

For most applications, DNA is now purified by using one of several commercially available kits, which are based on the adsorption of DNA to special resins. Apart from convenience and speed, the major advantage of these procedures is the effective removal of various inhibitors of the PCR reaction that often are present in food samples. However, the relative performance of the kits depends on the food commodity [10,11], and for large-scale applications different kits should be compared.

Heating for prolonged periods destroys DNA, which especially hinders the DNA-based species identification of extremely heated meat and bone meal. However, bovine DNA could be amplified from meat subjected to the most common cooking procedures with the exception of panfrying for 80 min [12].

A promising approach is the binding and subsequent sequence analysis of highly fragmented DNA to beads, followed by emulsion PCR and high-throughput sequencing. This advanced technology has been used for the partial sequence analysis of Neanderthal DNA extracted from fossil remains [13].

27.3.2 Polymerase Chain Reaction

27.3.2.1 Design of Polymerase Chain Reaction

Any PCR reaction critically depends on the design of the primers. With only a few exceptions, primers for animal species identification target variable regions in the mitochondrial DNA (mtDNA). Mitochondrial DNA is more variable than nuclear DNA, but its high copy number increases the sensitivity relative to the PCR of single-copy nuclear sequences. However, because of its maternal origin, mtDNA may not be representative if samples originate from hybrids between species [2].

Remarkably, earlier species identification methods [1,2] were based on hybridization to species-specific repetitive elements, which combine a high copy number with often absolute specificity for a species, suborder, order, or higher taxon. In general, a centromeric satellite DNA sequence is confined to one species, whereas homologous satellites from related species can be differentiated by a restriction fragment length polymorphism (RFLP) assay [1,2]. Further, the dispersed short interspersed nuclear elements (SINE) are specific for mammalian order or suborder, which is useful, for instance, in detection of ruminant DNA [14,15]. However, repetitive elements must be characterized for each species, which is not practical for exotic animals. Furthermore, standardization of the PCR across species with several nonhomologous repetitive elements will be more difficult than for mitochondrial DNA.

Several different strategies for the PCR-based species detection are being adopted.

1. One strategy relies on the design of universal primers in conserved regions that amplify a DNA fragment from all species to be detected (Section 27.3.2.2). Subsequent analysis of the PCR product then allows the determination of the species origin (Section 27.3.2.3).
2. In another strategy, PCR primers match the sequence of a single species. Species identification follows from the presence or absence of an amplification product (Section 27.3.2.4). If different components have to be detected, primers can be combined in a multiplex reaction, often with one common forward primer and for each species a specific reverse primer (Section 27.3.2.5).
3. Several methods are available for the generation of fingerprints by PCR. The resulting patterns depend on the species and thus allow their detection (Section 27.3.2.6).
4. The latest development is real-time quantitative PCR, which often is able to differentiate low levels of target DNA from insignificant background signals (Section 27.3.2.7).

27.3.2.2 Universal Primers

A seminal paper in 1989 [16] described a number of universal mtDNA primers. These or similar primers often allow the sequencing or detection of various mtDNA segments from known or unknown species [2,17]. However, with species not previously tested, the matching of the primers and the amplification should be checked. Further, even for the most common meat species [16], matching to the mtDNA target sequences is incomplete [2]. This may necessitate a low annealing temperature, but then invites nonspecific amplification of, for example, nuclear mtDNA copies. In addition, it is likely to cause uneven amplification of different targets with samples of mixed-species composition.

For purposes of detecting all animal DNA in foodstuffs, primers specific for the 16S mtDNA gene were designed that (with two ambiguities) matched completely to species from all mammalian orders [18,19]. In the same gene, other primers were designed to generate a short amplicon

from mammalian and avian species for real-time PCR [20]. Primers in the mtDNA *ATP8* gene were designed to be specific for nonhuman mammalian DNA [21] or ruminant DNA. However, as can be checked by a Genbank search, these mammalian primers match completely only to bovine DNA, whereas the ruminant primers match only four ruminant species. As a consequence, it is not likely that DNA from all targeted species will be amplified with the same efficiency, if at all. Similarly, other universal primers [22,23] match most completely to ruminant DNA and indeed appeared to amplify only ruminants and horse [22].

Different primer pairs in the mtDNA *12S rRNA* gene designed to match the cattle, sheep, and pig sequences [24] also matched to several other mammals. Curiously, the 3' end of the reverse primer [24] does not match any mammalian mtDNA sequence, including the Genbank sequences used for the design of the primer. Other primers with cross-species specificity in the same gene were used for PCR-RFLP of several ruminants [25,26] or for quantitation of mammalian DNA [27]. However, the amplicon of 425 bp [27] is rather long for this purpose.

Trading sensitivity for broad specificity, universal primers may be derived from nuclear genes. Primers specific for an intron in an actin gene were found to be suitable for species identification by sequence analysis in a wide range of species [17]. Mammalian primers have also been based on the myostatin [28] or growth hormone [29] genes. Truly universal eukaryotic primers have been derived from the nuclear *18S rRNA* gene to serve as positive control of species-specific PCR reactions [30,31].

The nucleotide database now contains mitochondrial and genomic DNA sequences of most, if not all, species that are used for meat production. However, more often than not, allegedly universal primers have not been aligned with all relevant homologous sequences to check their taxonomic range. Further, the implicit assumption that in a sample of mixed-species origin the primers target the different components with the same efficiency has in most cases not been validated.

27.3.2.3 *Determination of Polymerase Chain Reaction Products*

For samples with single-species origin, sequencing of the PCR product is the most straightforward way of species identification. It is especially useful if it is not known beforehand which species is to be expected, for instance with game species. For this, the mtDNA cytochrome *b* gene is the most popular target [32–35], since this gene has been used frequently for phylogenetic studies. If the sample is derived from an exotic species for which no sequence data are available, a basic local alignment search tool (BLAST) search in the nucleotide database will turn up a number of related species. Other genes suitable for species identification are the mtDNA *12S rRNA* [36], *16S rRNA*, and *ND4* genes [17], or the nuclear actin genes [17].

A simple way to determine the species origin of PCR products is digestion by a restriction enzyme that cleaves at a species-specific (diagnostic) site. Although RFLP for restriction enzyme length polymorphism formally refers to a genetic polymorphism within a species, the term “PCR-RFLP” is now commonly used to denote the procedure to detect the species-specific restriction sites. The method requires only simple equipment and is most practical if few samples have to be tested. In general, admixtures of 1% can be detected. Table 27.1 summarizes a number of PCR-RFLP assays, most of which use the original universal primers [16]. Most of these reports confirm or add other species to the report of Meyer et al. [37]. Maede [38] gives the most complete list of species and restriction patterns and also describes a number of species-specific primers.

Apart from preferential amplification by the use of the original universal primers, another caveat is that the diagnostic site can be polymorphic with the consequence that the assay does not detect all individuals from a species [2]. This can be circumvented by testing for more than one

Table 27.1 PCR-RFLP Systems for Species Identification

Reference	Target Gene	Primers	Detected Species	Remarks
39	mt <i>cytB</i>	Universal [16]	Cattle, sheep, goat, roe-, red deer	
40	mt <i>cytB</i>	Universal [16]	Cattle, fallow, roe-, red deer, pig, chicken, turkey, quail, Muscovy duck	
41,42	<i>cytB</i>	Universal [16]	Pig	
38	mt <i>cytB</i>	Universal [16]	24 mammalian and avian species	Several enzymes
		Horse-specific	Horse, donkey	
		Poultry-specific	Chicken turkey, mallard duck, Muscovy duck, goose	
		Deer-specific	Red-, roe-, fallow deer, elk	
38	Growth hormone	Cattle-specific	Cattle, water buffalo, etc.	Amplification of other related species not excluded
		Sheep/goat specific	Sheep, goat, etc.	
43	mt <i>cytB</i> , <i>CO2</i>	Bovine-specific	Cattle, zebu, gayal, banteng	Several enzymes
	Satellite IV			
	Satellite 1.711b			
26	mt <i>12S rRNA</i>	Ruminants	Cattle, sheep, goat, red-, roe-, fallow deer	
44	mt <i>12S rRNA</i>	Ruminants	Chamois, ibex, mouflon	
	mt D-loop	Sheep, mouflon	Sheep, mouflon	
45	mt <i>12S rRNA</i>	Universal [16]	Cattle, water buffalo, sheep, goat	
46	mt <i>cytB</i>	Dog-, cat-specific	Dog, cat	
47	mt <i>cytB</i>	Universal [16]	Two ostrich species, chicken, turkey	
48	mt <i>12S rRNA</i>	Universal [16]	Chicken, mallard duck, turkey, guinea fowl, quail	
49	mt <i>12S rRNA</i>	Universal [16]	Peacock, chicken, turkey	Two enzymes
50	mt <i>cytB</i>	Turtle-specific	Ten turtle species	
51	mt <i>12S rRNA</i> , <i>16S rRNA</i>	Snail-specific	Two snail species	

Note: *ATPase6*, gene for ATPase subunit 6; *ATPase8*, gene for ATPase subunit 8; *CO1*, gene for cytochrome oxidase subunit I; *CO2*, gene for cytochrome oxidase subunit II; *cytB*, cytochrome *b* gene; mt, mitochondrial; *ND5*, gene for NADH dehydrogenase subunit 5; *t-Glu*, tRNA^{Glu} gene; *t-Lys*, tRNA^{Lys} gene; *t-Phe*, tRNA^{Phe} gene; and *t-Val*, tRNA^{Val} gene.

diagnostic site. Further, the taxonomic range of the diagnostic site should be checked in alignment with homologous sequences of closely related species. For instance, it is relevant to know if a bovine pattern is the same in zebu, bison, and water buffalo and which of the several deer species share a diagnostic site.

Alternatively, species can be detected by hybridization of PCR products to immobilized species-specific probes. For analysis of feed, mtDNA cytochrome *b* fragments generated by ruminant-specific primers were spotted on polyester cloth and hybridized to probes specific for cattle, sheep, goat, elk, and deer [23]. Using newly developed cytochrome *b* primers, PCR products were hybridized to microarrays containing probes for cattle, sheep, goat, pig, chicken, and turkey [52]. The commercially available kit CarnoCheck (http://www.jainbiologicals.com/PDF/carno_cryo.pdf) has been developed for use with the original universal cytochrome *b* primers [16]. Hybridization of amplicons to an array of probes targeted to the detection of cattle, sheep, goat, pig, horse, donkey, chicken, and turkey allows the detection of admixtures of 1% or less.

27.3.2.4 *Species-Specific Amplification*

Although most universal primers are a compromise of specificity and taxonomic range, primers targeted at a single species potentially offer better selectivity, that is, a more sensitive and specific detection in the presence of a complex and dominating background of other components in the sample. Several of these methods have been developed for the detection of bovine or ruminant material in feed to prevent a further spread of transmissible spongiform encephalopathy, but are equally applicable for analysis of processed meat products.

Specific primers have been described in several publications (Table 27.2). Although the design of these primers for any species-variable sequence on the basis of an alignment of homologous sequences is straightforward, published data will lend credibility to test results in the event of prosecution.

27.3.2.5 *Multiplex Polymerase Chain Reaction*

Often, only a limited number of species is to be expected in a sample. This obviously applies to dairy products, but also to meat products if possible adulterations likely originate from the available livestock species. Further, with game species, the number of species that can be present in a sample is in practice limited by their geographical distribution.

To detect these species, species-specific primers can be combined in one multiplex reaction (Table 27.3). However, increasing the number of primers also increases the chance of nonspecific amplification. This can be reduced by combining one common forward primer with a specific primer for each species to be detected [80]. Amplification products can be differentiated either by gel electrophoresis (see the various references in Table 27.3) or by their melting temperature [81,82].

27.3.2.6 *Fingerprinting*

PCR amplification with random primers [17,94], or primers specific for an ancient mammalian repetitive element [95], generate a fingerprint pattern that is specific for the species. Although this would allow the detection of several different species with one protocol, these methods suffer the disadvantages of problematic reproducibility and exchange of patterns between institutes. Further, the methods are not very well suited for the detection of a species against a background

Table 27.2 Species-Specific PCR Amplifications

Reference	Target Gene	Detected Species	Detection Limit (w/w)
53	Lactoferrin	Cattle	0.02% in foodstuff
54	mt <i>CO1</i>	Cattle	0.5% in water buffalo cheese
55	mt <i>cytB</i>	Cattle	0.025%
56	mt <i>ATPase8</i> [57]	Cattle, sheep, pig	0.1% in animal feed, ring trial
58	mt <i>ATPase8</i> [56]	Cattle	0.006–0.03% in feed
59	mt <i>12S rRNA</i>	Cattle	0.1% in sheep or goat cheese
60	mt <i>12S rRNA</i>	Goat	1% goat milk in sheep milk
21	mt <i>ATPase 8</i>	Ruminants, cattle, sheep, goat	0.1–0.01% meat and bone meal in vegetable meal
61	mt <i>cytB</i>	Pig	
	mt <i>ATPase8</i> [57]	Cattle	0.1%
62	mt <i>12S rRNA</i>	Ruminants, pig, poultry	0.125–0.5% in fish meal
63	mt <i>12S rRNA</i>	Cattle, sheep, goat	0.1% in feedstuff
64	mt <i>t-Lys, ATPase8,</i> <i>ATPase6</i>	Cattle, sheep, pig, chicken	0.01% meat and bone meal in grain concentrates
65	mt <i>12S rRNA, 16S</i> <i>rRNA</i>	Cattle, sheep, goat, deer, ruminant	0.05% in vegetable meal
66	mt D-loop	Chamois, ibex, mouflon	0.1% in pork after sterilization
25	mt <i>12S rRNA</i>	Red-, roe-, fallow deer	
67	mt <i>cytB</i> [68]	Pig	
69	mt D-loop	Dog	0.05%
70	<i>cytB</i>	Tiger	
71	<i>cytB</i>	Chicken, turkey	
72	mt <i>12S rRNA</i>	Chicken, turkey, mule duck, goose	0.1% in oats
73	mt <i>12S rRNA</i>	Four duck species	0.1–1% in goose meat
		Muscovy duck	0.1–1% in goose meat
74	α -Actin	Mule duck, goose	1% duck in goose <i>foie gras</i>
75	mt <i>cytB</i>	Goose	
76	mt <i>cytB</i>	Ostrich, emu	
77	mt <i>cytB</i>	Chinese alligator	
78,79	mt <i>cytB</i>	Basking shark	

Note: For abbreviations, see Table 27.1. Different primers were developed for each species or taxon listed in the third column.

of other species. However, a qualitative PCR with species- or taxon-specific primers will not target all DNA components in a mixture and will not always differentiate trace amounts or contamination from a complete species substitution. In this case, a species-specific pattern would yield additional evidence for the species origin of a sample.

27.3.2.7 Real-Time Polymerase Chain Reaction

Quantification of species composition is mainly relevant if low but significant levels of a species must be differentiated from an insignificant signal, which, for instance, may originate from

Table 27.3 Multiplex PCR Amplifications for Species Identification

<i>Reference</i>	<i>Target Gene</i>	<i>Detected Species</i>	<i>Detection Limit (w/w)</i>
Two primers per species			
62	mt 12S rRNA	Ruminants, pig, poultry	0.25%
83	mt cytB	Cattle, water buffalo	
84	mt 16S rRNA	Cattle	0.002–0.004% in maize
	mt 12S rRNA— <i>t-Val</i>	Pig	
	mt 12S rRNA	Fish, poultry	
81	mt <i>t-Glu</i> — <i>cytB</i>	Cattle, horse	1% cattle, 5% horse by melting temperature analysis
		Cattle, wallaroo	5% cattle, 5% wallaroo by melting temperature analysis
		Pig, horse	5% pig, 1% horse by melting temperature analysis
		Pig, wallaroo	60% pig, 1% wallaroo by melting temperature analysis
One primer per species and one common primer			
80	mt cytB	Cattle, sheep, goat, pig, horse, chicken	ca. 10%
85	mt 12S rRNA, 16S rRNA	Cattle, sheep	0.1% bovine milk in ovine cheese
86	mt 12S rRNA, 16S rRNA	Cattle, goat	0.1% bovine milk in goat cheese
87	mt 12S rRNA, 16S rRNA	Cattle, sheep, goat	0.5% in cheese
88	mt cytB	Cattle, water buffalo	1% in cheese
89	mt cytB [80]	Pig, horse	
90	mt 12S rRNA	Cattle, sheep, goat, pig	1% for monoplex reactions
68	α -Actin	Chicken, pork	0.1% in goose and mule duck <i>foie gras</i>
91	5S rDNA	Mule duck, goose	
92	mt 12S rRNA	Pig, chicken, turkey, mule duck, goose	1% in <i>foie gras</i>
93		Pig, goose	
82	mt <i>t-Phe</i> —12S rRNA	Six Tasmanian carnivores	

Note: For abbreviations, see Table 27.1. One or two primers were developed for each species or taxon listed in the third column.

nonspecific side reactions or from contaminations of the reagents. For instance, qualitative PCR reactions described earlier would not be suitable for a sensitive yet specific detection of potentially pathogenic ruminant material in animal feed or for traces of porcine material in food for Jewish or Islamic consumers. For these applications, quantification has already been accomplished by competitive PCR [96–98]. However, much more accurate and convenient is real-time PCR

Table 27.4 Real-Time PCR Amplifications for Species Identification

Reference	Target Gene	Detected Species	Detection Limit (w/w)
SYBR Green detection			
15	Bov-B SINE	Ruminants	0.1% ruminant material in processed chicken feed samples
81	mt <i>t-Glu—cytB</i>	Cattle, pig, horse, wallaroo	0.04 pg pig, wallaroo DNA, 0.4 pg cattle, horse DNA
99	Satellite DNA	Cattle	0.005%
	PRE-1 SINE	Pig	0.0005%
100	Bov-tA2 SINE	Ruminants	
	Cr1 SINE	Chicken	0.05%
	SINE and LINE elements	Birds, rodents, horse, dog, cat, rat, hamster, guinea pig, rabbit	0.1–100 pg
104	mt <i>cytB</i>	Tiger	0.5%
TaqMan detection			
105	mt <i>ATPase8</i>	Cattle	0.0001% bovine material in meat and bone meal
106	mt <i>12S rRNA</i>	Goat	0.6% goat milk in sheep milk
107	mt <i>12S rRNA</i>	Cattle	0.6% cow milk in sheep milk
14	Bov-A2 SINE	Ruminants	10 fg bovine DNA
108	mt <i>16S rRNA</i>	Ruminants	
29	Growth hormone	Cattle	
		Mammals	
101	mt <i>t-Lys—ATPase8</i>	Cattle, pig	0.1% in compound feeds
109	mt <i>cytB</i>	Cattle, sheep, chicken	35 pg bovine DNA
110	mt <i>t-Lys, ATPase8, ATPase6</i>	Cattle, sheep, pig, chicken	0.01% in grain concentrates
111	mt <i>cytB</i>	Cattle, sheep, pig, chicken, turkey	0.5%
112	Prion protein	Cattle + sheep + goat, pig, chicken	10 pg DNA after heating
30	mt <i>t-Glu—cytB</i>	Cattle, sheep, pig	1% pig, 5% cattle, lamb in binary mixtures
	mt <i>ND5</i>	Chicken, ostrich, turkey	1% chicken, turkey
28,113,114	<i>18S rRNA</i>	Eukaryotes	
	Phosphodiesterase	Cattle, sheep, goat	0.1% in processed food
	Ryanodin	Pig	
	Interleukin-2 precursor	Chicken, turkey, duck	
	Myostatin	Several mammals and birds	
27	mt <i>12S rRNA</i>	Pig, mammals	0.5% pig in beef
115	mt <i>cytB</i>	Horse, donkey	1 pg donkey DNA, 25 pg horse DNA

(Continued)

Table 27.4 (Continued)

Reference	Target Gene	Detected Species	Detection Limit (w/w)
116	<i>MC1R</i>	Dog	
117	mt <i>cytB</i>	Mallard duck, Muscovy duck	
118	mt <i>12S rRNA</i>	Mule duck, mule duck + goose	1% duck in goose <i>foie gras</i>
FRET (Lightcycler)			
119	mt <i>ATPase8</i> [21]	Cattle	0.001% bovine gelatin in gelatin
120	mt <i>cytB</i>	Cattle	0.001% bovine material in cattle feed
121	mt <i>cytB</i>	Cattle, sheep	0.05% cattle MBM, 0.1% sheep MBM in feed
20	Chloroplast <i>rpoβ</i>	Plants (positive control)	
	mt <i>16S rRNA</i>	Mammals + birds	
	mt D-loop	Cattle (scorpion reverse primer)	0.1%

Note: For abbreviations, see Table 27.1. Separate assays were developed for each species, combination of species, or taxon listed in the third column.

that can be based on the binding of the fluorescent reporter SYBR Green to double-stranded probe, on relieving the quenching of fluorescence by the 5' nuclease degradation of an internal probe (the TaqMan procedure), or on fluorescence resonance energy transfer (FRET) between two internal probes (often performed in a Lightcycler apparatus). In fact, because of its closed-tube format without post-PCR steps, real-time PCR is now becoming the method of choice for species identification.

As for the qualitative PCR methods, most published real-time PCR protocols (Table 27.4) exploit the high copy number of mtDNA or DNA repetitive elements [14,15,99,100]. Short amplicons (150 bp or shorter) are most suitable [101,102]. Hird et al. [103] give a few hints for deriving species-specific real-time PCR primers from alignments of homologous sequences. However, for most assays of common livestock species, no information is available about results with closely related species, either in the wild or kept locally as domesticates.

Detection limits (Table 27.4) are variable, but most assays appear adequate to detect significant adulterations or potentially harmful trace amounts.

27.4 Conclusion

The technical progress of the methodology of species identification mirrors the fast and continuing progress in DNA technology. As a consequence, several methods have been replaced before being put in practice and validated by routine testing. Quite often, the same authors successively publish various methods for the detection of the same species without an explicit evaluation of the relative merits of the different approaches.

Quantitative real-time PCR is now accessible to most laboratories and is likely to dominate the field during the coming years. Future progress is likely to come from bead-based technologies, which are now being established in single nucleotide polymorphism (SNP) typing, microbial typing, and high-throughput sequencing.

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Chapter 28

Residues of Food Contact Materials

Emma L. Bradley and Laurence Castle

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28.1 Introduction

This chapter deals with the topic of the transfer of chemical residues from contact materials into processed meat and poultry products. This topic is important because if chemicals transfer to the product they may cause taint or odor problems, and if the transfer is high enough, it may even make the product unsafe to eat. Therefore, understanding how this can be tested for and kept under control by the proper selection and use of packaging materials is important. This chapter aims to give the reader such an understanding and includes some examples that, although by no means exhaustive, are illustrative of the main scientific and technical issues. It will start with the chemical and physical processes that underlie this transfer process, which is called chemical migration. This is because it is the migration phenomenon that makes testing of processed meats and poultry for residues of food contact materials (FCMs), and testing the FCMs themselves, a special topic.

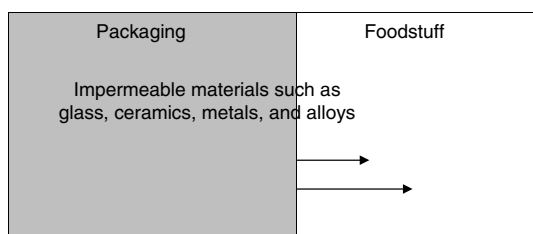
28.2 Food Contact Materials and Chemical Migration

The term FCM describes any material that may come into contact with a foodstuff. The most obvious example is food packaging, but the term also encompasses materials (and articles) used in food processing, transport, preparation, and consumption. A distinction is often made by the cognoscenti between materials and articles. Materials include films and sheets that require fabrication into their final usable form, and articles such as boxes and pouches that are in final form. In this chapter we shall refer to both as “materials.” These materials may be made from plastic, paper/board, rubber, metal, glass, or ceramics. Chemicals are needed to give these materials desirable properties. Any chemical constituents present have the potential to transfer to the foods with which they come into contact. In addition, the chemicals present in any adhesives, coatings, or printing inks applied to these substrates also have the potential to transfer. This transfer is known as chemical migration. Chemical migration is defined as “the mass transfer from an external source into food by sub-microscopic processes.” The extent to which any substance migrates into a foodstuff is controlled by diffusion processes that are subject to both kinetic and thermodynamic control. These processes can be described by Fick’s second law, and the extent of any chemical migration is dependent on

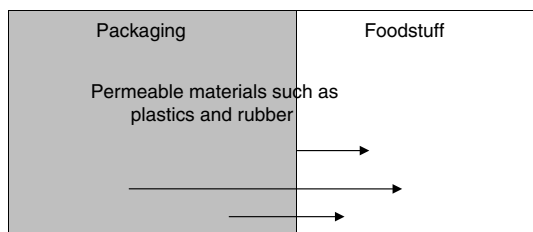
- The nature of the FCM
- The nature of the foodstuff
- The nature of the migrating substance
- The nature, the extent, and the type of contact between the FCM/article and the foodstuff
- The duration of the contact
- The temperature of the contact

28.2.1 *The Nature of the Food Contact Material*

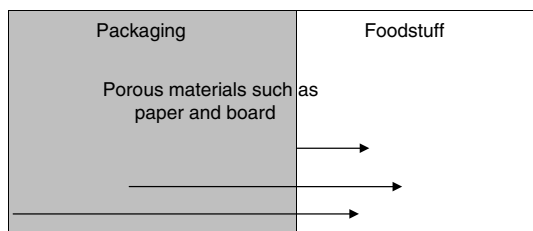
Any chemical migration is dependent on the concentration of the substance in the FCM (i.e., if only a low concentration of a given substance is present, then the maximum migration that can



Migration can occur from the food contact surface only.



Migration of substances can occur from within the polymer as well as those at the surface.



Migration of substances occurs from the food contact surface, from within the material as well as any substances contained in inks and coating applied to the non-food contact surface. Porous substrates offer practically no resistance to chemical migration.

Figure 28.1 Depiction of the effect of the nature of the FCM on chemical migration.

occur is also low) and its diffusion characteristics. Migration from a material occurs at the interface with the food. For a material with low diffusivity, the speed with which the surface is replenished with the migrant will be slower than that of a high-diffusivity material. As a result, the rate of the migration will be reduced for the low-diffusivity material. Migration from materials such as glass, ceramics, or metal occurs only from the surface of the material; no diffusion of migrants will occur from within these materials to the food contact surface. Plastic materials exhibit diffusivity to different extents depending on the structure, crystallinity, and other factors. However, in all cases diffusion of migratable substances from within the plastic to the food contact surface can occur. More porous materials such as paper and board provide practically no resistance to the movement of the migratable substances within the matrix. This is depicted in Figure 28.1.* Multilayer packaging materials in which a barrier layer such as aluminum foil is included in the

* Migration of substances occurs from the food contact surface, from within the material, and from any substances contained in inks and coating applied to the nonfood contact surface. Porous substrates offer practically no resistance to chemical migration.

packaging structure are also commonplace. In these cases, any migratable substances on the non-food side of the aluminum foil layer will not be able to pass through this barrier layer, and therefore migration of such substances into the foodstuff will not occur by this mechanism. However, if a material has been rolled (reeled) or stacked such that the food contact surface is stored in contact with the nonfood contact surface, then transfer of chemicals between the two can occur. In such cases, even the presence of a functional barrier such as a layer of aluminum foil is not sufficient to ensure that no migration will occur. This transfer process is known as set-off, and it is especially important when evaluating inks.

28.2.2 The Nature of the Foodstuff

When considering migration, foodstuffs are conventionally split into five categories: aqueous, acidic, alcoholic, fatty, and dry. The solubility of the migrating substance in the foodstuff will influence the extent of the migration. Lipophilic (“fat-loving”) substances will have a greater solubility in fatty foods or foods with free fat on the surface, and the migration of such substances into these food types will be greater than that into an aqueous foodstuff. Conversely, polar molecules are more soluble in aqueous media and less soluble in fatty foods. Figure 28.2 shows the effect of the fat content of minced pork meat on the migration of the polar molecule caprolactam, tested in our laboratory. Caprolactam has the chemical structure $\text{cyclo} -[(\text{CH}_2)_5 - \text{CO} - \text{NH}] -$, and it is the main chemical (a “monomer”) used to make nylon-6. Because polymerization processes are not 100% complete, there are always low levels of residual, unreacted monomers in plastics. In this example, as the fat content of the meat product increases, the solubility of the polar caprolactam

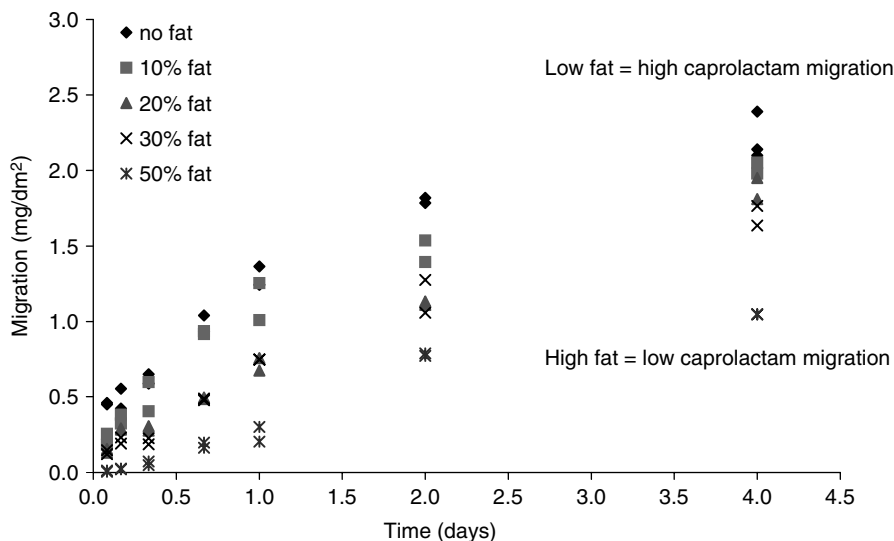


Figure 28.2 Kinetics of the migration of caprolactam into minced pork meat with different fat content held in contact with nylon at 4°C.

monomer in the foodstuff is reduced, and as a result, both the rate and the extent of the migration in a given time period are reduced.

28.2.3 *The Nature of the Migrating Substance*

Any substance that is incompatible with the FCM type will “bloom” to the surface, resulting in it being readily available to transfer to the foodstuff. Conversely, any strong interaction that occurs between a substance and the material containing it will slow down the mass transfer process.

28.2.4 *The Nature, Extent, and Type of Contact between the Food Contact Material and the Foodstuff*

Both the nature of the FCM and the nature of the foodstuff will influence the partitioning between the two. If the foodstuff interacts strongly with the FCM, it can cause swelling at the surface, which increases the rate at which chemicals are released. The greater the surface area of the material in direct contact with the foodstuff, the greater the potential for migration. Similarly, where intimate contact is made as opposed to point contact—for example, liquid or semisolid foods, including sauces and pastes, as compared to solid foods—the potential for migration also increases.

28.2.5 *The Duration of the Contact*

The longer the material is in contact with the foodstuff, the greater the extent of the migration that will occur. Migration kinetics are normally first order, which means that the extent of any migration increases relative to the square root of the contact time.

28.2.6 *The Temperature of the Contact*

Similarly, as migration is a diffusion process that occurs more rapidly at elevated temperature, the extent of the migration increases with increasing contact temperature.

28.3 Why Test for Residues of Food Contact Materials

By the diffusion processes described above, any substances present in a material placed into contact with a foodstuff has the potential to migrate. This migration can impact on the safety of the food, because some substances used to make FCMs may be harmful if consumed in sufficient amounts. Migration can also impact on the quality of the food, because the transfer of sensorially active substances may impart a taint or odor to a foodstuff, such that it is no longer appealing to the consumer. The need to control the effects of FCMs on both of these aspects has been considered in legislation. In the European Union, The Framework Regulation (EC) No. 1935/2004 is the basic legislation that covers all FCMs. It states in the general requirements of Article 3 that

Materials and articles, including active and intelligent materials and articles, shall be manufactured in compliance with good manufacturing practice so that, under

normal or foreseeable conditions of use, they do not transfer their constituents to food in quantities which could:

- (a) endanger human health; or
- (b) bring about an unacceptable change in the composition of the food; or
- (c) bring about a deterioration in the organoleptic characteristics thereof.

A similar philosophy on the need for controls operates in the United States, Japan, and other countries, although the detailed legislative and technical instruments used differ.

28.4 What Residues Need Testing

A range of different chemicals are needed to make materials intended for food contact. There are several thousand chemicals in inventory lists used by producers, and of these probably several hundred find regular use. They include monomers and other starting substances needed to make plastics, catalysts, and production aids to make plastics and paper, additives to modify the properties of the finished products, and ingredients of inks and adhesives. Because chemical migration is a diffusion phenomenon, it is the small, low molecular weight substances that tend to migrate fastest. This is certainly true for the monomers used to make high-volume plastics such as vinyl chloride, butadiene, acrylonitrile, and styrene. Additives, on the other hand, must remain in the finished material to have a technical effect, and so they tend to be higher molecular weight substances to prevent their loss. Finally, as producers strive to make materials with lower migration properties, they incorporate so-called polymeric additives of molecular weight of 1000 Da or more. This means that the full range of analytical methods are deployed in testing for these residues, with headspace gas chromatography–mass spectrometry (GC-MS) for the volatiles, GC-MS for the semivolatiles, and increasingly LC-MS for the nonvolatiles and the polar residues. The detection level needed depends on the toxicological or organoleptic properties of the substances, but typically it is in the range of a few parts per million (ppm, mg/kg) down to ca. 10 parts per billion (ppb, µg/kg) in the food.

28.5 Testing Strategies

The food itself can be tested for undesirable chemical residues. Alternatively, the packaging material can be tested before it is used to ensure that it does not contain residues that can migrate at levels that could cause problems. Finally, uniquely for FCMs, the packaging can be tested for its suitability before use by employing food simulants that are intended to mimic the migration properties of different categories of foods.

28.5.1 Overall Migration and Total Extractables

By way of an example, the EU Plastics Directive imposes an overall migration limit to ensure that materials do not transfer large quantities of substances that, even if they are not unsafe, could bring about an unacceptable change in the food composition amounting to adulteration. The total amount of all migrating substances is limited to 60 mg/kg of food. This is tested for using food simulants, and a set of test methods is available as European standards. Because a test for overall migration using food simulants is entirely conventional—that is, the test result depends on the method used—the standard test procedures have to be used and followed exactly. In countries

such as the United States and Japan, suitability end-tests of materials may use extraction solvents rather than food simulants.

28.5.2 Specific Migration Limits

Again by way of an example, the EU Plastics Directive 2002/72/EC, as amended, contains a positive list of monomers and additives permitted for use in the manufacture of plastic for food contact. This list contains any limits on the migration of individual substances—limits that have been assigned following the toxicological assessment of these substances. Similar lists exist in Europe and other countries for the chemical ingredients used to make paper, silicones, inks, adhesives, coatings on metal, etc. The form of any restrictions—such as specific migration limits or limits on the extractable substance or on the total content in the material—differs from country to country and for the different material types.

28.5.3 Extraction Tests Followed by Estimation of Migration Levels

Compliance of a material with a specific migration limit or some other migration restriction can be tested for by extracting the material to determine the concentration of the substance(s) of interest. Then the expected migration into food can be estimated either by assuming total mass transfer (worst case 100% migration scenario) or by using mathematical models. The measured concentration in the packaging ($c_{p,0}$) may also be available from formulation details provided by the producer. A number of commercial software packages (e.g., Migratest[®] Lite, SMEWISE, and EXDIF v 1.0) are available to predict the extent of migration from the $c_{p,0}$ value. They have been validated mainly for plastics. All are based on diffusion theory and a consideration of partitioning effects. The underlying key parameters are the diffusion coefficient of the migrant in the plastic (D_p) and the partition coefficient of the migrant between the plastic and the food or food simulant $K_{p,F}$. These models have been tuned to provide an overestimation of migration in the majority of cases, so that they can be used with confidence in compliance testing.

28.5.4 Using Food Simulants

Food simulants are an important tool for testing the suitability of materials for the food that are intended to be placed in contact with. Again, the EU system for plastics is taken as an illustrative example. Simulants intended to mimic the migration from plastics into foods were introduced in the early 1980s (Directive 82/711/EEC, as amended), along with the rules for using simulants (Directive 85/572/EEC, as amended). Simulants are specified for the five food categories described earlier:

<i>Food Type</i>	<i>Food Simulant</i>
Aqueous foods of pH >4.5	A—distilled water
Acidic foods of pH <4.5	B—3% acetic acid solution
Alcoholic foods	C—10% ethanol solution (or higher)
Fatty foods	D—rectified olive oil or similar
Dry foods and frozen foods	No migration testing is specified

Processed meat and poultry products, such as ham, salami, and bacon, are fatty foods. As such, they are mimicked by using both simulant A (water; to represent the aqueous phase) and simulant

D (oil; to represent the fatty phase of the food). Oil simulant is considered too severe compared to the foods, that is, it elicits higher migration levels. Therefore, a reduction factor of 4 is applied to the test result using oil. Clearly this is a conventional approach, because a factor of 4 cannot be strictly correct for all types of processed meat and poultry products, for all types of different plastics, for different substances, etc. (e.g., see Section 28.2 describing the influence of these different parameters on the migration process).

Simulants were introduced at a time when analytical instrumentation and methods were not available to test foods for all the substances of interest at ppm to ppb levels. Simulants also provide a means to test for broad food categories rather than having to test individual food items. However, as methodology and instrumentation have advanced, it becomes clear that in some circumstances the simulants may not overestimate migration (as designed) but may underestimate migration into foods. The recent case of ITX (2-isopropylthioxanthone) illustrated that, for example, the organic particulate matter in cloudy fruit juices and the fat content of milk gave these foods a greater solubility for ITX (the packaging–food partitioning dimension of kinetic migration, see above) than the simulant specified for these products. The case also illustrated that not only the direct FCM needs to be evaluated. In this case, the ITX originated from an external printing ink on a paper/foil/plastic laminate, and had set-off from the printed outer layer onto the inner food contact layer when the laminate was stored on reels. Many processed meat and poultry products are packed in multilayer materials, and both set-off and transfer through one layer to another must be considered.

Migration testing into food simulants should be performed under exposure conditions equivalent to the worst foreseeable contact with foods. For example, exposure conditions of 10 days at 40°C are defined (Directive 82/711/EEC, as amended) for a plastic packaging of food intended for long-term storage at ambient temperature. For processed meat and poultry products that have a long shelf life because they have chemical preservatives added or are packed aseptically, or are sterilized in pack by retorting cans or pouches, it is likely that these accelerated laboratory test conditions of 10 days/40°C may be inadequate.

28.5.5 Testing for the Unexpected

As well as testing for known ingredients used to make FCMs, a proper safety assessment must go further. For example, the fourth amendment to Directive 2002/72/EC [1] includes the explicit provision that there is a general requirement to assess the safety of all potential migrants. This includes what have become known as the nonintentionally added substances (NIAS), such as impurities, reaction, and breakdown products. The onus is placed on the business operator to do so. Again, although this directive is applicable to plastics, it can also be used as a guide for other FCMs. To demonstrate their safety, these “nonlisted substances” should be assessed in accordance with international risk assessment procedures. Such a risk assessment should have three components (a) the identification of the substances present in the material, (b) an estimation of their migration level leading to an estimate of possible consumer exposure, and (c) a risk assessment that considers the potential exposure in context with any hazard (nature and potency) posed by the chemical. This requirement to identify substances places emphasis on the information-rich separation techniques using MS as the detection system, i.e., GC-MS and LC-MS. Increasingly, testing laboratories will turn to LC-TOF-MS (where TOF—Time-of-flight) to get accurate mass information on molecular ions and fragment ions to gain further confidence in substance identification.

28.6 Packaging Formats of Relevance to Processed Meat and Poultry Products

A study examining the marketplace for meat and poultry packaging identified rigid packaging (boxes, trays, cans, plastic containers, paperboard sleeves, etc.), flexible packaging (film, bags, pouches, paper, and foil), and packaging accessories (labels, absorbent pads, etc.). Specifically, the following packaging materials were identified:

- Plastics
- Paper/cartonboard
- Metal cans to which a polymer coating has been applied to the food contact surface
- Glass jars with lacquered metal lids and polyvinyl chloride (PVC) gaskets
- Multilayer flexible packaging materials including inks and adhesives
- Active and intelligent packaging (meat pads, oxygen-absorbing films, microwave susceptor materials)
- Surface-active biocides

Testing these packaging formats is described in more detail in the following, using selected examples.

28.6.1 *Plastics*

Plastics are the most commonly used material type for packaging foodstuffs. Examples to package processed meat and poultry products include trays made of polystyrene, polypropylene, polyethylene terephthalate, or PVC for cooked meat and poultry and for convenience ready-meals. The film lidding materials used with these trays is usually polyester or ethylene vinyl acetate copolymer. Nylon or polyvinylidene chloride (PVDC) films are used for sausage casings, and nylon/polyethylene laminates are used for boil-in-the bag and microwaveable pouches. Polyethylene is used for general-purpose food bags. Nylon and polyester films are used for meat and poultry roasting bags.

The different types of plastics and the typical monomers and additives used in their production have been reviewed elsewhere [2]. The migration of some of these substances into foods has been monitored in national surveillance campaigns. For example, the migration of the nylon-6 monomer caprolactam into foods (including processed meat and poultry products) was determined in a U.K. survey [3]. Caprolactam was detected and confirmed in nine of the 50 food samples, in the range 2.8–13 mg/kg [4]. The presence of caprolactam was indicated in further 15 samples, in the range 0.8–11 mg/kg, but these samples did not meet all of the confirmation criteria applied in that survey. The confirmation criteria used were typical for food contaminant analysis; they were: relative retention times, ion ratios, and full scan mass spectra. All migration levels (both confirmed and unconfirmed) were below the European specific migration limit for caprolactam, which is 15 mg/kg. All of the samples with detectable caprolactam migration were for applications involving heating the food in the packaging. They were packs of, for example, sausage meat for which the food would have been heat processed in the nylon casing, or they were nylon pouches for heating foods by boiling, microwaving, or roasting.

28.6.2 *Paper/Cartonboard*

Several standard methods have been published for the testing of paper and board intended to come into contact with foods. These include methods for paper-making chemicals, for contaminants

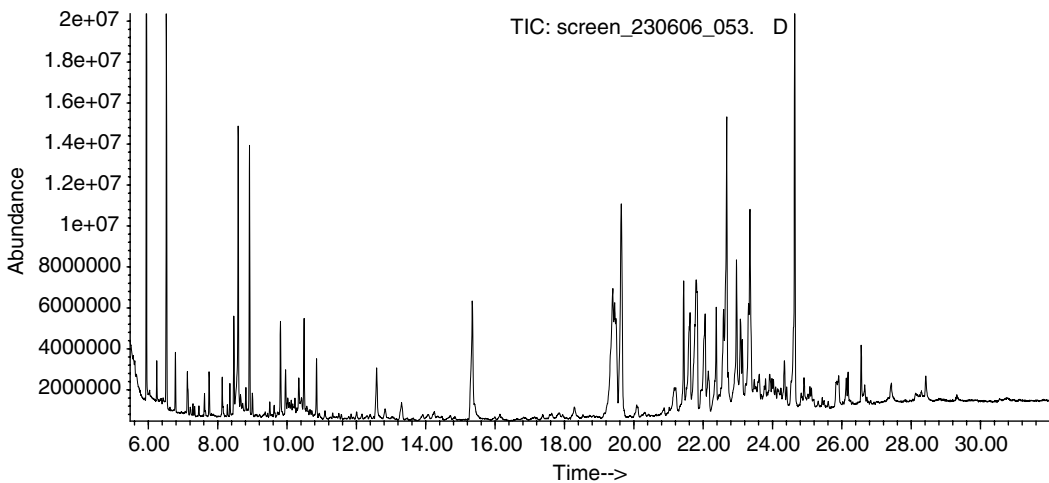


Figure 28.3 GC-MS total ion chromatogram obtained from the analysis of an ethanol extract of a food contact paper/board.

such as those that may be introduced by recycling, and for taint and odor transfer. Paper and board materials are chemically complex systems with special challenges regarding their safety evaluation. Figure 28.3 shows a GC-MS total ion chromatogram of a solvent extract of a food contact paperboard studied in our laboratory. Numerous peaks (individual substances) are detected. Limited peak identification can be performed by comparison of the mass spectra of each peak with those spectra contained in spectroscopic libraries. Typically, many of the substances detected remain unidentified. This is because many are derived from the woods, rosins, etc. used to make paper, and consequently they are not in standard libraries of spectra. Laboratories expert in paper analysis have over the years built up their own libraries of commonly encountered substances.

Given the natural source of the paper, its variability (e.g., different wood species), and the use of recycled fibers with their attendant contaminants that a water-based process for recycling paper may not remove completely, the safety evaluation of paper is difficult using chemical analysis alone. Therefore, an approach has been proposed that complements chemical analysis. This involves the application of a battery of short-term bioassays to extracts of paper and board, to assess the toxicity of the total migrate. Within the BIOSAFEPAPER project (<http://www.uku.fi/biosafepaper>) a battery of cytotoxicity tests was applied to extracts of paper and board materials. These assays correctly identified a nonfood grade board as being unsuitable for contact with food. This approach, that is, assessing the toxicity of the whole migrate, may also be applicable to other materials, particularly in cases where a number of unintentionally added substances are present in the finished material, and for which the toxicity of the individual substances is not known.

Direct contact with plain paper/cartonboard only occurs for frozen meat and poultry products such as frozen beef/chicken burgers and frozen sausages. A method has been developed for testing paper and board using modified polyphenylene oxide (MPPO) as a simulant for dry, nonfatty foodstuffs and for testing paper and board intended for baking purposes. In other applications, the fat and water present in the product means that direct contact with a porous substrate such as paper and board renders it unsuitable for this type of contact. In such cases, the cartonboard

may be laminated to a polymer film, with the cartonboard providing the rigidity required of the packaging, and the polymer film protecting the cartonboard from the foodstuff. The barrier properties of the laminated polymer will determine whether or not any migration of chemicals derived from the cartonboard will occur. For frozen foods, it is often assumed that migration does not occur at the low temperature. However, migration into frozen foods has been reported [5]. Paper and board materials are porous substrates within which the diffusion of low and medium molecular weight chemicals readily occurs originating both from the paper/board itself and from any printing inks and coatings applied to the external (nonfood contact) surface. The migration of the printing ink photoinitiator benzophenone through cartonboard substrates into frozen foods has been reported [5–6]. Unintentional contact with cartonboard may occur for meat-based ready-meals that are packaged in a plastic or aluminum foil tray held within a cartonboard box. In such cases, the migration of volatile substances can transfer by vapor phase diffusion from the cartonboard with adsorption onto the surface of the foodstuff.

28.6.3 Metal Cans with Polymeric Internal Coatings

Products such as corned beef, spam, hotdog sausages, stews, soups, tongue, and meat-based baby foods are often packed in metal cans with a polymer coating inside. This coating is intended to form a barrier between the food and the metal of the can. In this way, the coating protects the food from the metal substrate, as well as protecting the metal substrate from the potentially corrosive foodstuff contained within. The major types of can coatings are made from epoxy resins. These coatings exhibit a combination of toughness, adhesion, formability, and chemical resistance under the conditions that the coated metal is subjected to. The most widely used epoxy resins are based on bisphenol A diglycidyl ether (BADGE), itself synthesized by the reaction of bisphenol A with epichlorohydrin. The migration of bisphenol A and BADGE is well documented [e.g., 7–10]. Although much work has been carried out to determine the migration levels of these substances, there are many other potentially migratable substances in the coatings. In addition to epoxy resins, hardeners such as acid anhydrides, aminoplasts, or phenoplasts may also be included in the formulation, as well as additives, such as pigments, fillers, wetting and flow aids, defoamers, and lubricants, and any reaction/breakdown products formed from these starting materials. As mentioned previously, migration is influenced by both contact temperature and time. Most canned foods are sterilized (e.g., at 121°C for 1 h) and also have long shelf lives (up to 3–5 years is not uncommon); therefore, the migration conditions in canning are severe. Consequently, coatings manufacturers are constantly striving to produce “cleaner coatings” with fewer low molecular weight migratable substances [11].

28.6.4 Glass Jars with Lacquered Metal Lids and Polyvinyl Chloride Gaskets

Examples of processed meat and poultry products packaged in glass jars include pastes, cooking sauces, and baby foods based on meat or poultry. Whereas the glass containers themselves are generally considered to be inert, they need a metal closure—a lid—that will be coated (see Section 28.6.3) and will have an integral plastic sealing gasket. These PVC gaskets contain high levels (typically 40–45%) of plasticizers to make them soft enough to form an air-tight and microbiologically safe seal against the rim of the glass jar. With the high temperatures used to

sterilize meat and poultry products in the jars, there can be extensive migration from the gasket—even though the surface area of gasket exposed to food is generally small. This can be especially marked if the jar is sterilized in a rotating head-over-heels retort (rather than a static retort), which brings the gasket into intimate contact with the hot food contents and also fouls the gasket, promoting further migration during long-term storage.

Plasticizers used in gaskets include phthalic acid esters, adipic acid esters, epoxidized soybean oil, and acetyl tributyl citrate. Some of these are complex mixtures, and no standard methods exist for testing either foods or food simulants, although several research groups have published in this area (see, e.g., [12–16]). In addition to these additives, other substances used to make the gaskets may migrate. One recent example is semicarbazide formed as a breakdown product of azodicarbonamide used as a blowing agent [17]. Azodicarbonamide is added to the gasket formulation, and when heated it decomposes to liberate gases, primarily nitrogen and carbon monoxide together with some carbon dioxide and ammonia. These gases turn the PVC into a closed-cell foam, which helps it to make an effective seal. Semicarbazide was an unexpected and unwanted side product formed at very low yields. Nevertheless, the decomposition product semicarbazide can migrate into the food, and in Europe the use of azodicarbonamide as a blowing agent for food contact plastics is now prohibited. Bicarbonate is the normal replacement used.

28.6.5 Multilayer Packaging Materials

Many types of packaging materials consist of more than one layer. This is especially true for the flexible packaging films used for processed meat and poultry products, where the combinations of toughness for protection, barrier properties against gases (e.g., modified atmosphere packaging [MAP]) or odor, printability, heat-sealability, economy, etc., can be provided by combining two and sometimes several layers in a multilayer structure. The layers may be joined by coextrusion processes or by lamination using adhesives. In addition to the potential migrants derived from the individual materials that make up the different layers, the potential also exists for migration of components present in any adhesive used. Typically, reactive adhesive systems are used for this purpose. These include polyurethanes and to a lesser extent epoxy adhesives, which are polymerized *in situ*. A very common multilayer film would be nylon or polyester (for toughness and barrier properties) laminated using reactive polyurethanes to a polyethylene film (for heat-sealability) and printed on the outside or reverse-printed with inks inside the laminate sandwich (for decoration and consumer information). Any residues of incomplete polymerization of the adhesive or reaction by-products may remain in the FCM, and may then migrate into a foodstuff on contact. Polyurethanes are formed by the reaction between polyhydroxy compounds and isocyanates.

Examples of polyhydroxy substances used in the polyurethane adhesives include monoethylene glycol and diethylene glycol, 1,1,1-trimethylolpropane, 1,2-propylene glycol, 1,4-butanediol, and neopentyl glycol. Higher molecular weight polyhydroxy substances may also be used, up to several thousand daltons in size. Low molecular weight oligomers of polyols may be detected in polyurethane resins, and these compounds can migrate [18].

Some isocyanates can be sensitizing agents. Isocyanates also react with water to form amines. Some primary aromatic amines derived from aromatic isocyanates may be toxic. Spectrophotometric and a high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection methods of analysis for the measurement of primary aromatic amines in food simulants are currently being standardized within Comité Européen de Normalisation (CEN). A further LC-MS/MS method has also been described [19].

About 5% of the market for plastic-based flexible packaging laminating adhesives is based on epoxy resins (migration from these resins is discussed earlier). The main reason some epoxy adhesives are used is that when used between two barrier layers, they do not give rise to carbon dioxide bubbles, as most polyurethane adhesives do. In addition to concerns deriving from the migration of any residual epoxy monomer into the foodstuff, any polymerization reaction by-products may also migrate into the food. Previous work investigating boil-in-the bag [20] and microwave susceptor laminates [21] demonstrated that adhesive components can be highly reactive, and the reaction products can migrate into foods. For isocyanates, these included isocyanate dimers, prepolymerization oligomers, aromatic and aliphatic diamines, and carboimides.

28.6.6 *Active and Intelligent Packaging*

One of the most innovative developments in food packaging in recent years is the use of active and intelligent packaging. Active packaging materials can be defined as “food packaging that has an extra function, in addition to that of providing a protective barrier against external influence.” It is intended to change the condition of the packed food, to extend shelf life, or to improve sensory properties while maintaining the freshness and the quality of the food. This can be achieved through the removal (scavenging) of substances that have a detrimental effect on food quality. Examples of active absorbers and scavengers include

- Oxygen scavengers
- Moisture absorbers
- Ethylene and off-flavor scavengers
- Acetaldehyde scavengers
- Amine scavengers
- Sulfide scavengers
- Bitter taste removers

Alternatively, the active packaging systems can emit substances that improve the foodstuff. Examples of active releasing substances include

- Carbon dioxide–regulating systems
- Antimicrobial-releasing systems
- Nitrogen releasers
- Antioxidant releasers
- Sulfur dioxide releasers
- Flavor releasers

Intelligent packaging materials can be defined as “Concepts that monitor to give information about the quality of the packed food.” Examples of monitoring systems used in food contact applications include

- Time and temperature indicators
- Freshness and ripening indicators
- Oxygen indicators
- Carbon dioxide indicators

Consequently, for active packaging, the packaging is intended to influence the food and for intelligent packaging, the food is intended to influence the packaging.

In addition to using scavenging and releasing systems to maximize the shelf life of fresh and processed meat and poultry products, vacuum packaging, controlled atmosphere packaging, and MAP can also be used. Oxygen in the air increases the rate of both the chemical breakdown and microbial spoilage of many foods. Vacuum packaging removes air from packages and produces a vacuum inside. MAP and controlled atmosphere packaging (CAP) help to preserve foods by replacing some or all of the oxygen in the air inside the package with other gases, such as carbon dioxide or nitrogen, thereby reducing the oxidative damage. These systems are often used alongside oxygen-absorbing, carbon dioxide-regulating systems, working together to maximize product shelf life.

In most countries, any active substance emitted into the food is considered to be a direct food additive, and food additive rules and regulations apply. Therefore, the food should be tested for the additive using the available methods, for example, preservatives (see Chapter 5), flavors (Chapter 6), or colors (Chapter 7). Any chemical migration of other components of the delivery system of the active ingredient (e.g., SO₂/sulfite sorbed onto an inorganic reservoir), the holding system for the scavenging ingredient (e.g., a separate sachet of iron oxide as an O₂ scavenger), or the intelligent components (e.g., an impregnated plastic time/temperature strip) should be tested for migration of ingredients, breakdown products, and impurities, in the normal way for conventional packaging materials.

28.6.7 Surface-Active Biocides

A number of products have come onto the market in recent years with surface biocidal properties. These include conveyor belts, meat cutting boards, the inside linings of commercial and domestic refrigerators, and the plastic parts of complex food-processing machinery such as poultry lines. These surface-active biocidal materials should not be confused with active packaging (see Section 28.6.6), because there is no intention that the biocidal agent has any preservative effect on the food. Rather, the intention is that the biocide remains in the FCM, perhaps concentrated at the surface, and improves the surface hygiene and cleanability. Surface-active biocidal materials may have benefits especially for food-processing machinery parts that are awkward to clean *in situ*.

A common biocide used for this is silver in a number of chemical forms. It is generally accepted that silver ions are antimicrobial to all microbial species that are likely to be found in a food environment, including Gram-negative bacteria, Gram-positive bacteria, molds, and yeast. Another biocide used is 2,4,4'-trichloro-2'-hydroxydiphenyl ether, which seems to have a less uniform activity against bacteria, molds, and yeast.

Although these surface-active biocides are not intended to migrate into the food and exert any preservative effect, some level of migration is inevitable, and should be tested for as for any other substance used in FCMs. In the two examples given, the inorganic silver compounds may be expected to migrate mostly into aqueous and acidic foods, whereas the organic substance 2,4,4'-trichloro-2'-hydroxydiphenyl ether is expected to migrate more into fatty foods.

The unavoidable migration level should not be high enough to exert any preservative effect on the food. This can be checked by calculation, that is, by comparing the migration concentration against the minimum inhibitory concentration (MIC) values for common food-related microbes. But these calculations can be difficult to interpret because, first, the MIC values are usually recorded in pure buffer media and may change significantly in the presence of food components.

This is especially true for silver ions, which can be sequestered. Second, for solid and semisolid foods such as processed meat and poultry products, the migration will be concentrated at the food contact surface, and of course the surface of the food is most prone to microbiological spoilage. So the migration concentration at the surface could be much higher than when calculated as an average for the whole mass of the food. For these reasons, there is a need to conduct real food trials to ensure that indeed there is no preservative effect exerted.

There does seem to be two areas where test methods are lacking. The first is a test to demonstrate whether these materials are effective under the particular conditions of recommended use. Simple film tests examining surface inhibition against different organisms seem to be the only laboratory tool available at present. The alternative is real-life factory trials with full microbiological audits. Because these materials are intended to complement and not replace normal cleaning and hygiene procedures, hard facts and data are difficult to find. The second, related area, is testing for how long these biocidal materials retain their efficacy. Again, laboratory tests seem to be inadequate to simulate the resistance of the biocidal agent to loss through repeated washing, exposure to caustic cleaning agents, repeated contacts with food, etc., during the service life of the material. These research and development needs for test methods should be addressed.

28.7 Conclusion

Chemical residues in processed meat and poultry products may occur as a result of chemical migration from FCMs, of which food packaging materials are the most important example. Analysis of the food for these chemical residues uses basically the same chemical analytical methods that are in food analysts' armory. What makes the topic special is the added dimension of also needing to analyze the food packaging materials themselves (to indicate what chemicals may migrate), as well as food simulants used to test materials for their suitability for contact with different types of foods.

Further Reading

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Chapter 29

Polychlorinated Biphenyls: Environmental Chemical Contaminants in Muscle Food

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29.1 Introduction

Safety is a fundamental prerequisite of food quality and a factor that indisputably influences the level of consumer health [1]. Among the chemical compounds that can affect the safety of foods, several classes of chemicals are intentionally used to improve food production (including veterinary drugs and pesticides) or can be added to improve several food properties (stability, sensorial, and rheological characteristics, etc.). The use of these chemicals is strictly regulated in many countries; doses and conditions for their use are defined, and if necessary “maximum” or “safety residues levels” in food are established. However, other toxic chemicals can also come in contact with foods unintentionally, and can be accumulated during the processes of production, packaging, transport, storage, and consumption. These “chemical contaminants” can be classified according to their chemical nature, the source of contamination, and the route of incorporation into food.

The first group of chemical contaminants are the naturally occurring toxins, such as mycotoxins (patulin, ochratoxin A, Fusarium-toxins, aflatoxins), histamine, alkaloids, and marine toxins. Other chemical contaminants can be released in the environment as a consequence of industrial and human activities, and can enter the food chain through air, water, and soil [2]. Typical examples are persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), dioxins, heavy metals, and perchlorate.

A third group of chemical contaminants can be generated as a consequence of the interaction between food constituents and several technological treatments, such as heating, fermentation, or cooking. This group can include nitrosamines, polycyclic aromatic hydrocarbons (PAHs), acrylamide, dibenzofurans, perfluorooctanoic acid (PFOA), and residues of cleaning and sanitizing agents. The accumulation of these contaminants can be partially controlled during technological processes, but cannot be completely avoided. Because many of these contaminants have only recently been detected or considered in foods, they are also called “emerging food contaminants.”

Chemical contaminants could be defined as the group of unintended compounds that can be present in foods through the different steps of food production and processing [3–6]. The environment is a source of several important contaminants. The use of chemicals in agriculture and industry provokes the release of compounds into the environment and uptake by living organisms. The most dangerous compounds are characterized by their toxicological properties and persistence in the environment. Also, hydrophobic compounds have a high rate of bioaccumulation that implies higher concentrations in the tissues of animals. Another important aspect to be considered is the capacity of the metabolic pathways to produce metabolites having a low toxicity and to eliminate the compound from the tissues.

Chemical food contaminants generally possess (provoke) potential adverse health effects, so their level in foodstuffs should be monitored to minimize the risk to consumers. For this reason, many countries have developed legislation based on scientific advice and the principle that contaminant levels must be kept as low as possible, following good working practices.

The PCBs are a class of chemical contaminants the toxicity of which was clearly established several years ago, and maximum levels have been established in many countries for these contaminants. PCBs refer to a wide group of chlorinated aromatic hydrocarbons, composed of 209 different congeners, that are highly lipophilic and persistent. The general chemical structure of PCBs is shown in Figure 29.1; the multiple combinations in the number and position of 10 possible chlorine atom substituents account for the high number of possible congeners. All congeners are highly soluble in nonpolar solvents, oils, and fats, and their molecular weights range from 188.7 to 498.7.

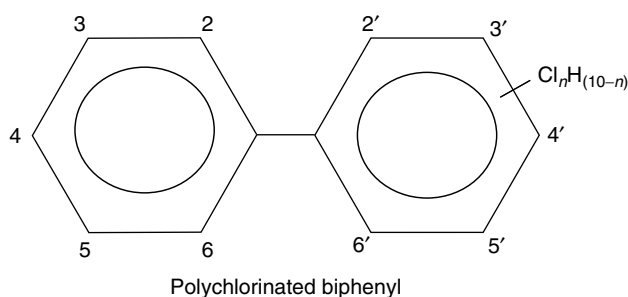


Figure 29.1 Polychlorinated biphenyl.

For their chemical and thermal stability, PCBs were much valued industrial compounds, and have been applied widely in industry as nonflammable dielectric fluids or plasticizers in different formulations since 1929 [7]. The original technical PCB formulations were composed of complex mixtures, containing different proportion of the possible congeners. The best-known commercially available products were Kaneclor (KC-300, KC-400, KC-500, KC-600), Aroclor (Ar1016, Ar1221, Ar1232, Ar1242, Ar1254, Ar1260, Ar1262, Ar1268), and Clophen (A30, A40, A50, A60). Since the late 1970s, the use of PCBs has been progressively banned in industrialized countries due to their toxicity and bioaccumulative effects. Even so, their persistence in the environment poses considerable hazards, as the result of the continuous cycling from soil to atmosphere and back to soil again. Currently, further amounts of PCBs can be released into the environment as by-products of several industrial activities involving chlorine, such as water chlorination and thermal degradation of chlorinated organics [8].

29.1.1 Toxicological Effects

PCBs provoke several short-term and long-term toxicological effects such as eye irritation, chloracne, skin rashes, skin discoloration, excessive body hair, mild liver damage, and the risk of cancer [9,10]. Furthermore, PCBs are endocrine disruptors [11] and environmental estrogens, and can induce cancer, neurobehavioral changes, cognitive dysfunction, reproductive and developmental defects, and immunological abnormalities [12–16].

Among the PCB congeners, the nonortho (77, 81, 126, 169) and mono-ortho (105, 114, 118, 123, 156, 157, 167, 189) PCBs show toxicological properties similar to those of the dioxins, and are potent inducers of the cytochromes CYP1A1 and CYP1A2 and of the Aryl hydrocarbon receptor (AhR) [17]. These congeners are therefore termed “dioxin-like PCBs.”

For these congeners, a toxic equivalency factor (TEF) has been established (by the World Health Organization [WHO]), with the most toxic dioxin having a TEF of 1 (Table 29.1). Single TEFs are multiplied for the concentration of the corresponding toxic congeners to give the toxic equivalent (TEQ) [7,18], which is a global estimation of the dioxin-like PCBs’ toxicity.

Tolerances for PCBs for feed and foodstuffs can be found in the title 21 of the Code of Federal Regulations (21CFR 109.30 and 21CFR 509.30) from the U.S. Government, as well as in the European Union (EU) directives [19–21]; the FDA, in conjunction with the EU and the United States Department of Agriculture (USDA), is addressing both international and domestic dioxin and PCB concerns (Table 29.2) [19–22].

Table 29.1 WHO—TEF for Dioxin-Like PCBs and Some Dioxins

<i>Congener</i>	<i>TEF Value</i>
Non-ortho PCBs	
PCB 77	0.0001
PCB 81	0.0001
PCB 126	0.1
PCB 169	0.01
Mono-ortho PCBs	
PCB 105	0.0001
PCB 114	0.0005
PCB 118	0.0001
PCB 123	0.0001
PCB 156	0.0005
PCB 157	0.0005
PCB 167	0.00001
PCB 189	0.0001
Dioxins (PCDDs)	
2,3,7,8-TCDD	1
1,2,3,7,8-PeCDD	1
1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-HxCDD	0.1
1,2,3,7,8,9-HxCDD	0.1
1,2,3,4,6,7,8-HpCDD	0.01
OCDD	0.0001
Dibenzofurans (PCDFs)	
2,3,7,8-TCDF	0.1
1,2,3,7,8-PeCDF	0.05
2,3,4,7,8-PeCDF	0.5
1,2,3,4,7,8-HxCDF	0.1
1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-HxCDF	0.1
OCDF	0.0001

Source: Adapted from Council Regulation No. 2375/2001 of November 29, 2001, *Official Journal of European Communities* L32 (2001) 1.

Table 29.2 Maximum Levels for Dioxins and Dioxin-Like PCBs (pg/g Fat) WHO-PCDD/F-TEQ and WHO-PCDD/F-PCB-TEQ

<i>Food</i>	<i>Sum of Dioxins (pg/g TEQ)</i>	<i>Sum of Dioxins and Dioxin-Like PCBs (pg/g TEQ)</i>
Meat and meat products (excluding edible offal) of the following animals		
Bovine and sheep	3.0	4.5
Poultry	2.0	4.0
Pigs	1.0	1.5

Source: Adapted from Council Regulation No. 2375/2001 of November 29, 2001, *Official Journal of European Communities* L32 (2001) 1.

29.2 Analysis of Polychlorinated Biphenyls in Muscle Foods

29.2.1 Sample Pretreatment

The bioaccumulation of PCBs in meat follows the general rule that the higher the fat content, the higher the expected concentration of PCBs, due to the fact that PCBs show a polarity very close to the neutral lipids (triacylglycerides). Thus, the procedures to extract PCBs from meat samples are similar to those applied for the extraction of the lipid fraction. Nevertheless, meat possesses a high content of water (60–80%), which makes the extraction of the lipophilic compounds difficult, and therefore a partial reduction of the water content in the matrix is generally required to facilitate the action of the organic solvents.

Water must be removed at a low temperature, mixing the sample with anhydrous Na_2SO_4 or by freeze-drying, to avoid the loss of the more volatile PCBs. The use of Na_2SO_4 seems to produce the best results, but is a long and complicated procedure, and a considerable amount of Na_2SO_4 (20–40%) is necessary to obtain a dry sample. This hindered the automatic sample handling, and lengthened the pretreatment step, making it more difficult.

29.2.1.1 Extraction Procedures

The use of the Soxhlet system is one of the most efficient and common techniques to extract PCBs from meat samples. The Environmental Protection Agency (EPA) method 3540C [23] relies on the Soxhlet extraction. The typical organic solvents employed are hexane alone or hexane mixed with dichloromethane or diethyl ether; the use of more polar solvents is not necessary because of the very high hydrophobicity of PCBs. This method allows obtention of an exhaustive extraction of the analytes with slight selectivity by modulating the composition of solvent mixture.

The Soxhlet equipment requires large volumes of organic solvent (about 50–200 mL/1–10 g of sample), which produces a significant dilution of the extracts. This implies that solvents must be evaporated to concentrate the analytes before determination.

Another disadvantage is the long extraction time (hours), although several commercial systems can provide partial automation of the process or the extraction of multiple samples at the same time. The evaporation of the extract can be performed more effectively if the solvents have a low boiling point. This allows the application of low temperatures in this process. The efficiency of the extraction is improved by repeating the procedure several times. The coextraction of other compounds among other lipids, pesticides, and other hydrophobic compounds implies the need to apply cleanup procedures to isolate PCBs from interferences.

The application of the accelerated extraction system (ASE) can help to obtain good recoveries of PCBs in a short time. The use of temperature to help the extraction could produce the possible degradation of some compounds, but PCBs are very stable during heat treatment.

A first simplified procedure is the use of ultrasonic waves to accelerate the extraction. The solid sample is dispersed in an organic solvent (the same used in Soxhlet extractors) and then sonicated in an ultrasonic bath or by using an ultrasonic homogenizer. The most important factors are the time of the treatment and the intensity (energy). The use of supercritical fluid extraction (SFE), typically CO_2 , has been proposed to improve the speed and the selectivity of the extraction.

An important problem is the evaporation of the extract obtained after extraction and before applying the cleanup step. Some of the PCBs can be evaporated in soft evaporation condition. The use of the injection mode of large volumes in gas chromatography (GC) analysis can help to reduce

the losses of some PCBs by avoiding the concentration step in some cases. However, if low concentrations of PCBs are present in the sample, it is more difficult to avoid the concentration step.

29.2.1.2 Cleanup Procedures

Extract cleanup is always required before the GC analysis. However, different cleanup strategies can be defined according to the detection techniques and the level of sensibility of the analysis. The identification and quantification of nonortho “dioxin-like” PCBs, detected at picogram per gram levels, require an exhaustive cleanup procedure to remove interfering compounds and to concentrate the extracts.

The use of GC-MS (where MS—mass spectrometry), GC-HRMS (where HRMS—high resolution mass spectrometry), and GC-MS/MS can help to reduce the problem of the possible overlapping of peaks between PCBs and some chlorinated pesticides, but some problems arise if the extract is not free from potential interferences.

29.2.1.2.1 Sulfuric Acid Purification

The simplest procedure is the use of concentrated sulfuric acid; this allows the elimination of the organic matter as well as the majority of interfering compounds.

29.2.1.2.2 Alkaline Treatment

Another alternative is the saponification of the extract obtained by using potassium hydroxide in ethanol. To reduce the possible losses of the chloride atoms, it is better to use low temperatures during the procedure. One advantage of this technique is that 4,4'-dichlorodiphenyltrichloroethane (4,4'-DDT) and 2,4'-DDT are converted to dichlorodiphenyldichloroethylene (4,4'-DDE) and 2,4'-DDE, and the possible overlapping of those peaks is eliminated [24].

29.2.1.2.3 Solid Phase Extraction

Another approach is applying solid phase extraction (SPE) to obtain different fractions to separate the PCBs from more polar compounds and to achieve a separation on the basis of different PCBs structures. These procedures use mainly polar stationary phases, such as florisil, alumina, and silica. The parameters to be evaluated are size of column (mass of adsorbent), activity (water content), pores diameter, and particle diameter. One important parameter is the quantity of lipid content that can be loaded onto a column packed with these materials. In general, 2–5% of the mass of adsorbent is the typical value. Therefore, a column containing 5 g of florisil could be loaded with a volume of extract with 200 mg of lipids. This fact is an important factor in the case of extracts with high lipid concentrations, because they require large columns that need more volume of solvent to elute the desired compounds. In muscle samples, if the sample taken does not contain intermuscular fat, the quantity of fat is limited, and it is easier to use low mass adsorbent columns. However, in the case of muscles with a high content of fat or including intermuscular fat, it is necessary to apply a previous cleanup step called acetonitrile partition. In this case, the extract obtained in hexane is mixed with acetonitrile to remove the excess of lipids by liquid–liquid extraction. Several extractions are necessary to remove most of the lipid material and recover PCBs in the acetonitrile phase, which is then mixed with water and coextracted with hexane to obtain the definitive extract of PCBs to be applied to the adsorbent column.

Florisil is the most used adsorbent to obtain PCBs separated from interferences. It is possible to separate planar PCBs and polychlorinated dibenzo-*p*-dioxins/polychlorinated dibenzofurans (PCDDs/PCDFs) from nonplanar PCBs. The column is eluted with different solvents of increasing

polarity: (a) hexane and (b) dichloromethane. Fraction I contains nonplanar PCBs and Fraction II planar PCBs and PCDDs/Fs. However, this procedure does not allow the possible presence of chlorinated pesticides in the two fractions, mainly in the second fraction. Another possibility is the use of porous graphite carbon to separate planar PCBs from PCDDs/Fs [25,26].

29.2.1.2.4 Gel-Permeation Chromatography

This is a method that allows the separation of the interference matrix compounds on the basis of size of molecules or molecular weight. A typical stationary phase is SX-3 Biobeads. The size of the columns is relatively large, 500×20 or 35 mm i.d., and in this case the use of a large volume of solvents is required to elute the different compounds. First, compounds containing lipids with high molecular weights are eluted, but free fatty acids, which have a molecular weight below 500 Da, remain in the column. After this step, PCBs and other compounds can be eluted. One alternative to eliminate free fatty acids, if they are present, is the application of alkaline treatment to produce the saponification. In some cases, another gel permeation chromatography (GPC) procedure is applied to remove more lipids, if the first GPC could not achieve this goal completely, but in muscle samples this procedure is not necessary. Nonplanar PCBs can be isolated from lipids by this procedure. This method facilitates the application of the extracts containing high quantities of lipids, because the size of the column allows loading a large volume or quantity of sample.

29.3 Determination of Polychlorinated Biphenyls by Capillary Gas Chromatography

29.3.1 Efficiency, Selectivity, and Stationary Phases

The separation of PCBs is a complex task, because of the large quantity of congeners that have to be separated. This fact has been an important factor in establishing the analytical procedure to improve the analysis of PCBs and also the analysis of PCDD/Fs. Long columns greatly improve the efficiency in terms of plate number (N); however, values above $N = 100,000$ do not improve the separation, because the increase of Chromatographic resolution (R_s) is low. Therefore, there is a limit in the use of very long columns; the typical length of the columns is in the range of 30–60 m. The selectivity can be managed by the use of different stationary phases; the most typically used is 5–8% phenyl/95–92% methyl polysiloxane (DB-5ms, HP-5ms, CP-Sil 8, Zebron 5-ms, SLB-5ms). Other stationary phases can produce better selectivity: HT-8 (8% phenyl-polycarbonate-siloxane), CP-Sil 88 for dioxins, CP-Sil 5/C18 CB for PCBs, DB-XLB. However, it is very difficult to separate the total profile of PCB congeners with a capillary column [27,28]. There are several critical pairs of peaks: 28/31, 56/60, 149/118, 105/153/132, and 170/190.

Because of this problem, efforts have been applied in the cleanup step to reduce the number of compounds to be separated, but this approach has very important drawbacks. PCBs are coeluted with many similar compounds (4,4'-DDE, 2,4'-DDE, lindane, and HCHs, 4,4'-DDT, dichlorodiphenyldichloroethane (4,4'-DDD), 2,4'-DDD, 2,4'-DDT, chlordane, etc.). This fact implies that the performance of the column, in terms of separation number (number of peaks between two alkane peaks), to separate all the compounds is not sufficient. One method of solving this problem is the use of a very complex cleanup procedure to obtain fractions containing specific compound classes: (a) PCBs, and (b) planar PCBs and dioxins. Despite this method, the complete separation of PCBs cannot be achieved by capillary gas chromatography in one dimension. The application

of GC × GC (comprehensive gas chromatography) could help to solve this problem, but this technique requires the use of specific instruments due to the fast peaks produced. Therefore, in the case of the use of GC × GC-MS, the analyzer's time of flight (TOF) should have a very rapid scan speed [29,30]. Another possibility is 2D GC, which entails the use of a Deans switch that allows the coupling of two capillary columns. A Deans switch was used with heart cut seven PCB indicators: 28, 52, 101, 118, 138, 153, and 180. The primary column was a DB-XLB and the secondary was a DB-200; specific retention windows from the first column were transferred to the secondary column to obtain a complete resolution of the target congeners. For this application, it is necessary to use two electron capture detector (ECD) detectors. The application of the back flushing technique allows the elimination of the nonvolatile compounds that can contaminate the column and improves the analysis. This procedure can simplify the sample treatment and reduce the global time of the analysis.

29.3.2 Inlet Procedures for the Analysis of Polychlorinated Biphenyls by Gas Chromatography

The injection of compounds in GC is a critical step in the chromatographic separation. The injector design must avoid changes in the composition of the solution injected. The best mode of injection is the cold on-column, as it avoids the thermal degradation and discrimination due to the different boiling points of solutes. This is typical in hot injections, such as split and splitless injection. Another alternative is the use of a programmed temperature vaporizer (PTV) injector in the modes of cold splitless or large-volume injection (based on solvent-venting injection). This last procedure allows the injection of volumes higher than 1 μL, in the range 5–100 μL. This is possible because the solvent is vented from the injector at low temperatures (60°C) and high flow rates in the split valve (50–100 mL/min), the solutes are trapped in the liner of the injector, and after the split valve is closed the injector is heated to the temperature necessary to evaporate the solutes.

In the first instance, PCBs are very stable to heat, and their thermal degradation can be minimal. However, DDTs are not stable to heat, and can suffer chemical degradation in the injection steps. If they are present in the extract, the original profile can be modified, and they can produce the overlapping of some peaks of PCBs with the by-products of 4,4'-DDT and 2,4'-DDT: 4,4'-DDE and in minor quantities, 2,4'-DDE, 4,4'-DDD, and 2,4'-DDD. Cold injections must be applied to avoid this problem. Another problem that can be reduced by cold injections is the discrimination between the PCB congeners that are more volatile (less Cl substitution) and those that are less volatile (higher Cl substitution); this is very important for obtaining a good quantification of the different PCB congeners.

Because of the very low concentrations of dioxin-like PCBs, the application of large-volume injection is a very useful technique to improve the sensitivity of the global analytical method and the quantification.

29.3.3 Detection in Gas Chromatography Analysis

There are two possibilities: ECD and MS. ECD is a simple method that can be used for screening purposes, because it is not possible to obtain the identification of the compound based on retention time despite the selectivity of the ECD. On the other hand, ECD is very sensitive for chlorinated compounds, but structure dependent. However, if the result obtained by using ECD is below the concentration on the basis of the TEQ values, it is possible to use this detector to determine the number of samples to be analyzed by GC-MS to assure the identification and the correct quantification.

GC-MS/MS and GC-HRMS are the most suitable techniques for analyzing dioxin-like PCBs and dioxins. HRMS is the preferred method, because of the high mass resolution of the MS analyzers used. In the case of GC/MS/MS, two systems can be used: ion trap (IT) and triple quadrupole (QqQ). The quantification is based on the method of isotope dilution. $^{13}\text{C}_{12}$ -labeled compounds (congeners: 28, 52, 118, 153, 180, 194, and 208, or more) are used as surrogates and internal standards to obtain data about the recovery; in some cases other Cl isotopes can be used [30,31]. GC/MS/MS using an IT allowed the detection of 60 femto-gram (fg), 300 fg, and 200 fg of the congeners 77, 126, and 169, in the nonresonant mode using the parent ions 292 (congener 77), 326 (congener 126), and 360 (congener 169) [31]. In GC/MS/MS, it is very important to define the conditions for each group of congeners or a specific compound. The parent ion could be common for groups of PCBs having the same number of chloride atoms; however, this can produce problems for isolated parent ions if overlapping of different groups of PCBs occurs. HRMS at a resolution of 10,000 in the mode of selected ion monitoring (SIM) is the best choice for the analysis of dioxin-like PCBs and dioxins. The ions selected are the molecular ion M and the ions $M+2$ and $M+4$, and the isotopic ratio is used to identify the compounds [32]. The tolerance of this ratio is in the range of $\pm 15\%$ of the theoretical value. The more suitable procedure is described in the EPA method 1668A. Gauthier et al. [33] described an interlaboratory study to evaluate the use of HRMS in PCBs and dioxins analysis. The results showed a good agreement between laboratories, but in some cases some compounds were not reported, or differences were important for PCB congeners 105 and 156. HRMS is an expensive technique that requires maintenance tasks and calibrations more complex than low resolution mass spectrometry (LRMS) (IT and quadrupole). The concentrations below 0.1 ng/kg are critical for the quantitative analysis, because the relative standard deviation (RSD) (%) is very high and fits the equation relative standard deviation (RSD) (%) = $ac^{-1} + b$, where c is the concentration (nanogram per kilogram).

The high cost of using this analysis to perform a statistical evaluation of a relatively large number of samples is problematic. Another problem arises from the fact that the commercial products used were different mixtures of PCBs containing specific percentages of chloride. These products are called Aroclor, followed by a number related to the chloride content: Aroclor 1221, Aroclor 1232, Aroclor 1016, Aroclor 1242, Aroclor 1248, Aroclor 1254, Aroclor 1260, Aroclor 1262, and Aroclor 1268. The content of 3,3',4,4',5-Pentachlorobiphenyl (congener 126) with a TEQ of 0.1 is higher in Aroclor 1248 (98 $\mu\text{g/g}$ TEQ). Aroclor 1248 and Aroclor 1254 presented higher global TEQ concentrations: 15 and 21 $\mu\text{g/g}$, respectively. However, the profiles of commercial Aroclor cannot be used to define the concentration of these commercial products in samples [34]. The interaction with the environment has produced changes in the initial profile by evaporation, adsorption, and metabolism. On the other hand, in the environment combinations of the different Aroclors make the identification of them more difficult. For this reason, the analysis of congeners is the preferred method, avoiding the definition of Aroclor profiles. If a sample shows a profile very close to a specific Aroclor, it could be associated with contamination by the use of commercial products, rather than with an environmental contamination.

29.4 Distribution of Polychlorinated Biphenyls in Poultry Muscle

The uptake of PCBs and dioxins in poultry muscle could occur by two important means: feed and soil. Free-range chickens can incorporate PCBs from contaminated soils, which are an important

factor in several countries in Europe and elsewhere. However, it was observed that the uptake from feed was more important (90%) than the uptake from soil (40–60%) on the basis of a model developed by Eijkeren et al. [35].

An important episode in the contamination of poultry by PCBs occurred in January 1999. Five hundred tons of feed contaminated with ca. 500 kg of PCBs and 1 g of dioxins were used by animal farms in Belgium and in neighboring countries (the Netherlands, France, and Germany). The analysis of the feed and poultry samples showed a predominance of PCDF over PCDD congeners. The theoretical ratio dioxin/PCB was 1:50,000, and the PCBs profile was close to an Aroclor mixture. This was a key factor in concluding that the origin was a contamination by transformer oil rather than an environmental source. In this case, PCBs made a higher contribution to TEQ than dioxins. TEQ from dioxins was 170 (standard deviation [sd] 487.7) pg/g, and from PCBs it was 240 (sd 2036.9) pg/g [36]. It is important to note the high standard deviation of the results, which could be produced by the effect of animals or by the analytical difficulties related to the analysis of more toxic compounds from PCBs and dioxins. This episode reflects the need for control of animal feed, including the raw material used, and traceability of the origins and the distribution channels of feed ingredients.

The typical range of PCBs and dioxins in poultry muscle is lower than contamination produced by bad practices in animal feed production.

In a study by MeeKyung et al. [37], the concentrations of PCBs and dioxins in pork, beef, and poultry were compared. The profile was similar in the three species, but in pork fat the concentration of the congener PCB118 was higher. Only 2% of the total concentration of seven indicators were related to three coplanar PCBs. The compounds with the highest concentrations were PCB138 and PCB28, which was higher in chicken fat than in pork and beef fat.

An example of PCB analysis is given in Figure 29.2.

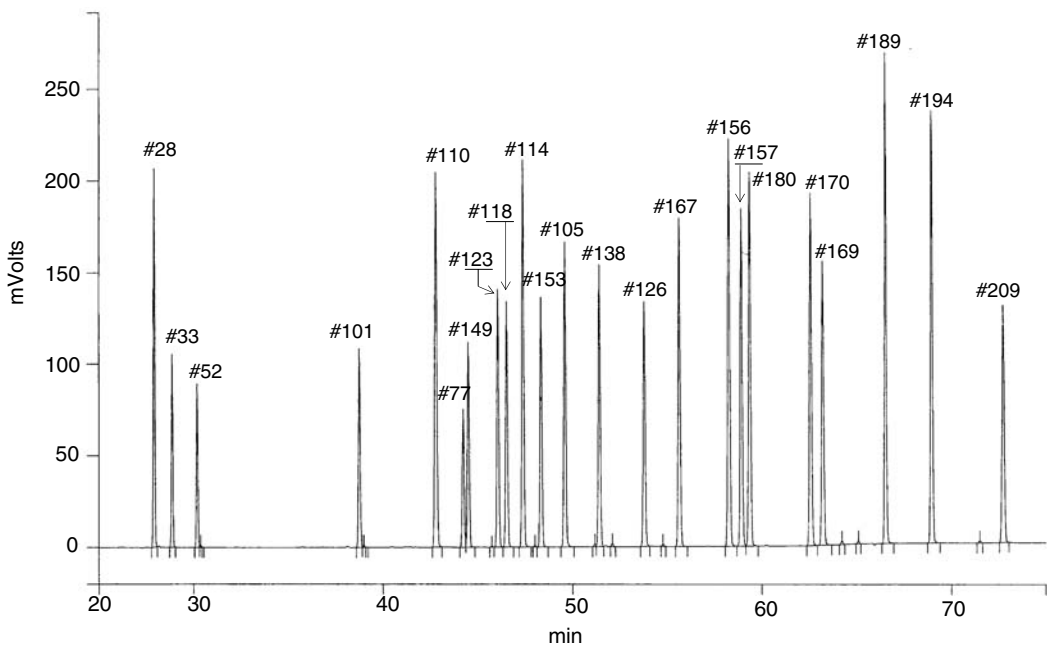


Figure 29.2 Polychlorinated biphenyl analysis.

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Chapter 30

Veterinary Drug Residues

Milagro Reig and Fidel Toldrá

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30.1 Introduction

Veterinary drugs are generally used in farm animals for therapeutic and prophylactic purposes; they include a large number of different types of compounds which can be administered in the feed or in the drinking water. In some cases, residues may come from contaminated animal feed-stuffs.¹ Many of these substances, along with others that have anabolic effects, may have other effects when administered to animals for purposes such as growth promotion. Some promoters may be administered in the United States to meat-producing animals, including, among others, estradiol, progesterone, and testosterone. The regulations in 21 Code of Federal Regulations (CFR) Part 556 list acceptable concentrations of residues of approved animal drugs that may remain in edible tissues of treated animals.²

Other countries allowing the use of certain growth promoters are Canada, Mexico, Australia, and New Zealand. However, the use of growth promoters is officially banned in the European Union since 1988 due to concerns about harmful effects on consumers.³

A primary effect of growth promoters is the increase in protein deposition, usually linked to fat utilization, decreasing the fat content in the carcass and increasing meat leanness.⁴ This allows greater efficiency in the feed conversion rate and thus leaner meat. In addition, the practice may involve the use of “cocktails” consisting of mixtures of small amounts of several substances such as β -agonists (clenbuterol), corticosteroids (dexamethasone), and anabolic steroids that have a synergistic effect, promoting growth but reducing the margin for effective analytical detection.⁵ The residues of these substances or their metabolites in meat and other foods of animal origin may cause adverse effects on consumers’ health, as described in the following text.

The presence of residues and their harmful health effects on humans makes the control of veterinary drug residues an important measure in ensuring consumer protection. The use of veterinary drugs in food animal species is strictly regulated in the European Union and, in fact, only some of them are permitted for specific therapeutic purposes under strict control and administration by a veterinarian.⁶

Sanitary authorities in a number of countries are concerned about the presence of residues of veterinary drugs or their metabolites in meat because they may have adverse toxic effects on consumers’ health. The European Food Safety Authority has recently issued an opinion about substances with hormonal activity, specifically testosterone and progesterone, as well as trenbolone acetate, zeranol, and melengestrol acetate. Exposure to residues of the hormones used as growth promoters could not be quantified. Although epidemiological data in the literature provided evidence for an association between some forms of hormone-dependent cancers and red meat consumption, the contribution of residues of hormones in meat could not be assessed.⁷ Other substances such as β -agonists have shown adverse effects on consumers. This was the case with intoxications in Italy, with symptoms described as gross tremors of the extremities, tachycardia, nausea, headaches, and dizziness after consumption of lamb and bovine meat containing residues of clenbuterol.⁸

Meat quality is also affected by the use of substances used as growth promoters.⁴ There is an increase in connective tissue production as well as in the collagen cross-links, making the meat tougher.^{9–11} Furthermore, muscle proteases responsible for protein breakdown in postmortem meat are inhibited.^{9,12} The lipolysis rate and breakdown of triacylglycerols are accelerated.^{13–14} The result is reduced tenderness and juiciness which, of course, affects the quality of meat products. When cocktails of clenbuterol and dexamethasone are used, meat quality is also affected, though it has been reported to be less tough than when using clenbuterol alone.¹⁵

Antibiotics have been extensively used as growth promoters to improve feed conversion and reduce toxin formation, thus promoting animal growth and improving productivity. Some typical antibiotics are chloramphenicol, nitrofurans, and enrofloxacin. Furazolidone, which is one of the major metabolites of nitrofurans, has mutagenic and carcinogenic properties.¹⁶ Low levels of chloramphenicol may cause an irreversible type of bone marrow depression that might lead to aplastic anaemia.¹⁷ It has been reported that sulfamethazine produces tumors in rodent bioassay, and there is some evidence on the toxicity of sulphonamides on the thyroid gland.¹⁸ Enrofloxacin may give rise to allergic reactions as well as promote the emergence of drug-resistant bacteria.¹⁹ In fact, the main concerns regarding the presence of antibiotics residues in foods of animal origin are related to the selection of resistant bacteria in the gastrointestinal tract and disruption of the colonization barrier of the resident intestinal microflora.²⁰ Intestinal flora may vary depending on the diet and thus is subject to large variations in the proportion of major bacterial species.²¹

This flora is essential for human physiology, food digestion, and metabolism of nutrients.^{22–23} Antibiotics residues present in meat or meat products may cause alteration of the intestinal microflora, changing its density and composition, contributing to the development of antibiotic resistance of any species of the indigenous microflora as well as impairing colonization resistance, which can increase susceptibility to infection by pathogens such as *Salmonella* spp. and *Escherichia coli*.²⁴

Anticoccidials are used in poultry to prevent and control coccidiosis, a contagious infection caused by parasites that causes serious effects such as bloody diarrhea and loss of egg production. A wide range of coccidiostats have been developed to avoid drug resistance in the parasites; their extensive use has led to the presence of coccidiostat residues in poultry products.²⁵ Anticoccidials may also be used as a growth promoter, though its safety margin is narrow since humans may be susceptible to its toxic effects. Some effects due to ionophores, including specific dilatation of coronary artery, have been observed in dogs. Even when a withdrawal period is allowed before slaughter, coccidiostat residues in poultry products might constitute a risk for consumers.²⁶

Meat products may contain different types of toxic compounds, some originating in the processing but others, like veterinary drugs residues, originating in the meat used as raw material.²⁷ Control strategies must include sampling of the meat to be used in the factory as raw material before processing. This chapter reports the main types of analysis that can be performed for the control of veterinary drug residues in meat products and poultry.

30.2 Control of Veterinary Drug Residues

The monitoring of residues of substances having hormonal or thyreostatic action as well as β -agonists is regulated in the European Union through Council Directive 96/23/EC,²⁸ which governs measures to monitor certain substances and residues in live animals and animal products. The European Union member states have set up national monitoring programs and sampling procedures following this directive.

The major veterinary drugs and substances with anabolic effects are listed in Table 30.1: Group A includes unauthorized substances having anabolic effects, while group B includes veterinary drugs, some of which have established maximum residue limits (MRL). Commission Decisions 93/256/EC²⁹ and 93/257/EC³⁰ give criteria for the analytical methodology regarding the screening, identification, and confirmation of these residues. Council Directive 96/23/EC²⁸ was implemented by the Commission Decision 2002/657/EC,³¹ which has been in force since September 1, 2004. This directive provides rules for the analytical methods to be used in testing of official samples and specific common criteria for the interpretation of analytical results of such samples by official control laboratories. The identification of a substance is based on a minimum number of identification points that are found, depending on the analytical technique used. For instance, when using mass spectrometric detection, substances in group A would require 4 identification points while those in group B would only require a minimum of 3. The relative retention of the analyte must correspond to that of the calibration solution at a tolerance of $\pm 0.5\%$ for GC and $\pm 2.5\%$ for LC. The guidelines given in this directive also imply new concepts such as the decision limit ($CC\alpha$), which is defined as the limit at and above which it can be concluded with an error probability of α that a sample is noncompliant. Also included is the detection capability ($CC\beta$), which is defined as the smallest content of the substance that may be detected, identified, and quantified in a sample with an error probability of β . Together these limits permit the daily

Table 30.1 Lists of Substances Having Anabolic Effects Belonging to Groups A and B According to Council Directive 96/23/EC²⁸**Group A: Substances Having Anabolic Effect**

Stilbenes
 Antithyroid agents
 Steroids
 Androgens
 Gestagens
 Estrogens
 Resorcylic acid lactones
 Beta-agonists
 Other compounds

Group B: Veterinary Drugs

Antibacterial substances
 Sulfonamides and quinolones
 Other veterinary drugs
 Antihelmintics
 Anticoccidials, including nitroimidazoles
 Carbamates and pyrethroids
 Sedatives
 Nonsteroidal antiinflammatory drugs
 Other pharmacologically active substances

control of the performance of a specific method when used with a specific instrument and under specific laboratory conditions, thus contributing to the determination of the level of confidence in the routine analytical result.

30.3 Sampling and Sample Preparation

The preparation of samples for analysis is very important for improving the sensitivity of the tests used.³² Typical procedures include cutting, blending, and homogenization of the meat product or the poultry tissue, followed by liquid extraction and solid-phase extraction for sample cleanup and concentration. Previously, the residues may have been bound or conjugated (i.e., as sulphates or glucuronides) and needed further cleavage by treatment with the juice of the snail *Helix pomatia*, which has sulfatase and β -glucuronidase enzymes able to release the free analytes.

30.4 Methods for Cleanup and Extraction of Veterinary Drugs and Their Residues

Extraction is mainly performed to remove interfering substances while retaining most of the analyte. Extraction solvents must be carefully chosen, depending on pH, polarity, and solubility in different solvents. For instance, polar extraction methods for the determination of anabolic steroids in beef are used because they avoid some cleanup problems following nonpolar extraction, but they are insufficient. It has been reported that polar extraction followed by nonpolar extraction gives better results.³³ Supercritical fluid extraction of meat with unmodified supercritical CO₂ has also been used for certain residues such as steroids.³⁴

Matrix solid-phase dispersion consists in the mechanical blending of the sample with a solid sorbent, which progressively retains the analyte by hydrophobic and hydrophilic interactions. The solid matrix is then packed into a column and eluted with an appropriate solvent.

Solid-phase extraction is extensively used for the isolation of the group or class of analytes. The type of extractant and cartridge depends on the target analyte.³⁵ Small cartridges are commercially available at reasonable prices; these have low affinity and specificity but high capacity. Furthermore, they can be performed in parallel, and thus they allow the simultaneous extraction of a good number of samples.

Immunochemistry is based on the antigen–antibody interaction, which is very specific for a particular residue. The columns are packaged with a specific antibody bound to the solid matrix, usually a gel. These chromatographic columns are highly specific and are only limited by potential interferences (i.e., substances that may cross-react with the antibody) that must be checked. These columns are rather expensive and can only be reused up to ten times. Due to the nature of the specific antibody when preparing the immunosorbent material, an in-depth assessment is necessary before considering their use in a routine analytical method.³⁶

There are several methods based on molecular recognition mechanisms for cleanup. Molecular imprinted polymers (MIPs) have shown promising results for the isolation of small amounts of residues found in meat. These are cross-linked polymers prepared in the presence of a template molecule that may be a β -agonist. When this template is removed, the polymer offers a binding site complementary to the template structure. MIPs have better stability than antibodies because they can support high temperatures, larger pH ranges, and a variety of organic solvents. The choice of appropriate molecule as template is the critical factor for a reliable analysis.³⁷ The extracted residues are then analyzed by LC-MS; they have shown good quantitative results for cimaterol, ractopamine, clenproperol, clenbuterol, brombuterol, mabuterol, mapenterol, and isoxsurine, but not for salbutamol or terbutaline.³⁸

30.5 Screening Methods

The wide variety of veterinary drugs and residues potentially present in a meat sample makes it necessary to use screening procedures for routine monitoring. Screening methods are used to detect the presence of a suspect analyte in the sample at the level of interest. If the searched residue has an MRL, then the screening method must be capable of detecting the residue below this limit. These controls are based on the screening of a large number of samples and thus must have a large throughput, low cost, and enough sensitivity to detect the analyte with a minimum of false negatives.³⁹ Compliant samples are accepted while suspected noncompliant samples must be further analyzed using confirmatory methods. According to the Commission Decision 2002/657/EC,³¹ the screening methods must be validated and must have a detection capability ($CC\beta$) with an error probability (β) lower than 5%.

30.5.1 Immunological Techniques

Immunological methods are based on the antigen–antibody interaction, which is very specific for a given residue. The most well-known and extensively used technique is the enzyme-linked immunosorbent assay (ELISA). Today, there are a wide variety of commercially available assay kits with measurement based on color development. ELISA kits are available either for a specific

residue (e.g., sulphametazine) or a group of related compounds (e.g., sulfonamides). ELISA kits have shown good performance in the analysis of antibiotic residues such as tylosin and tetracycline,^{40–43} chloramphenicol,⁴⁴ and sulfonamides,⁴⁵ as well as sedatives.⁴⁶ New enzyme immunoassays are continuously reported in the literature, including (1) an ELISA assay for the simultaneous detection of five banned antibiotics (bacitracin, tylosin, spiramycin, virginiamycin, and olaquinox) with a detection capability below 1.5 mg/kg,⁴⁷ (2) an ELISA kit for semicarbazide, the banned nitrofurantoin veterinary antibiotic nitrofurazone, with a detection capability (CC β) of 0.25 ng/g,⁴⁸ and (3) an ELISA kit for nitroimidazoles.⁴⁹ In this case, the detection capability (CC β) is below 2 ppb for dimetridazole, 10 ppb for metronidazole, 20 ppb for ronidazole and hydroxydimetridazole, and 40 ppb for ipronidazole. In some cases, the possibility of interferences by cross-reactions with other substances must be taken into account. Other immunologically based techniques consist of radioimmunoassay (RIA), which is based on the measurement of the radioactivity of the immunological complex; dipsticks based on simple membrane strips, with receptor ligands on the surface able to develop color that can be easily measured with color detectors;⁵⁰ and the use of luminiscence or fluorescence detectors to improve the sensitivity.⁵¹

30.5.2 Biosensors

The need to screen a large number of meat samples in a relatively short time has prompted the development of biosensors based on an immobilized antibody that interacts with the analyte in the sample, and optical or electronic detection of the resulting signal.^{52–53} Biosensors can simultaneously detect multiple veterinary drug residues in a sample⁵⁴ with no need for sample cleanup.⁵⁵ There are various types of biosensors, including (1) surface plasmon resonance (SPR), which measures variations in the refractive index of the solution close to the sensor⁵⁶ and has been successfully applied to the detection of various veterinary drug residues^{57–58} and (2) biosensors based on the use of biochip arrays that are specific for a certain number of residues.^{59–60}

30.5.3 Chromatographic Techniques

High-performance thin-layer chromatography (HPTLC) has been successfully used for multi-residue screening purposes in meat. Samples are injected onto the plates and the residues eluted through the plate with the appropriate eluent. Once eluted, residues can be viewed under ultraviolet (UV) or fluorescence, or visualized by spraying with a chromogenic reagent. HPTLC has been applied to meat to screen various residues including agonists,^{61–62} nitroimidazole,⁶³ sulfonamides,^{64–66} and thyrostatic drugs.^{67–68}

Gas chromatography (GC) and HPLC are powerful separation techniques able to separate the analyte from most interfering substances by varying the type of column and elution conditions.⁶⁹ In some cases, the analyte can be detected after appropriate derivatization.⁷⁰ In addition, these techniques can be used for multi-residue screening. The recent development of ultraperformance liquid chromatography systems and new types of columns with packagings of reduced size offer valuable improvements for residue detection, with a considerable reduction in elution times and the possibility of a larger number of samples per day.^{71–72} HPLC has been applied to meat for detection of a wide variety of veterinary drug residues,^{73–76} anabolic steroids,^{77–78} quinolone residues,⁷⁹ and corticosteroids.^{80–83}

Additional advantages of GC and HPLC are automation and the possibility of coupling the chromatograph to mass spectrometry (MS) detectors for further confirmatory analysis.

30.6 Confirmatory Analytical Methods

Confirmatory methods are preferentially based on MS because these provide direct information on the molecular structure of the suspect compound and thus an unambiguous identification and confirmation of the residue in the meat or poultry product. However, these methods are costly in terms of time, equipment, and chemicals. When the target analyte is clearly identified and quantified above the decision limit for a forbidden substance (i.e., substances of group A) or exceeding the MRL in the case of substances having an MRL, the sample is considered noncompliant (unfit for human consumption). A suitable internal standard must be added to the test portion at the beginning of the extraction procedure. If no suitable internal standard is available, the identification of the analyte can be performed by cochromatography.

GC with MS detection has been used for many years in residue analysis of foods of animal origin. However, derivatization (e.g., silyl or boronate derivatives) is required when analyzing some nonvolatile residues such as agonists, but derivatization constitutes a serious limitation that adds time and cost to the analysis.

In recent years, the rapid development of MS coupled with liquid chromatography has expanded its applications in this field, especially for nonvolatile or thermolabile compounds. Tandem mass spectrometry (MS-MS) has shown high selectivity and sensitivity, allowing the analysis of more complex matrices like meat with easier sample preparation procedures. LC-MS-MS allows the selection of a precursor m/z , which is performed first. This contributes to eliminating any uncertainty about the origin of the observed fragment ions, eliminating potential interferences from the meat sample or from the mobile phase, and reducing the chemical noise and increasing the sensitivity.⁸⁴

The interface technology has been developing rapidly. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces are the sources of choice depending on the polarity and molecular mass of analytes.⁸⁵ The ESI technique facilitates the analysis of small to relatively large and hydrophobic to hydrophylic molecules.^{70,86,87} An important limitation of LC-MS-MS quantitative analysis is its susceptibility to matrix effects, which are dependent on the ionization mode, type of sample, and sample preparation. APCI ionization has been reported to be less sensible than ESI to matrix effects and has been proposed as the choice for quantitative analysis.^{88–90} The use of liquid chromatography (ESI-LC/MS/MS) in the negative ion mode coupled to an ion trap analyzer has been reported to be effective for the identification and quantitation of chloramphenicol.^{17,91} Four nitrofurans (furazolidone, furaltadone, nitrofurantoin, and nitrofurazone) have also been successfully analyzed by the same technique with positive ESI.⁹² A list of recent reports on the analysis of growth promoters and veterinary drug residues in meat products and poultry is shown in Tables 30.2 and 30.3.

Quadrupole time-of-flight (Q-TOF) has been proposed as a useful instrument for the confirmation of the identity of residues. It has been reported to have high sensitivity, high resolution, and a high mass accuracy for both precursor and product ions.¹⁰⁷ Its main application would be for qualitative analysis, especially the detection and identification of unknowns in complex mixtures (i.e., illegal cocktails of anabolic steroids). This instrument would be complementary to other well-established techniques in residue analysis such as triple quadrupole or ion-trap.

The ion suppression phenomenon in LC-MS must be taken into account. This is due to matrix effect problems and the presence of interfering compounds that affect analyte detection. A wide-ranging review about ion suppression phenomenon and its consequences for residue analysis has been published recently.¹⁰⁸ The main mechanism for ion suppression involves the presence of matrix interfering compounds that appear to reduce evaporation efficiency. The consequences

Table 30.2 Examples of Recent Methods of Analysis of Growth Promoters in Liver and Poultry

Analyte	Matrix	Extraction	Column	Detector	C α (ng/g)*	C β (ng/g)	Recovery (%)	Reference
Clenbuterol	Liver	Enzymatic hydrolysis SPE C18	Synergi MAX-RP 80 A, 4 μ m	MS/MS ESI ⁺	0.08	0.27	—	93
Ractopamine	Liver	Enzymatic hydrolysis SPE C18	Synergi MAX-RP 80 A, 4 μ m	MS/MS ESI ⁺	0.15	0.32	—	93
Zilpaterol	Liver	Enzymatic hydrolysis SPE C18	Synergi MAX-RP 80 A, 4 μ m	MS/MS ESI ⁺	0.13	0.52	—	93
Trenbolone	Poultry meat	MSPD	Alltima C18, 5 μ m	MS/MS APCI ⁺	—	0.13	99	94
Testosterone	Poultry meat	MSPD	Alltima C18, 5 μ m	MS/MS APCI ⁺	0.03	0.21	97	94
Melengestrol acetate	Poultry meat	MSPD	Alltima C18, 5 μ m	MS/MS APCI ⁺	0.03	0.26	90	94
Progesterone	Poultry meat	MSPD	Alltima C18, 5 μ m	MS/MS APCI ⁺	0.21	0.16	96	94
α -Zeranol	Poultry meat	MSPD	Alltima C18, 5 μ m	MS/MS	0.08	0.87	90	94
α -Estradiol	Poultry meat	MSPD	Alltima C18, 5 μ m	TIS ⁻	0.11	0.85	100	94
Diethylstilbestrol	Poultry meat	MSPD	Alltima C18, 5 μ m	TIS ⁻	0.04	0.33	80	94
Hexestrol	Chicken meat	Liq. extraction SPE C18	DB-5, 30 m, 0.25 μ m	MS/MS TIS ⁻	LOD 0.2	—	104.3	95
Diethylnestilbestrol	Chicken meat	Liq. extraction SPE C18	DB-5, 30 m, 0.25 μ m	MS/MS TIS ⁻	LOD 0.1	—	85.7	95
Androsterone	Chicken meat	Liq. extraction SPE C18	DB-5, 30 m, 0.25 μ m	MS/MS TIS ⁻	LOD 0.2	—	87.3	95
Estradiol	Chicken meat	Liq. extraction SPE C18	DB-5, 30 m, 0.25 μ m	MS/MS TIS ⁻	LOD 0.1	—	95.2	95
Zeranol	Chicken meat	Liq. extraction SPE C18	DB-5, 30 m, 0.25 μ m	MS/MS TIS ⁻	LOD 0.1	—	90.2	95
α -Zearalenol	Chicken meat	Liq. extraction SPE C18	DB-5, 30 m, 0.25 μ m	MS/MS TIS ⁻	LOD 0.1	—	101.3	95
17 α -Hydroxyl- progesterone	Chicken meat	Liq. extraction SPE C18	DB-5, 30 m, 0.25 μ m	MS/MS TIS ⁻	LOD 0.4	—	100.7	95

* LOD given when C α is non-available.
 Note: APCI = Atmospheric pressure chemical ionization, ESI = Electrospray ionization, TIS = Turbo ion spray, MSPD = Matrix solid phase dispersion.

Table 30.3 Examples of Recent Methods of Analysis of Antibiotics, Coccidiostats and Anthelmintics in Different Matrices

Analyte	Matrix	Extraction	Column	Detector	C α (ng/g or ng/mL)	C β (ng/g or ng/mL)	Recovery (%)	Reference
Amoxicillin	Broiler muscle	KH ₂ PO ₄ extraction Ultrafiltration	PLRP-S column, 100 Å	MS/MS ESI ⁺	51.6	57.4	—	96
Amoxicillin	Broiler liver	SPE C18 KH ₂ PO ₄ extraction Ultrafiltration	PLRP-S column, 100 Å	MS/MS ESI ⁺	51.6	56.1	—	96
Amoxicillin	Broiler kidney	SPE C18 KH ₂ PO ₄ extraction Ultrafiltration	PLRP-S column, 100 Å	MS/MS ESI ⁺	51.9	57.2	—	96
Chloramphenicol	Kidney	SPE C18 Enzymatic hydrolysis	Nucleodur C18, 5 µm	MS/MS ESI ⁻	0.05	0.09	—	97
Chloramphenicol	Poultry muscle	SPE NH ₂ Liq. extraction	—	Surface Plasmon biosensor	0.005	0.02	98	98
Chloramphenicol	Poultry meat	Liq. extraction deproteinization	Synergi MAX RP, 4 µm	MS/MS ESI ⁻	0.15	0.22	80–100	91
Chloramphenicol	Poultry meat	Liq. extraction SPE C18	—	+CD-IMS	—	LOQ = 19.7	63–72	99
Furazolidon	Poultry meat	Liq. extraction SPE C18	—	+CD-IMS	—	LOQ = 12.5	43–52	99
Enrofloxacin	Poultry meat	Liq. extraction SPE C18	—	+CD-IMS	—	LOQ = 13	48–62	99
Minocycline	Bovine muscle	Different extraction protocols Liq. extraction SPE	Inertsil ODS, 3.5 µm	HPLC-DAD	46.53	53.57	10–104	100
Tetracycline	Bovine muscle	Different extraction protocols Liq. extraction SPE	Inertsil ODS, 3.5 µm	HPLC-DAD	27.98	31.83	15–112	100
Oxytetracycline	Bovine muscle	Different extraction protocols Liq. extraction SPE	Inertsil ODS, 3.5 µm	HPLC-DAD	29.18	30.97	15–90.8	100
Chlortetracycline	Bovine muscle	Different extraction protocols Liq. extraction SPE	Inertsil ODS, 3.5 µm	HPLC-DAD	45.95	51.69	10–115	100

(Continued)

Table 30.3 (Continued)

Analyte	Matrix	Extraction	Column	Detector	C α (ng/g or ng/mL)	C β (ng/g or ng/mL)	Recovery (%)	Reference
Doxycycline	Bovine muscle	Different extraction protocols Liq. extraction SPE	Inertsil ODS, 3.5 μ m	HPLC-DAD	42.97	47.26	10–106.7	100
Albendazole sulphoxide	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	1303	1556	50–73	101
Albendazole sulphone	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	1164	1343	20–27	101
Thiabendazole	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	116	132	55–96	101
Oxfendazole/ Fenbendazole	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	561	627	57–85	101
Hydroxy-mebendazole	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	481	544	60–103	101
Fenbendazole sulphone	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	587	670	55–87	101
Oxibendazole	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	242	281	61–120	101
Mebendazole	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	467	520	59–100	101
Flubendazole	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	493	558	53–85	101
Albendazole	Benzimidazoles (anthelmintics) in liver	Liq. extraction SPE C18	XTerra C18, 3.5 μ m	HPLC-DAD	1216	1397	36–63	101
Sulfadiazine	Muscle	Liq. extraction SPE cation exchange	C 8 5 μ m	HPLC-DAD	109.3	120	73	18
Sulfathiazole	Muscle	Liq. extraction SPE cation exchange	C 8 5 μ m	HPLC-DAD	116.2	134.9	55	18

Sulfapyridine	Muscle	Liq. extraction SPE cation exchange	C 8 5 µm	HPLC- DAD	110.2	121.7	80	18
Sulfamerazine	Muscle	Liq. extraction SPE cation exchange	C 8 5 µm	HPLC- DAD	105.2	110.9	79	18
Sulfamethazine	Muscle	Liq. extraction SPE cation exchange	C 8 5 µm	HPLC- DAD	106.6	113.9	80	18
Sulfamonomethoxine	Muscle	Liq. extraction SPE cation exchange	C 8 5 µm	HPLC- DAD	106.6	114.1	81	18
Sulfachlorpyridazine	Muscle	Liq. extraction SPE cation exchange	C 8 5 µm	HPLC- DAD	108.8	119.1	72	18
Sulfamethoxazole	Muscle	Liq. extraction SPE cation exchange	C 8 5 µm	HPLC- DAD	107.1	115.0	76	18
Sulfaquinolaline	Muscle	Liq. extraction SPE cation exchange	C 8 5 µm	HPLC- DAD	107.8	116.4	77	18
Sulfadimethoxine	Muscle	Liq. extraction SPE cation exchange	C 8 5 µm	HPLC- DAD	107.0	114.4	75	18
Halofuginone	Poultry liver	Extraction enzymatic (trypsin) hydrolysis SPE-Oasis	Prodigy C18, 5 µm	MS/MS	35.4	43.6	—	102
Halofuginone	Chicken liver	Trypsin hydrolysis Liq. extraction SPE C18	Lichrosorb RP18 5 µm	HPLC-UV	LOD: 50	—	78.8–82.2	103
Nitrofurantoin	Nitrofuran metabolites in chicken meat	Acid catalyzed reaction Liq–liq extraction SPE	Symmetry C18, 3.5 µm	MS/MS ESI ⁺	0.21	0.36	85–122	92
Furaltadone	Nitrofuran metabolites in chicken meat	Acid catalyzed reaction Liq–liq extraction SPE	Symmetry C18, 3.5 µm	MS/MS ESI ⁺	0.12	0.21	85–122	92
Furazolidone	Nitrofuran metabolites in chicken meat	Acid catalyzed reaction Liq–liq extraction SPE	Symmetry C18, 3.5 µm	MS/MS ESI ⁺	0.11	0.19	85–122	92
Nitrofurazone	Nitrofuran metabolites in chicken meat	Acid catalyzed reaction Liq–liq extraction SPE	Symmetry C18, 3.5 µm	MS/MS ESI ⁺	0.20	0.34	85–122	92
Spiramycin	Chicken meat	Extraction cation exchange SPE	Hypurity Elite C18 5 µm	MS/MS ESI ⁺	LOD: 23	—	83	104

(Continued)

Table 30.3 (Continued)

Analyte	Matrix	Extraction	Column	Detector	C α (ng/g or ng/mL)	C β (ng/g or ng/mL)	Recovery (%)	Reference
Tylosin tartrate	Chicken meat	Extraction cation exchange SPE	Hypurity Elite C18 5 μ m	MS/MS ESI ⁺	LOD: 8	—	93	104
Oleandomycin phosphate	Chicken meat	Extraction cation exchange SPE	Hypurity Elite C18 5 μ m	MS/MS ESI ⁺	LOD: 6	—	57	104
Erythromycin	Chicken meat	Extraction cation exchange SPE	Hypurity Elite C18 5 μ m	MS/MS ESI ⁺	LOD: 3	—	65	104
Tilmicosin	Chicken meat	Extraction cation exchange SPE	Hypurity Elite C18 5 μ m	MS/MS ESI ⁺	LOD: 1	—	56	104
Kitasamycin	Chicken meat	Extraction cation exchange SPE	Hypurity Elite C18 5 μ m	MS/MS ESI ⁺	LOD: 4	—	70	104
Josamycin	Chicken meat	Extraction cation exchange SPE	Hypurity Elite C18 5 μ m	MS/MS ESI ⁺	LOD: 1	—	67	104
Diclozauril	Poultry meat	Liq. extraction	Simmetry C18 5 μ m	MS/MS ESI ⁻	0.5	0.6	111	105
Nifursol	Turkey meat	Extraction Derivatization Clean up	Symmetry C8 3.5 μ m	MS/MS ESI ⁺	0.47	0.65	—	106
Furazolidone	Turkey meat	Extraction Derivatization Clean up	Symmetry C8 3.5 μ m	MS/MS ESI ⁺	0.14	0.68	—	106
Furaladone	Turkey meat	Extraction Derivatization Clean up	Symmetry C8 3.5 μ m	MS/MS ESI ⁺	0.01	0.02	—	106
Nitrofurantoin	Turkey meat	Extraction Derivatization Clean up	Symmetry C8 3.5 μ m	MS/MS ESI ⁺	0.01	0.10	—	106
Nitrofurazone	Turkey meat	Extraction Derivatization Clean up	Symmetry C8 3.5 μ m	MS/MS ESI ⁺	0.02	0.03	—	106

Note: APCI = Atmospheric pressure chemical ionization, ESI = Electrospray ionization, TIS = Turbo ion spray, CD-IMS = corona discharge ion mobility spectrometry.

are reduced detection capability and repeatability. Ion ratios, linearity, and quantification are also affected. It could even lead to failure to detect an analyte, underestimation of its concentration, or nonfulfillment of the identification criteria.¹⁰⁸ Prevention of this phenomenon involves an improved purification and cleanup of the sample as well as the use of an appropriate internal standard. Another strategy involves modifying the elution conditions so that analytes elute in an area unaffected by ion suppression.¹⁰⁸

According to the Commission Decision 2002/657/EC,³¹ a system of identification points is used for confirmatory purposes, with a minimum of 4 points required for the substances of group A and a minimum of 3 for group B substances. Thus, 1 identification point can be earned for the precursor ion with a triple quadrupole spectrometer and 1.5 points for each product ion. A high-resolution mass spectrometer acquires 2 identification points for the precursor ion and 2.5 for each product ion. Variable window ranges for MS peak abundances are also established in the new decision.³¹ Thus, the relative intensities for the 4 ions must be >50, >20 to 50%, >10 to 20%, and ≤10%. In the case of electronic impact, the maximum permitted tolerances are ±10, ±15, ±20, and ±50%, respectively, while in the case of chemical ionization, GC-MSⁿ, LC-MS, and LC-MS,ⁿ tolerances are ±20, ±25, ±30, and ±50%, respectively.

Other methods are allowed for group B substances.³¹ Thus, liquid chromatography–full scan diode array (LC-DAD) can be used as a confirmatory method if specific requirements for absorption in UV spectrometry are met. The absorption maxima of the spectrum of the analyte must be at the same wavelengths as the calibration standard within a margin of ±2 nm for diode array detection. Furthermore, the spectrum of the analyte above 220 nm must not be visibly different (at no point greater than 10%) from the spectrum of the calibration standard.

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Chapter 31

Biogenic Amines

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31.1 Introduction

31.1.1 Biogenic Amines: Origin and Classification

Biologically active amines, also known as biogenic amines, are nitrogenous compounds of basic nature that show biological activity. They are synthesized and degraded by animal, plant, and microbial metabolisms, and consequently are found in a wide variety of food products [1–3]. On the basis of their chemical structure, the biogenic amines most commonly found in food are grouped as

- Aromatic monoamines—tyramine and phenylethylamine
- Heterocyclic amines—histamine and tryptamine
- Aliphatic diamines—cadaverine and putrescine
- Aliphatic polyamines—agmatine, spermidine, and spermine

Classically, biogenic amines are defined as “biogenic” or “endogenous/natural,” depending on their synthesis. However, sometimes there is no clear division between these two categories [4]. The former result from the activity of decarboxylase enzymes against precursor amino acids. Within this group, tyramine, phenylethylamine, histamine, tryptamine, cadaverine, putrescine, and agmatine originate from the decarboxylation of tyrosine, phenylalanine, histidine, tryptophan, lysine, ornithine, and arginine, respectively. The decarboxylase enzymes responsible for the synthesis of these biogenic amines in food are mainly of bacterial origin and usually inducible by certain environmental conditions (e.g., unfavorable acidic pH). Although bacterial decarboxylases are generally specific for one amino acid, in some cases they may have activity, although with a lower affinity, against other amino acids of a similar chemical structure, such as tyrosine and phenylethylamine [5] or ornithine and lysine [4].

The so-called endogenous or natural amines are formed as a result of the intracellular metabolic processes of animals, plants, and microorganisms. The aliphatic polyamines spermine and spermidine are the most relevant amines within this category, the synthesis of which follows other reactions apart from the decarboxylation of arginine during the early stages of the biosynthetic pathway. Small amounts of putrescine, as a precursor of polyamines, can also be considered of endogenous origin [4]. In addition to putrescine, several other biogenic amines, such as cadaverine and agmatine, may occur in certain foods both endogenously and as microbial metabolic products. When these biogenic amines are present in low concentrations, it is difficult to differentiate their true origin, and it is difficult to know the significance of their occurrence in food products.

31.1.2 Relevance of Biogenic Amines in Food

Interest in biogenic amines is related to both food safety and food quality issues. Traditionally, these compounds have been regarded as undesirable toxic components of food. Tyramine, histamine, and to lesser extent phenylethylamine, are the main dietary biogenic amines associated with several acute adverse reactions in consumers. Interaction with monoamine-oxidase-inhibitor (MAOI) drugs, histaminic intoxication, food intolerance related to enteral histaminosis, and food-induced migraines may occur following the ingestion of biologically active amines [4,6–9]. These compounds trigger vasoactive and psychoactive reactions. The vasoconstrictive properties of tyramine and phenylethylamine have been reported to be directly responsible

for increases in blood pressure, and may also cause headaches, sweating, vomiting, and pupil dilatation, among other effects. Histamine causes vasodilatation and subsequent hypotension as well as other dermal (flushing and pruritus), gastrointestinal (diarrhea, cramps, vomiting), and neurological (headache, dizziness) effects [7,8,10]. The severity of the disorders associated with biogenic amines varies depending on individual sensitivity, but, in general, reactions are mild and medical attention is rarely required [11]. It is precisely the mild nature of the symptoms together with misdiagnosis and the lack of a mandatory or adequate system for reporting these food diseases that explain the poor statistics on the incidence of intoxications caused by dietary amines.

In spite of compelling evidence that biogenic amines are the causative agents of adverse food reactions, the toxic dose is difficult to estimate. Not only do the concentrations of biogenic amines vary greatly among food products, but the amounts ingested also vary greatly among consumers, who in turn show a wide range of inter- and intraindividual clinical responses to a given amount of these dietary compounds [7,8,12]. Moreover, there are numerous potentiating factors of dietetic-, physiological-, and pharmacological nature that contribute to the variability of the response to biogenic amines in food. The toxicity of tyramine may be of special concern for individuals taking MAOI drugs, which may increase the vasoconstrictive effects of this dietary amine. Nevertheless, according to literature, amounts from 50 to 150 mg of tyramine are well tolerated by patients under a new generation MAOI treatment [13–15]. According to a review by Shalaby [8], ingestion of 8–40 mg of histamine causes slight toxicity, over 40 mg moderate toxicity, and over 100 mg severe poisoning. Although these doses are repetitively cited in the literature, no toxicological studies supporting them are available. In fact, histamine food poisoning incidents are related to fish containing high concentrations (usually above 600 mg/kg) of this biogenic amine [12,16]. Therefore, if an average fish portion weighs 200–300 g, the toxicological effects of histamine would appear after ingestion of more than 120–180 mg of this biogenic amine. However, histamine intolerance by sensitive individuals has been described after the intake of variable amounts of this biogenic amine, ranging from 50 µg accompanied by wine to 75 mg of pure histamine [17].

The diamines putrescine and cadaverine, although not considered toxic individually, may enhance the absorption of vasoactive amines as a result of the saturation of intestinal barriers through competition for mucin attachment sites, and detoxification enzymes [6,18].

Biologically active amines present in food products can also act as precursors of nitroso compounds with potential carcinogenic activity, thereby constituting an indirect additional risk. Nitrosamines result from the action of nitrite on secondary amines, which in turn may be formed from primary amines (such as the aliphatic diamines and polyamines) by a cyclization reaction under certain circumstances [1,8,19]. Some aromatic amines, such as tyramine, have also been proposed as possible precursors of diazotyramine, which shows mutagenic activity [20]. The occurrence of nitrosating agents (i.e., nitrites and nitrates), mild acidic pH, and high temperatures during food manufacture favor nitrosamine formation. Cured and cooked or smoked meat products (such as cooked and fried bacon) are sources of nitrosamines.

Given the potential effects of biogenic amines on health and their microbial origin, the occurrence of these substances in food is relevant from the technological and food quality standpoints. Indeed, the accumulation of biogenic amines can be associated with fermentation processes but also with spoilage. In this regard, dietary biogenic amines are of particular interest, because they can be used as chemical indicators or monitors of the hygienic quality of raw materials and manufacturing conditions.

31.2 Biogenic Amines in Meat and Meat Products

In general, all protein-rich food subjected to conditions that allow bacterial development and activity (e.g., storage, maturation, fermentation) is expected to accumulate certain amounts of biogenic amines, in addition to those present naturally. Meat and meat products contain moderate or high amounts of these compounds. Apart from spermine and spermidine, the main origin of notable amounts of biologically active amines in food in general, and in meat products in particular, is widely attributed to the action of bacterial decarboxylase enzymes [1,2]. However, there is no common origin for all biogenic amines, and the final type and content will depend on the conditions of manipulation, treatment, and storage as well as microorganism activity.

31.2.1 *Aminogenic Microorganisms Associated with Meat and Meat Products*

Several bacterial groups associated with meat and meat products can generate biogenic amines. The capacity to decarboxylate certain amino acids has generally been attributed to specific bacterial families or genera. For instance, enterobacteria are frequently histamine and diamine (cadaverine and putrescine) producers, and although fewer studies have addressed *Pseudomonas*, they have also been reported as notably aminogenic [2,21,22]. Among Gram-positive bacteria, lactic acid bacteria, especially enterococci and certain lactobacilli such as *Lactobacillus curvatus*, are usually associated with tyramine production. In contrast, staphylococci are much less frequently reported as powerful aminogenic organisms [21,23–25]. Despite these general rules of thumb, the capacity to produce one or more biogenic amines simultaneously is strain-dependent [21], thus explaining why the biogenic amine content of a given product cannot always be statistically correlated with the global counts of specific bacterial groups in the same product.

31.2.2 *Occurrence of Biogenic Amines in Meat and Meat Products*

31.2.2.1 *Fresh Meat and Fresh Meat Products*

In freshly slaughtered meat, spermine, and spermidine are the main biogenic amines [26]. Apart from small amounts of putrescine, the other amines are usually undetectable and appear only under conditions that allow bacterial activity. The contents of spermine and spermidine may vary widely in meat. In contrast to vegetable products, meat and products of animal origin contain higher amounts of spermine than spermidine, with a ratio of approximately 10:1 [27]. Concentrations of 15–50 mg/kg of spermine and 1–5 mg/kg of spermidine are commonly reported [2,27–31]. The animal species does appear to be a determinant of this variability, because the differences between pork, beef, and poultry products, for example, are not as wide as between organs or parts of the same animal or another animal of the same species. One of the factors influencing the cellular levels of polyamines is the metabolic activity of the tissue. The synthesis *de novo* and the accumulation of polyamines are particularly stimulated in tissues and organs that show rapid growth or in phases with a considerable cellular regeneration rate [32]. This observation could explain, at least in part, the range of polyamine concentrations detected in meat from distinct animals or even from different parts of the same animal.

31.2.2.2 Cooked Meat Products

In heat-treated meat products (cooked ham, cooked meat sausages, etc.), spermine and spermidine are the only biogenic amines usually detected. The levels of these polyamines in these products are in general slightly lower than in fresh meat. This fact is attributed to a dilution effect produced when lean meat is mixed with fat and other ingredients included in the product formula [30]. Although polyamines are considered heat-resistant, a small reduction of these compounds has also been reported during thermal treatments of products [33–35].

The contents of other biogenic amines in cooked products are much more variable than those of polyamines. In general, concentrations of tyramine, histamine, and diamines are quite low, with some punctual exceptions. Rarely are phenylethylamine and tryptamine detected. In some particular cooked meat products, a short maturation/fermentation step is applied before cooking, for instance for bologna sausage, Catalan sausage (*butifarra*). In this case, the activity of aminogenic organisms can be notable, and may result in a significant accumulation of biogenic amines [36].

31.2.2.3 Cured Meat Products

The manufacture of cured products involves large pieces or whole muscle parts without mincing or mixing. Common salt is an essential ingredient not only for product safety but also for the development of the organoleptic characteristics during ripening at relatively low temperatures [37]. Although the pH does not drop, under these conditions microbial growth is strongly limited and only halophile bacteria, such as Gram-positive catalase-positive cocci (staphylococci, micrococci, and *kocuria*) grow, with counts ranging from 10 to 10⁶ colony-forming units (cfu)/g. Yeast and some lactic acid bacteria may also develop to a lesser extent. Consequently, the contents of biogenic amines, such as tyramine, histamine, cadaverine, and putrescine, in this type of product are quite low (with median values from 2 to 80 mg/kg), with only particular exceptions [30,36,38–40]. The occurrence of significant amounts of phenylethylamine and tryptamine has not been described in cured meat products.

The length of ripening is a critical factor that determines the extent of biogenic amine accumulation, especially tyramine [40]. In contrast, a considerable formation of diamines, especially cadaverine, during dry-cured ham manufacture has been reported to depend on the type of ripening [41]. Short (rapid) ripening allows greater accumulation of biogenic amines in comparison to a long (slow) ripening process. These findings are attributed to the higher temperatures applied during drying in the former. The proteolytic phenomena occurring during ripening increase the concentration of precursor amino acids and correlate with biogenic amine formation [40,42].

31.2.2.4 Fermented Meat Products

Fermented sausages and cheese are foods that register the highest biogenic amine contents. However, fewer studies have addressed the former. According to the literature [30,36,43–47], biogenic amine levels vary greatly between fermented sausages of diverse types, between manufacturers, and also between samples from distinct batches of the same kind of product and from the same producer. In retail fermented sausages, tyramine is usually the most frequent and most abundant biogenic amine. The literature describes an average tyramine content of 140 mg/kg

(relative standard deviation [RSD] of 89%) in these meat products. The diamines, putrescine and cadaverine, are also quite common, though with a higher variability (RSD of 145% for putrescine and 187% for cadaverine). Most samples of fermented sausages show relatively low amounts of diamines; however, some may accumulate large amounts, which may exceed the tyramine content. As a consequence of this variability, the mean values of 89 mg/kg for putrescine and 44 mg/kg for cadaverine are much higher than the corresponding median values (36 and 8 mg/kg, respectively). This variability is even more pronounced for histamine (median value of 4 mg/kg and RSD of 222%), which is not detected in most retail fermented sausages, but in some particular samples may reach quite high levels, usually accompanied by high amounts of other biogenic amines. Similarly, the contents of phenylethylamine (RSD of 206%) and tryptamine (RSD of 170%) are relatively low (median of 2 and 4 mg/kg, respectively) in these meat products. These two amines could be considered minor amines in fermented sausages and their accumulation appears to depend on the occurrence of high concentrations of tyramine.

Fermented sausages are significant sources of physiological polyamines, although these amines have received less attention [3,31]. Polyamines are found naturally in raw meat, and therefore their levels are much less variable than those of biogenic amines of microbial origin. According to data in the literature, the average content of spermine in fermented meat products is 23 mg/kg, and that of spermidine is 7 mg/kg. Occasionally a decrease in polyamine content during meat fermentation has been reported [48–50], which is attributed to uptake by microorganisms as a nitrogenous source [4] or to deamination reactions [2].

31.2.3 Biogenic Amine Index

As a result of their microbiological origin, biogenic amines have been used as criteria to evaluate the hygienic quality and freshness of certain foods, especially fish, but also meat and a number of meat products.

31.2.3.1 Biogenic Amines to Evaluate the Loss of Meat Freshness

Biogenic amines in fresh meat and fresh meat products (such as hamburgers, raw sausages, and packaged fresh meat) are usually below the detection limit, except for the physiological polyamines spermine and spermidine. When monitoring aminogenesis during the storage of meat under aerobic conditions, several biogenic amines, such as cadaverine, putrescine, tyramine, and histamine, progressively increase to variable extents. The higher the storage temperature, the faster the accumulation of these compounds [36,51]. Significant accumulation of biogenic amines generally occurs before the appearance of sensorial signs of spoilage, when counts of aerobic mesophile bacteria reach 10^5 – 10^7 cfu/g [52,53]. In contrast, polyamines usually remain constant or may even decrease [53–55]. These observations have been attributed to consumption by microorganisms [4].

Therefore, biogenic amines individually or in combination have been proposed as objective chemical indexes to evaluate meat freshness. The biogenic amine index (BAI) put forward by Mietz and Karmas [56] (cadaverine + putrescine + histamine/1 + spermine + spermidine) to evaluate fish freshness was applied by Sayem El Daher et al. [34] to assess the hygienic quality of beef. A highly significant correlation between BAI values and microbial counts was detected in this meat. Maijala et al. [28] also used this index to compare the effect of pH on aminogenesis during meat spoilage. Several authors defend the use of only one biogenic amine for evaluation purposes, for instance putrescine [51,57], cadaverine [58–60], or both [52,61] for aerobically stored meat,

mainly pork or beef. However, although the use of one biogenic amine for evaluation is more straightforward, the application of a multiple amine index may increase specificity and selectivity. Tyramine increases considerably during meat storage; therefore, this biogenic amine should also be included, together with cadaverine, putrescine, and histamine, in a BAI. This is the case of the BAI of tyramine + cadaverine + putrescine + histamine, proposed by Wortberg and Woller [27] and Hernández-Jover et al. [53]. Wortberg and Woller [27] established a spoilage limit at 500 mg/kg, but, according to other findings on pork and beef meat, Hernández-Jover et al. [53] reported that spoilage is evident at 10-fold lower values.

In particular for poultry meat, cadaverine concentrations have been proposed for the monitoring of chicken meat spoilage by Vinci and Antonelli [60], whereas Patsias et al. [62] suggested tyramine and putrescine limits for precooked chicken meat. Other authors consider the sum of tyramine, cadaverine, and putrescine to be the most promising indicator for both storage time and temperature, as well as for the microbiological quality of modified atmosphere and aerobically packaged chicken meat [63,64].

In vacuum-packaged meat, bacterial flora varies with the environment in the package, and thus the pattern of biogenic amine formation in meat packed in this way differs from that packaged aerobically. Lactic acid bacteria become dominant in the microflora of vacuum-packaged meats from early storage. As a result, tyramine may be a better indicator of spoilage/acceptability of vacuum-packaged meat stored at chilled temperatures [65,66].

31.2.3.2 *Biogenic Amines to Monitor the Hygienic Quality of Raw Materials in Meat Products*

The heat treatments commonly applied by the meat industry inactivate microorganisms but do not reduce the contents of biogenic amines, because these compounds are thermoresistant. Moreover, cooking does not favor aminogenesis. Consequently, cooked meat products should contain only the physiological amines spermine and spermidine. The occurrence of other biogenic amines in these products would indicate the decarboxylation of amino acids by undesirable contaminant microorganisms before, during, or even after manufacture of the product. Although meat products made of blood or liver may contain certain amounts of histamine of endogenous origin, the concentrations from this source are much lower than those formed by bacterial activity.

Therefore, because biogenic amines are thermoresistant, BAIs have been considered useful to evaluate the quality of the raw material used and the hygienic conditions prevalent during the manufacturing processes, and contribute valuable information relevant to quality control processes [67]. Indeed, most retail samples of cooked meat products contain low levels of biogenic amines (optimally <5 mg/kg). Only occasionally do some show considerable amounts of tyramine, cadaverine, putrescine, and histamine, which allow producers to monitor the hygienic quality of raw materials used during manufacturing.

Cured meat products, such as cured ham or cured loin, are subjected to the action of brine and maturation, and their manufacture does not include a fermentation step or a cooking process. In this case, the halophilic microorganisms surviving high salt concentrations are not usually related to notable decarboxylase activity [21]. In general, no significant formation or degradation of biogenic amines is observed in cured meat products when these are manufactured following proper hygienic practices. Therefore, BAIs could also be applied to evaluate the hygienic quality of raw meat materials as well as conditions during maturation.

On the whole, the application of BAIs as criteria for quality evaluation of fermented meat products is more difficult because the formation of biogenic amines cannot be directly and exclusively associated with the quality of raw materials [36,55]. A number of microorganisms that produce biogenic amines, especially tyramine, have been reported in these meat products (such as salami, *salchichón*, and other dry sausages). It has been demonstrated that fermented sausages practically free from tyramine and other biogenic amines can be produced, for instance, through scrupulously hygienic conditions and the inoculation of selected starter cultures [46,68–69]. Abundant data are available on biogenic amine contents in retail fermented sausages as well as on biogenic amine accumulation during the manufacture of this type of product using raw materials with optimal hygienic quality [30,36,43,47]. The consequences of using raw materials of poor hygienic quality [70] and also the contribution of contaminant enterobacteria and lactic acid bacteria [71] to overall aminogenesis during sausage fermentation have been reported. On the basis of the results from these two studies, it could be inferred that meat fermentation leads to the accumulation of certain amounts of biogenic amines. In particular, tyramine is the most important amine associated with fermented sausages, registering average concentrations from 100 to 200 mg/kg. Putrescine and cadaverine can also be accumulated at concentrations below 50 mg/kg, but histamine is rarely found in fermented sausages manufactured under proper hygienic and manufacturing conditions. Therefore, biogenic amine accumulation above the levels described earlier could be considered the result of poor hygienic practices, and therefore biogenic amines could also be used to monitor the hygienic quality of fermented meat products.

31.3 Determination of Biogenic Amines in Meat and Meat Products

Several procedures have been developed and improved for the detection and determination of various biogenic amines in meat and meat products. From an analytical perspective, the measurement of biogenic amines and polyamines in food in general, and in meat and meat products in particular, is not a simple procedure, mainly because of (a) the diverse chemical structures of biogenic amines (aromatic, heterocyclic, and aliphatic); (b) the wide range of concentrations at which each biogenic amine can be present in the product; and (c) the complexity of the sample matrix (high protein content and often high fat content).

Analytical study of biogenic amines in meat products involves two well-differentiated phases: (1) extraction of amines from the solid food matrix, in some cases including a further purification or cleanup of the raw extract; and (2) the analytical determination of these amines, which can be carried out by means of a variety of approaches including enzymatic, spectrofluorometric, and chromatographic procedures.

31.3.1 *Biogenic Amine Extraction and Cleanup*

In solid samples, biogenic amines are extracted to a liquid phase and separated from potentially interfering compounds. This separation step is crucial for the accuracy of the methodology, because it is probably the most decisive factor for the analytical recovery of each amine. Although some authors have extracted amines from solid matrixes with water at room or higher temperatures, the most common extracting solvents used for this purpose include acid solutions, such as hydrochloric acid (e.g., 0.1 M), trichloroacetic acid (e.g., 5–10%), and perchloric acid

(e.g., 0.4–0.6 M), as well as organic solvents, such as methanol, acetone, acetonitrile–perchloric acid, or dichloromethane–perchloric acid [80].

The selectivity and recovery of the extraction is influenced by the type of acid used. Although several studies have compared the extraction capacity of distinct acids on amines, the results obtained are not always concordant or conclusive. In the case of meat products, because of sample turbidity and the occurrence of interfering substances, hydrochloric acid is not a suitable choice [72]. However, perchloric acid [29,73–75] or trichloroacetic acid, which show a high capacity to precipitate proteins, are recommended [80,76].

A cleanup of the extract before analysis of biogenic amines is required, depending on the final analytical technique applied. A number of approaches have been proposed to purify raw extracts, including column chromatography with alumina or ion-exchange resins [61,77] and solid-phase extraction [78,79]. Liquid–liquid extraction with organic solvents is also applied [78,72]. In this procedure, the raw extract is saturated with a salt, adjusted to an alkaline pH, and partitioned with an organic solvent (butanol, butanol/chloroform) that can selectively extract free amines and leave free amino acids in the aqueous layer. Because the pH optimum for extraction varies between amines, a strict control of this parameter is required to ensure satisfactory recovery and reproducibility. A pH of 11.5 is considered a suitable compromise for all biogenic amines [80]. A cleanup step increases the time required for the analysis and introduces a factor of uncertainty and variability as a result of sample handling. The use of an internal standard may help to address this limitation. However, several procedures for the extraction of biogenic amines from meat and meat products that do not include this step have been reported [29,60,81].

31.3.2 Analytical Procedures to Detect and Quantify Biogenic Amines

31.3.2.1 Chromatographic Quantification Procedures

The analytical methodologies to determine biogenic amines in meat and meat products are usually based on a chromatographic separation coupled with distinct detection techniques. Chromatographic procedures are the most extensively used methods because they provide high resolution, sensitivity, and versatility, and sample treatment is simple. Thin-layer chromatography [82,83], gas chromatography [84], and micellar liquid chromatography [85] have been applied for the analysis of biogenic amines in meat products. However, high-performance liquid chromatography (HPLC) with ion-exchange columns [39,86,87] or reverse-phase columns using ion pairs to separate biogenic amines as neutral [29,75] or nonneutral [76,81,88,89] compounds are the most frequently reported methods in the literature. Recent studies have addressed capillary (zone) electrophoresis [90,91].

Most biogenic amines, especially those of an aliphatic nature, have low absorption coefficients or quantum yields and require derivatization when the methods involve ultraviolet (UV)-visible (Vis) absorption or fluorescence detection. Chemical derivatization of these compounds can be carried out with a variety of reagents. The most often used are 5-dimethylamino-1-naphthalene-sulfonyl chloride (dansyl chloride [DnCl]), which forms stable compounds after reaction with both primary and secondary amino groups, and *o*-phthaldialdehyde (OPA), which reacts rapidly (i.e., 30 sec) with primary amines in the presence of a reducing agent such as 2-mercaptoethanol (ME) or *N*-acetylcyteine [92]. Figure 31.1 shows the representative derivatization reactions for biogenic amines with these reagents. Other alternatives for the formation of detectable amine

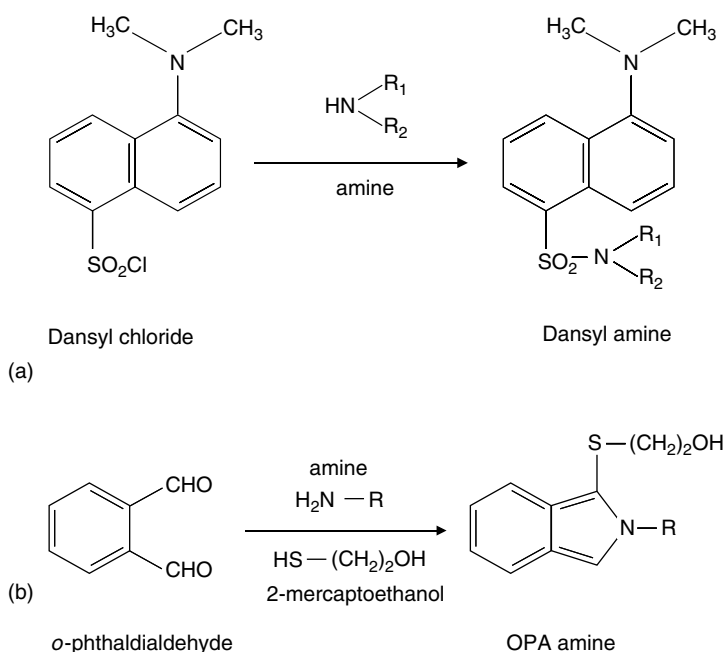


Figure 31.1 Representative derivatization reactions of biogenic amines with dansyl chloride (a) and *o*-phthalaldehyde (b).

derivatives include fluorescamine, fluorescein isothiocyanate (FITC), phenylisothiocyanate (PITC), 6-aminoquinoyl-*N*-hydroxysuccinimidyl-carbamate (ACCCQ), 2-naphthylloxycarbonyl chloride (NOC-Cl), benzoyl chloride, and ninhydrin [93,94].

Amine derivatives can be formed before (precolumn), during (on-column), or after (postcolumn) the chromatographic separation. Prederivatization comprises a series of time-consuming manual steps and may introduce imprecision to the overall analytical procedure. The use of an internal standard is critical to guarantee precision and accuracy (e.g., 1,7-diaminoheptane, 1,8-diaminooctane, or benzylamine have been described for the DnCl precolumn methodologies). Postcolumn derivatization has the advantage that it is automatically performed online, thereby avoiding sample manipulation and shortening the time required for the analysis. Moreover, changing the pH (to alkaline as required for derivatization reaction) is simple, easy, and quick with a postcolumn system. Nevertheless, it adds complexity to the instrumentation, because an extra pump is required. However, although postcolumn reactions have been criticized because of the occurrence of peak widening, this problem can be easily addressed using capillary connections and tubes.

Measurements of biogenic amines in meat and meat products have been taken by means of several techniques, such as fluorimetry [29,75,89], UV absorption [76,81], diode array-UV multi-channel [75,78], and mass spectrometry [39]. Most of these methods are related to pre-, post- or on-column derivatization.

Conductometry, as applied by Kvasnicka and Voldrich [95], does not involve a derivatization step, but uses chemical suppression of the eluent conductivity, which also leads to a loss of some analytes. This technique also detects common alkaline and alkaline-earth cations found in food matrices [87]. Pulsed amperometric detection, with dedicated wave-form [87] for complex

matrixes such as meat and meat products, is less affected by the already mentioned drawbacks, although electrode damage effects may arise.

Of the extraction alternatives for biogenic amines described, the most used for meat and meat products, as deduced from the literature, involve acid extraction of these compounds, followed either by (a) DnCl precolumn derivatization, reverse-phase HPLC separation coupled with UV detection (Figure 31.2) [80,81], or by (b) ion-pair reverse-phase HPLC with OPA-ME (post- or precolumn) derivatization coupled with fluorescence detection (Figure 31.3) [29,75]. The main conditions of these techniques are summarized in Table 31.1. The use of OPA instead of DnCl or fluorescamine is advantageous because of its greater selectivity for primary amines and the increase in method sensitivity as a result of fluorometric rather than spectrophotometric detection [76,96]. Moreover, DnCl and fluorescamine reactions result in several interfering by-products [80,93] that must be removed before chromatographic analysis to prevent coelution with biogenic amines. The addition of ammonia or proline [97] has been proposed for this purpose. The stability of OPA-amine derivatives is low, and postcolumn derivatization or an automated precolumn derivatization immediately before HPLC analysis is recommended. It has been reported that the natural polyamines spermidine and spermine can be analyzed only by means of DnCl derivatization, but not with OPA because the latter reacts only with primary amines [76]. However, several authors have described OPA-based methodologies that allow accurate measurement of these two polyamines (Table 31.1) [29,75,98]. In fact, spermidine and spermine bear primary amino groups and thus react with OPA-ME reagent as other biogenic amines do.

Few studies have compared the performance and the concordance between analytical methods for biogenic amines in meat and meat products. In an examination of Czech dry fermented sausages, HPLC procedures after precolumn derivatization of DnCl and OPA gave similar results in terms of detection limit, repeatability, recovery, and accuracy [76]. However, these authors reported that OPA derivatization was faster and much simpler in terms of sample pretreatment, which can be fully automated by the autosampler. In another study [25], the application of modifications of DnCl-based methodologies by three laboratories significantly affected the results obtained on biogenic amine accumulation in European fermented sausages. Two laboratories used 0.4 M perchloric acid as the extractant and 1,7-diaminoheptane as the internal standard, the derivatization was carried out for 40 min, after which the sample was dissolved in acetonitrile [81]. The third laboratory used acetone and 5% trichloroacetic acid as extractant solvent and 1,8-diaminooctane as internal standard. The derivatization was performed for a longer period (4 h), followed by a further extraction of the amines with diethyl ether before the sample was dissolved in acetonitrile. The amines most affected by the method of analysis were spermine and spermidine, for which this factor accounted for 43 and 83% of the total variance [25].

31.3.2.2 Rapid Screening Procedures

Alternatives to the instrumental procedures described earlier for the meat industry include the application of less expensive, less time-consuming, and simpler analytical techniques, especially for routine screening or controls.

An automated OPA derivatization and flow injection analysis for rapid (<1 min) histamine determination has been developed to screen fish and seafood products (though not tested for meat products) that does not include a sample cleanup other than extraction and crude filtration [99].

An enzymatic method has been described specifically for histamine determination. The procedure involves the use of amine-specific enzymes that recognize and rapidly transform the substrate into another measurable product. In the presence of oxygen, diaminoxidase (DAO) deaminates

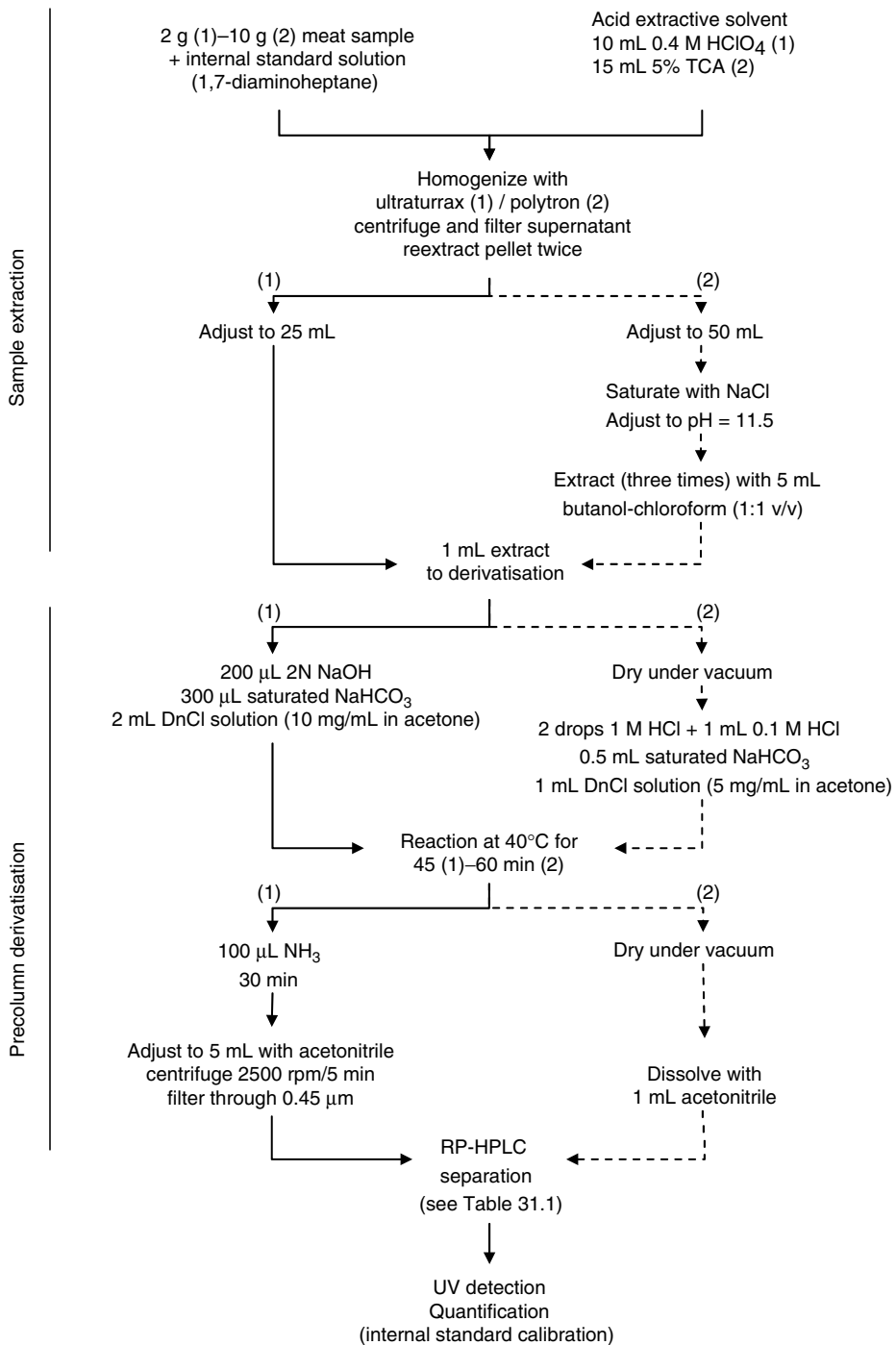


Figure 31.2 Schematic protocol for biogenic amine determination by dansyl chloride precolumn derivatization as described in (1) (Eerola, S., Hinkkanen, R., Lindfors, E. and Hirvi, T., *J. AOAC Int.*, 76(3), 575–577, 1993) and (2) (Moret, S., Conte, L. and Callegarin, F., *Ind. Aliment.*, 35(349), 650–657, 1996).

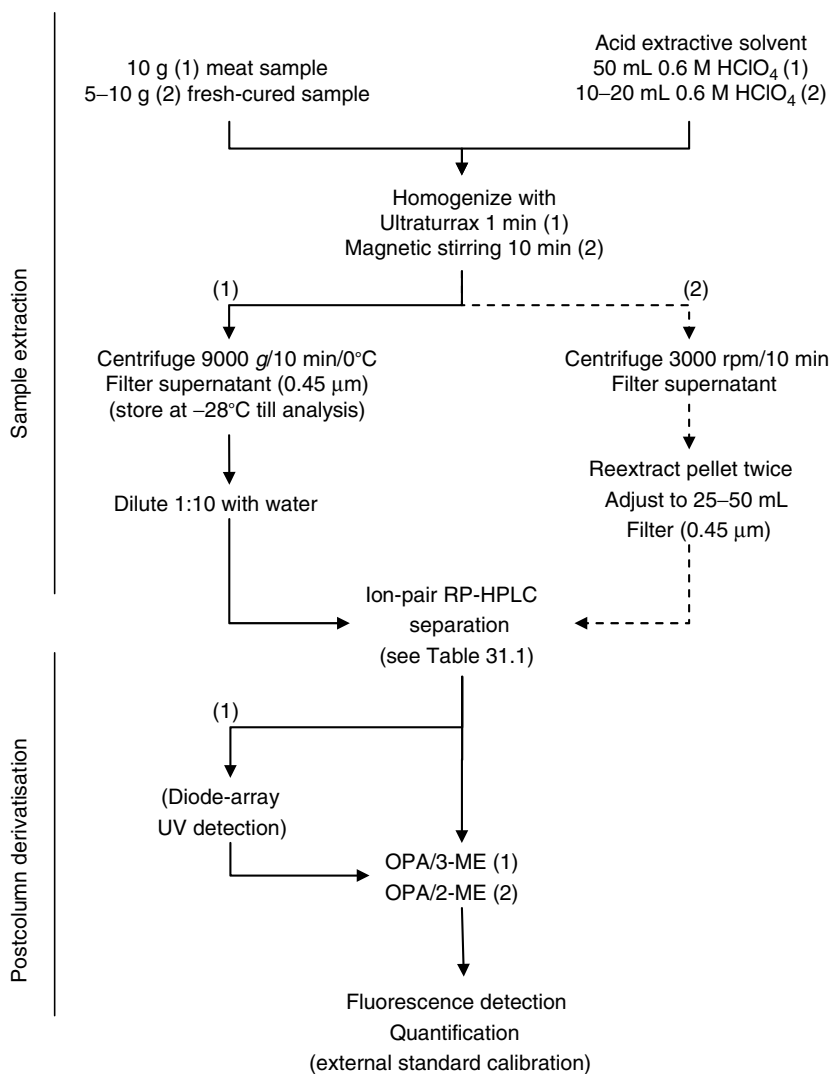


Figure 31.3 Schematic protocol for biogenic amine determination by *o*-phthaldialdehyde postcolumn derivatization as described in (1) (Straub, B., Schollenberger, M., Kicherer, M., Luckas, B. and Hammes, W.P., *Z. Lebensm.Unters. For.*, 197(3), 230–232, 1993) and (2) (Hernández-Jover, T., Izquierdo-Pulido, M., Veciana-Nogués, M.T. and Vidal-Carou, M.C., *J. Agr. Food Chem.*, 44(9), 2710–2715, 1996).

histamine, thereby forming hydrogen peroxide, which, coupled with horseradish peroxidase (HRP), converts a reduced dye (leucocrystal violet) to its oxidized form (crystal violet). The accompanying color development allows colorimetric quantification. This methodology was initially developed for detecting histamine in fish from a neutralized extract [100], and was reported to be suitable for routine analysis, providing simplicity, and speed. However, it tended to overestimate histamine concentrations below 10 mg/kg [101]. A number of limitations have been reported for this technique. Although DAO can also act on other biogenic amines, only little absorbance is developed by tyramine, and no change in the absorbance value for histamine is observed when

Table 31.1 Conditions of Some of the Most Used Chromatographic Procedures to Determine Biogenic Amines in Meat and Meat Products

	<i>Eero/la et al. [81]</i>	<i>Moret and Conte [80]</i>	<i>Straub et al. [75]</i>	<i>Hernández-Iover et al. [29]</i>
BA determined	TY, HI, PHE, TR, SE, PU, CA, SD, SM	TY, HI, PHE, TR, PU, CA, SD, SM ^b	TY, HI, PHE, PU, CA	TY, HI, PHE, TR, SE, OC, DO, PU, CA, AG, SD, SM
Meat sample assayed	1,7-Diaminoheptane (IS) Dry sausages	1,7-Diaminoheptane (IS) Salami	Minced meat (mixed lean pork, lean beef, and pork fat)	Fresh meat Cooked product Ripened (fermented) product
Sample preparation	Acid extraction	Acid extraction plus clean-up (liquid-liquid partitioning)	Acid extract	Acid extract
Precolumn derivatization	Dansyl chloride	Dansyl chloride, dry, and redissolve	—	—
Column	Reverse phase (Spherisorb ODS2)	Reverse phase (Spherisorb 3s TG)	Reverse-phase (Nucleosil 100 7C18)	Reverse phase (NovaPak C18)
Mobile phase	Solvent A (0.1 M ammonium acetate)	Solvent A (water)	Solvent A (0.05 M hexanesulfonic acid [ion pair] and 0.1 M KH ₂ PO ₄ , pH 3.5)	Solvent A (0.01 M sodium octanesulfonate [ion pair] and 0.1 M sodium acetate, pH 5.20)
Elution	Solvent B (acetonitrile)	Solvent B (acetonitrile)	Solvent B (solvent A/acetonitrile [3/1])	Solvent B (solvent C [0.2 M sodium acetate and 0.01 M sodium octanesulfonate, pH 4.5] and acetonitrile [6.6/3.4])
	Gradient (19 min + 10 min equilibration)	Gradient (12 min + equilibration)	Gradient (total time 49 min) (ca. 100 min to separate and resolve amino acids)	Gradient (54 min + 10 min equilibration)

	At 1.0 mL/min flow	At 0.8 mL/min flow	At 1.0 mL/min flow	Flow not specified	At 1.0 mL/min
Postcolumn derivatizing reagent	—	—	—	0.5 M borate buffer OPAV3-ME Brij 35	Borate buffer OPAV2-ME Brij 35
Detection	UV 254 nm	UV 254 nm	UV 254 nm	UV diodearray detector (before derivatization) Fluorescence after derivatization Ex 340 nm; Em 455 nm	Fluorescence after derivatization Ex 340 nm; Em 445 nm
Accuracy (percentage recovery)	TY, 98; HI, 96; PHE, 104; TR, 84; SE, 56; PU, 90; CA, 101; SD, 91; SM, 90	TY, 85; HI, 76; PHE, 87; TR, nq ^a ; PU, 2.9; CA, 82; SD, 67; SM, 72	TY, 98; HI, 96; PHE, 104; TR, 84; SE, 56; PU, 90; CA, 101; SD, 91; SM, 90	TY, 102; HI, 113; PHE, 96; PU, 111; CA, 108	TY, 96; HI, 98; PHE, 95; TR, 93; SE, 93; OC, 97; DO, 98; PU, 99; CA, 97; AG, 99; SD, 99; SM, 100
Precision (percentage [RSD], for Straub et al. is SD)	TY, 4; HI, 4; PHE, 6; TR, 4; SE, 5; PU, 18; CA, 7; SD, 9; SM, 5	TY, 2.5; HI, 3.2; PH, 2.6; TR, nq PU, 4.0; CA, 2.6; SD, 4.2; SM, 6.0	TY, 4; HI, 4; PHE, 6; TR, 4; SE, 5; PU, 18; CA, 7; SD, 9; SM, 5	TY, 9.9; HI, 10.8; PHE, 9.1; PU, 9.5; CA, 10.1	TY, 3.22; HI, 3.9; PHE, 3.1; TR, 4.8; SE, 4.2; OC, 3.7; DO, 6.1; PU, 4.1; CA, 4.7; AG, 5.1; SD, 3.4; SM, 3.1
Sensitivity	Determination limit 1 mg/kg (TY, CA, SD, SM)	Not studied	Determination limit 1 mg/kg (TY, CA, SD, SM)	Detection limit (0.5 mg/kg)	Determination limit <1.00 mg/kg (TY, HI, PHE, TR, OC, DO, PU, CA, AG)
	2 mg/kg (PHE, TR, PU) 5 mg/kg (SE)				<1.50 mg/kg (SE, SM)

^a Not quantifiable due to interfering peaks.

Note: TY, tyramine; HI, histamine; PHE, phenylethylamine; TR, tryptamine; SE, serotonin; OC, octopamine; DO, dopamine; PU, putrescine; CA, cadaverine; AG, agmatine; SD, spermidine; SM, spermine; IS, Internal Standard; Ex, Excitation; and En, Emission.

equimolar solutions of diamines and histamine are determined [102]. These limitations imply that although other biogenic amines give little interference, this technique is not useful to detect amines other than histamine. Alternative specific amine-oxidase enzymes have also been applied in rapid tests to screen for the presence of other biogenic amines. A specific biosensor for tyramine was constructed either with a monoamine oxidase (MAO), from *Aspergillus niger* and beef plasma immobilized in a collagen membrane [103], or with a tyramine-oxidase (from *Micrococcus luteus*) immobilized on porous microglass beads [58,66]. As a result, tyramine is oxidized to aldehydes, and the oxygen consumption is monitored amperometrically with an oxygen electrode detector. These tyramine biosensors have been used to estimate bacterial spoilage during meat storage.

DAO from porcine kidney immobilized onto a porous nylon membrane attached to an amperometric electrode has been used to estimate the total concentrations of histamine, cadaverine, and putrescine accumulated in fish fillets during storage [104]. DAO from peas (*Cicer arietinum*) seem to be more selective to the diamines putrescine and cadaverine [105] and could be used together with other sources of DAO and MAO to distinguish the spoilage pattern [106].

An enzyme sensor array has been developed to simultaneously determine histamine, tyramine, and cadaverine, with a combination of specific amine oxidases of distinct origin [90]. The cross-reactivities of these enzymes against several biogenic amines were characterized, and data were included in an artificial neural network for pattern recognition. The best discrimination was obtained for samples containing tyramine (91%), followed by histamine (75%) and putrescine (57%). The use of enzymes exhibiting higher specific activities would improve the biosensor system.

An alternative potentiometric (nonenzymatic) sensor to measure putrescine has been proposed for monitoring pork freshness [107]. Sample pretreatment is required before analysis of biogenic amines, and therefore this system cannot be used as an online sensor.

An immunological approach has also been developed for the analysis of histamine. This method is simple, rapid, and relatively low-cost in comparison with HPLC. Commercial kits are already available to analyze histamine from aqueous food extracts (e.g., fish, cheese, or sausage) through an enzyme immunoassay. However, the antibodies used in these tests require chemical derivatization of histamine before analysis (propionic acid esters), or require toxic reagents (*p*-benzoquinone), both of which are time-consuming. Alternatively, polyclonal antihistamine antibodies recognizing intact histamine have been included in commercial competitive direct enzyme-linked immunosorbent (CD-ELISA) test kits [108] (e.g., R-Biopharm GmbH, Darmstadt, Germany, or Veratox[®] histamine test from NEOGEN Corporation, Lausing, MI, USA). Because a number of nonpurified aqueous extracts can be analyzed simultaneously in a microtiter plate, the maximum daily throughput of the CD-ELISA method is much higher than by HPLC. The CD-ELISA for the detection of histamine in fish [108,109], cheese [110], and other dairy products [108] as well as in wine [111] is a suitable alternative method that provides results comparable with the official fluorometric or HPLC methods. However, no studies have addressed the application of CD-ELISA in meat and meat products.

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Chapter 32

Nitrosamines

Susanne Rath and Felix Guillermo Reyes Reyes

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32.1 Introduction

Nitrosamines are *N*-nitroso compounds that have received considerable attention worldwide during the past half century, since Barnes and Magee¹ first reported in 1954 the association between dimethylnitrosamine (NDMA) and liver damage in rats. Two years later, the same British scientists confirmed the induction of liver tumors in rats by feeding them NDMA.²

During the period of 1957–1962, liver disorders, including cancer, in various farm animals in Norway were attributed to herring meal that had been preserved by the addition of large amounts of sodium nitrite.³ Further investigations showed that the fishmeal was contaminated with NDMA, which was formed as a result of a chemical reaction between dimethylamine, a commonly occurring amine in this meal, and a nitrosating agent formed from sodium nitrite. This finding led to the idea that nitrosamines might also occur in human food through the interaction between naturally occurring or added precursor compounds. This was the beginning of a world-wide investigation of the presence of nitrosamines in several matrices, including foodstuffs. As a result, NDMA was detected by European scientists in beer.⁴ Since then, nitrosamines have been found in a large variety of products such as foods (in particular, cured meat products), alcoholic beverages, water, soil, air, tobacco, rubber products, pesticides, cosmetics, and drugs. Nowadays, it is well established that nitrosamines are potential carcinogenic compounds.⁵

Although the occurrence of nitrosamines in food products was reported before 1970, some of these early results are untrustworthy, due to the lack of a reliable analytical method available at that time that could identify and determine nitrosamines at the low concentration level required, because many of the methods then available had limits of detection above the levels of nitrosamines now known to be present in foods. This situation was overcome with the development of analytical methodologies for the determination of volatile nitrosamines by gas chromatography (GC) associated with thermal energy analyzer (TEA) or mass spectrometric (MS) detection devices. The number of scientific papers reporting the presence of volatile nitrosamines in meat products peaked in the 1980s. It is worth emphasizing that most of these studies were conducted in the United States, Canada, Germany, and Japan.

This chapter will provide some insight on the chemistry, formation, and occurrence of nitrosamines in meat products, as well as toxicological information, the main focus being analytical aspects.

32.2 Chemistry

N-nitrosamines are aliphatic or aromatic compounds, which have a nitroso functional group attached to nitrogen. The chemical and physical properties depend on the substituents (R_1 and R_2) on the amine nitrogen. Whereas the low molar mass dialkyl nitrosamines are water-soluble liquids, the high molar mass nitrosamines are soluble in organic solvents and food lipids. The chemical structures and physicochemical parameters of some nitrosamines commonly found in meat products are presented in Table 32.1.^{6,7}

In general, nitrosamines are stable compounds in neutral and strongly alkaline solutions, and are difficult to destroy once they are formed. Under ultraviolet (UV) radiation or strongly acidic conditions nitrosamines decompose with cleavage of the nitroso group.⁸

Nitrosamine formation in food generally is related to the nitrosation of secondary amines, where the main nitrosating agent is nitrous anhydride produced from nitrite (Equations 32.1–32.3).

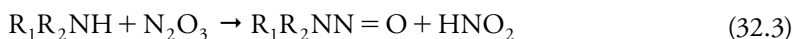
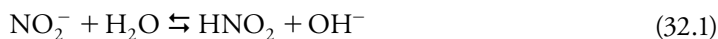
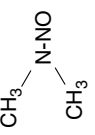
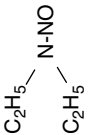
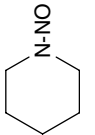
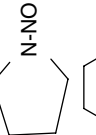
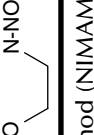


Table 32.1 Chemical Structures and Physicochemical Parameters of Some N-Nitrosamines Commonly Found in Meat Products

N-Nitrosamine	Chemical Structure	CAS	MM	sp gr (g/cm ³)	bp (°C)	vp (mm Hg)	Solubility (mg/mL)	General Description
N-nitrosodimethylamine (NDMA)		62-75-9	74.08	1.0048	151–153	2.7	>100 1000 ^a	Yellow oil
N-nitrosodiethylamine (NDEA)		55-18-5	102.14	0.9422	175–177	0.86	>100 106 ^a	Yellow liquid
N-nitrosopiperidine (NPIP)		100-75-4	114.2	1.0631	217–219	0.14	10–50 76.5 ^a	Yellow oil
N-nitrosopyrrolidine (NPYR)		930-55-2	100.14	1.085	214–216	0.06	1000 ^a	Yellow liquid
N-nitrosomorpholine (NMOR)		59-89-2	116.14	N/A	225–227	0.036	>100 861.5 ^a	Liquid/yellow crystals

^a NIOSH Manual of Analytical Method (NIMAM), Nitrosamines, Method 2522, Fourth edition, 1994.

Note: CAS: CAS registry number; MM: molar mass; sp gr: specific gravity; bp: boiling point; vp: vapor pressure; N/A: not available.
Source: Adapted from CAMEO Chemicals, <http://cameochemicals.noaa.gov/>, accessed June 2007.

The nitrosation rate is first and second order in terms of the amine (R_1R_2NH) and nitrite concentrations, respectively.⁹ The kinetics of the nitrosating reaction depends on the pH of the medium and the basicity of the amine. The optimum pH value lies between 2.5 and 3.5, conditions where the formation of nitrous acid (pK_a 3.35) is favored while molecules of amine still exist in their nonprotonated forms. This explains the fact that the reaction rate increases as the basicity of the amine decreases.¹⁰

Several conditions can contribute to an increase of rate or inhibition of the nitrosation reactions in food. It is well documented that the nitrosation of secondary amines is catalyzed by nucleophilic anions (thiocyanate, bromide, chloride), because the concentration of the available nitrosating agent is increased. The effectiveness of the catalysis is related to the nucleophilic strength of the anion. On the other hand, several compounds, such as ascorbic acid (vitamin C), erythorbic acid, and α -tocopherol (vitamin E), are well recognized as nitrite scavengers and, in consequence, act as inhibitors of the nitrosation reaction.¹¹

32.3 Formation and Occurrence in Meat and Meat Products

Several authors have reviewed the formation and occurrence of *N*-nitrosamine in meat products.¹²⁻¹⁵ The formation of nitrosamines in meat and meat products is a complex process, and several factors and substances could influence nitrosation reaction. The nitrosamine concentration in meat products depends on the residual nitrite concentration, presence of nitrosation catalysts and inhibitors, cooking method, cooking temperature and time, storage conditions, and presence of microorganisms, which are able to reduce nitrate to nitrite and promote degradation of proteins to amines and amino acids.

The food matrices that have received most attention are cured and smoked meats, because sodium nitrite is used as a food additive in the manufacturing process. Several model-system studies have been carried out to explain nitrosamine formation in meat products. The effect of the cooking process on nitrosamine formation in cured and smoked meat products was also extensively investigated. Accordingly, it has been postulated that NDMA is derived from creatine, a muscle constituent, through its breakdown to sarcosine, followed by the decarboxylation of its *N*-nitroso derivative. In the same manner, proline and lysine are considered to be the precursors of NPYR and NPIP in meat products, respectively.¹⁶

Pensabene and Fiddler were the first to associate the presence of *N*-nitrosothiazolidine (NTHZ) in bacon with smokehouse processing, indicating the nitrogen oxides generated during the smoking process and the residual nitrite in the bacon as the nitrosating elements.¹⁷ The nitrosable amine is formed by the condensation of cysteine with formaldehyde, a component of the wood smoke. In fried meat the nitrosating agent was identified as N_2O_3 , which could be formed during the heating of nitrite in meat, or to NO radical formed by dissociation of N_2O_3 at high temperature.^{18,19}

Byun et al.²⁰ verified that gamma irradiation (>10 kGy) reduced the content of volatile nitrosamines (NDMA and NPYR) in pepperoni and salami sausages during storage, and Rywotycki²¹ evaluated the nitrosamine content (NDMA and NDEA) in raw meat (gilts, sows, hogs, boars, heifers, cows, bullocks, bulls, calves, horses, rams, and goats) and verified that the nitrosamine level depended on the animal species, breeding factors, and the season of the year.

In general, the concentration of nitrosamines in meat products currently lies at levels lower than $30 \mu\text{g}/\text{kg}$, which demonstrates the efficacy of actions taken by the meat industry, such as the use of nitrosation reaction inhibitors and a decrease in the nitrite concentration used for the curing process.

32.4 Toxicological Aspects

The great majority of the over 300 *N*-nitroso compounds tested in laboratories, including nitrosamines, were found to be carcinogenic in a wide variety of experimental animals. In addition, they also present mutagenic and teratogenic activity.⁵

N-nitrosamines are readily absorbed from the gastrointestinal tract,^{5,22} do not undergo bioaccumulation, and require metabolic activation to exhibit their mutagenic and carcinogenic action. The initial step of the biotransformation involves hydroxylation of the α -carbon, which is catalyzed by the cytochrome P450 system, mainly CYP2E1^{23,24} and the cytochrome P450 isoform, CYP2A6.^{24,25} The resultant α -hydroxyalkylnitrosamine breaks down to an alkyldiazonium ion and the corresponding carbonyl compound. The diazonium ion could alkylate a variety of nucleophilic sites such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). This biotransformation is considered a fundamental step in cancer initiation.²⁶ The liver is the main organ of nitrosamine biotransformation, but other human tissues also have this capacity.²⁷

Carcinogenic effects induced by the nitrosamines have been reported in all the mammalian species tested, including monkeys, and *in vitro* studies suggest that *N*-nitrosamines present similar biologic activity in humans and experimental animal tissues.²⁶ Consequently, it is assumed that humans are susceptible to the toxic action of these compounds. In fact, the International Agency for Research on Cancer (IARC) concluded, for those *N*-nitrosamines evaluated by the agency, that although no epidemiological data were available, nitrosamines should be regarded for practical purposes as if they were carcinogenic to humans.²⁸

Volatile nitrosamines induce tumors in several organs including liver, lung, kidney, bladder, pancreas, esophagus, and tongue, depending on the animal species.^{29,30} Among the nitrosamines, the volatile nitrosamines show higher carcinogenic potential and, of those found in foods, NDEA is the one that shows the higher carcinogenic activity.²⁷ Tumor induction could occur in different organs, according to the chemical structure of the nitrosamine, the dose, the route of exposure, and the animal species, which makes difficult the extrapolation of the data obtained from experimental animals to humans.

Nitrosamines are more effective as carcinogenic agents to the experimental animals when administered at low repeated doses than in a higher single dose. This is the situation of human low-dose exposures (traces) to nitrosamines present in foods.¹⁶ Consequently, the presence of nitrosamines in foods, and particularly in meat, should be a matter of concern from the toxicological and public health standpoint.

32.5 Regulatory Aspects

Only a few countries have reported data related to the formation and to the presence of nitrosamines in foods, which would allow control of the nitrosamines to negligible levels to reduce exposure to levels that may not represent a higher risk to consumers.³¹ Moreover, only a few countries have specific legislation for the presence of nitrosamines in foods. Table 32.2 shows the maximum levels established in some countries for the presence of nitrosamines in foods.

It is worth emphasizing that the regulatory levels provide guidelines for the minimum required limit of determination of the analytical method to be used by the governmental agencies to conduct action on food surveillance.

Table 32.2 Maximum Levels Permitted in Some Countries for the Presence of N-Nitrosamines in Foods

Country	Level ($\mu\text{g}/\text{kg}$)	N-Nitrosamine	Food	Reference
United States	10	Total volatile N-nitrosamines	Cured meat products	22
Canada	10	NDMA, NDEA, NDBA, NPIP, NMOR	Meat products	32
	15	NPYR		
Chile	30	NDMA	Meat products	33
Russia	2	N-nitrosamines	Raw foods	34
	4	N-nitrosamines	Smoked foods	34
Estonia	3	NDMA, NDEA	Raw and smoked fish	19

32.6 Analytical Aspects

Traditionally, for analytical purposes the nitrosamines have been divided into nonvolatile and volatile compounds, the latter ones being considered a group of relatively nonpolar, low-molar mass nitrosamines, which present sufficiently high vapor pressure to be removed from the food matrix by distillation. Whereas long chain dialkyl nitrosamines, nitrosopeptides, and nitroso-amino acids possess lower vapor pressure and are considered nonvolatile compounds, short-chain dialkyl nitrosamines, such as NDMA, NDEA, and low molar mass cyclic compounds, such as NPYR and NTHZ, are considered volatile nitrosamines. The differences in their physicochemical properties hinder the establishment of analytical methods of general application.

During the 1970s intense research efforts were carried out toward development of analytical methodologies for the determination of volatile nitrosamines. As a consequence, there are a great number of scientific papers reported in the literature for the period of 1970–1990 on the presence of volatile nitrosamines in food matrices. In general, these methods recommend the extraction of nitrosamines from the food matrix by vacuum, steam, or mineral oil distillation with subsequent quantitation by GC-TEA. TEA was developed as a specific nitrosamine detector³⁵ and has been widely employed in the past half century for the determination of volatile nitrosamines in food. Usually, these methods are simpler and receive more attention than those required for the determination of nonvolatile nitrosamines, because they do not require sophisticated sample preparation before the quantitation step. Nowadays, it is well known that in foods, and in particular in meat products, among the nitrosamines the volatiles are certainly the compounds of main relevance, and for this reason in this chapter more attention will be devoted to these compounds.

In the past 10 years, novel analytical methodologies and techniques have been proposed, improving selectivity, detectability, analysis time, and cost. In addition, several analytical methodologies have been subjected to collaborative studies carried out under the auspices of the Association of Official Analytical Chemists (AOAC).³⁶

The extraction of the nitrosamine from the complex food matrices and the cleanup of the extract have been the critical points of the sample preparation step, and several approaches are documented in the literature, including distillation (steam, vacuum, or atmospheric), solvent extraction, solid-phase extraction, solid-phase microextraction (SPME), and supercritical fluid extraction. Thus, in this chapter, the analytical aspects of the determination of nitrosamines in meat products will be presented in terms of sample preparation procedures and quantitation steps.

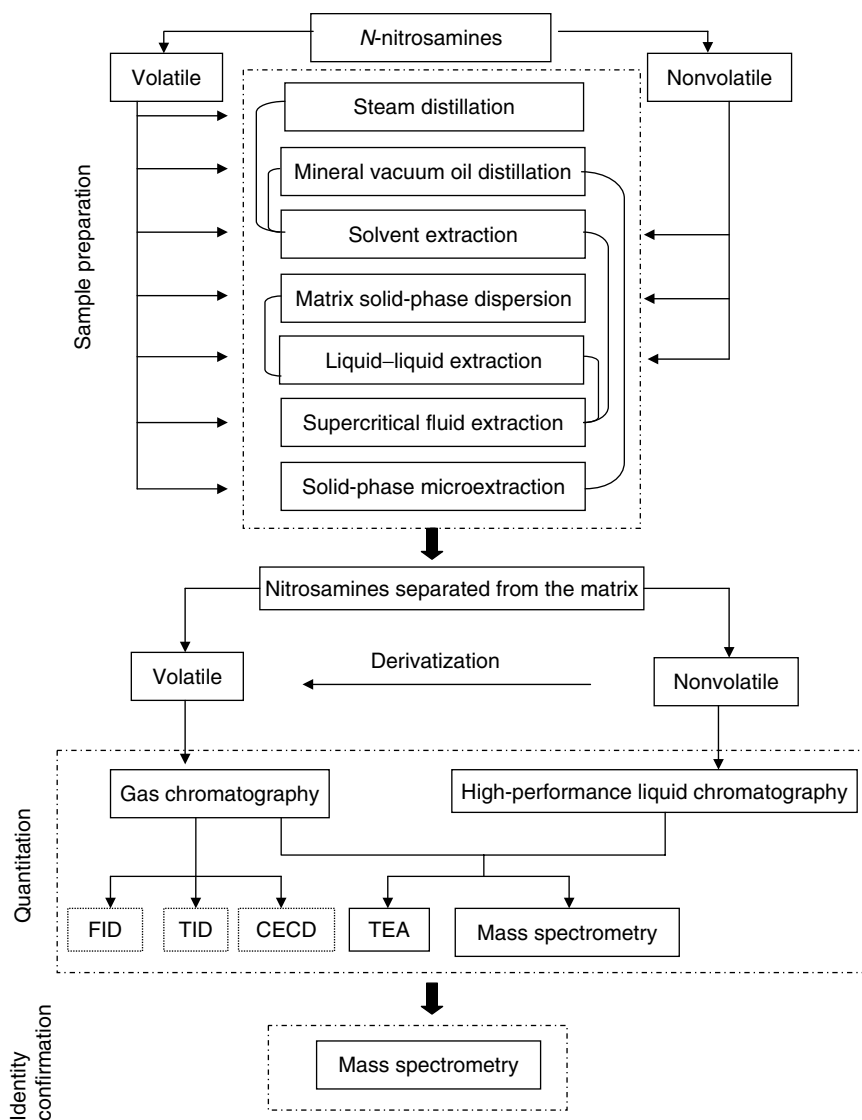


Figure 32.1 Analytical pathways for the determination of *N*-nitrosamines in meat and meat products. (FID, Flame ionization detector; TID, Thermoionic detector; and CECD, Coulson electrolytic conductivity detector.)

In addition, it should be mentioned that a worldwide single analytical method is not available; most of the methods comprise two or more clean-up steps, depending on the nitrosamine, the food matrix, and the detection device. Nonetheless, most of the methods recommend that artifactual nitrosamine formation during sample preparation should be inhibited by adding sulfamic acid, ascorbate, or other nitrosation inhibitors.

An overview of the possible steps in the analytical procedure for the determination of nitrosamines in foods is presented in Figure 32.1 and Table 32.3.

Table 32.3 Some Analytical Methods Reported for Nitrosamine Determination in Meat Products

Food	Nitrosamine	Sample Preparation	Analytical Technique	LOD ($\mu\text{g}/\text{kg}$)	Publ.	
					Year	Reference
Cooked bacon, cooked bacon fat	NDMA, NDEA, NPIP, NPYR	VD, LLE, C, LSE (alumina), K-D	GC-CECD, GC-MS, GC-TEA (Q)	N/A	1976	37
Bacon	NPRO	SE-LSE (anion exchange column), LLE, K-D	HPLC with photolysis (Q), GC-TEA (D, Q)	N/A	1977	38
Meat loaf, liver loaf, and bologna	NDMA, NPYR, NPIP	MOVD, LLE, K-D	GC-TEA (Q)	N/A	1978	39
Bacon, boiled ham, bologna	NHPYR	Celite, C, LLE, LSE (alumina +acidic cellulose), C	GC-MS (IC)	0.2 ng	1978	39
Ham, frankfurters, pork shoulder, canned meats	NDMA, NDEA, NDPA, NPYR, NPIP	Digestion in methanolic KOH, LLE, distillation, LLE, LSE (silica gel)	GLC-TEA (Q)	N/A	1972	40
Fried bacon, fried pork	NDMA, NDEA, NDPA, NDBA, NPIP, NPYR, NMOR	MOVD, LLE, K-D	GC-TEA (Q)	N/A	1980	41
Cooked bacon	NTHZ	MOVD, LLE, K-D or MSPD (Celite), + LSE (alumina column)	GC-TEA (Q)	N/A	1982	42
Fried bacon	NPYR	MSPD Celite (dry column), K-D	GC-TEA (Q)	N/A	1982	43
Fried bacon	NTHZ, NPYR	MSPD Celite (dry column), K-D, LSE (alumina column), K-D	GC-MS (Q)	N/A	1982	44
Smoked bacon	HMNTHZ	LLE, C, + SLE (alumina column), C	HPLC-TEA (Q)	1-2	1989	45
			GC-TEA (Q) (D)			
			GC-MS (IC)			

Bacon, smoked poultry	HMNTCA, HMINTHZ	SE, LLE, C, LLE, C, LSE (alumina column), LLE, C, deriv SFE, SPE (silica), C	HPLC-TEA (D, Q) GC-MS, deriv. (IC) GC-TEA (Q)	N/A	1992	46
Fried bacon	NPYR NDMA NDBA	Steam distillation, HS-SPME	GC-TEA (Q) GC-MS (IC) GC-TEA (Q)	1 0.3	1997 1997	47 48
Sausages	NDMA, NEMA, NDEA, NDPA, NMOR, NPYR, NPIP, NDBA	MSPD (Extrelut), K-D, SPE (Florisil), K-D	MEKC (Q) GC-MS (IC) GC-TEA	22.5–36.0 ^a N/A	2003 2004	50 20
Sausages	NDMA, NDEA, NMOR, NPIP, NPYR	VSD and SPE (activated carbon), C	MEKC (Q) GC-MS (IC) GC-TEA	22.5–36.0 ^a N/A	2003 2004	50 20
Fermented sausages	NDMA, NPYR	Steam distillation on a steam generator, LLE, K-D	GC-TEA (Q)	3	2005	51
Sausages	NDMA, NDEA, NPIP, NPYR	HS-SPME (DVB/PDMS)	GC-TEA (Q)	3	2005	51
Meat patties, gelatine (food model)	NDMA, NMEA, NDEA, NPYR, NMOR, NDPA, NPIP, NDBA, NDPhcA	SPME (DVB/PDMS)-DED	GC-TEA (Q/IC)	0.142–9.539 (Gelatine)	2006	52
Sausages	NDMA, NDEA, NMOR, NPIP, NPYR	SFE (CO ₂) + florisil trap	MEKC (Q) GC-MS (IC) GC-MS	Range mg/kg 0.09	2007 2007	53 19
Meat (raw, fried, smoked, grilled, pickled, and canned)	NDMA, NDEA, NPIP, NPYR, NDBA	SPE (Extrelut), C, SPE (Florisil), C	GC-MS (IC) GC-MS	0.09	2007	19

^a µg/L.

Note: N/A: not available; MOVD: mineral oil vacuum distillation; VSD: vacuum steam distillation; VD: vacuum distillation; MSPD: matrix solid-phase dispersion; LLE: liquid-liquid extraction; SE: solvent extraction; LSE: liquid-solid extraction; SFE: supercritical fluid extraction; SPME: solid-phase microextraction; HS: head space sampling; SPE: solid-phase extraction; K-D: Kuderna Danish concentrator; C: concentration; GC: gas chromatography; TEA: thermal energy analyzer; MS: mass spectrometry; MEKC: micellar electrokinetic chromatography; IC: identity confirmation; Q: quantitation; D: derivatization.

32.6.1 Sample Preparation

32.6.1.1 Distillation and Clean-Up Procedures

Distillation was extensively used in the past as the primary stage for the extraction of the volatile nitrosamines from food matrices, including steam distillation and mineral oil vacuum distillation (MOVD). The clean-up procedures that follow the extraction have included liquid–liquid extraction (LLE), liquid–solid extraction (LSE), and SPME. The concentration of the separated nitrosamines to a small volume before quantitation has generally been carried out using a Kuderna–Danish (K-D) evaporator.

The MOVD became the AOAC Official Method for the determination of volatile nitrosamines in fried bacon. For this purpose, 25.0 g of sample is added to 2 mL of 0.2 mol/L NaOH and 25 mL mineral oil. The mixture is introduced into a pumping and distillation assembly, vacuum (<2 torr) is applied, and the temperature is increased from ambient temperature to 120°C in 55–60 min. The distillate is collected in a vapor trap inserted in a Dewar flask containing liquid nitrogen. The nitrosamines are removed from the distillate by LLE using dichloromethane. The final extracted volume is reduced to 1.0 mL in a K-D flask, and the quantitation is carried out by GC-TEA.⁵⁴

Although the volatile nitrosamines are efficiently extracted from foods by vacuum distillation, this sample extraction procedure presents limitations, such as long analysis time, being work-intensive, possibility of contamination, loss of the analyte during the concentration process, formation of emulsions during LLE, and environmental problems related to discarding solvents.

A combination of vacuum steam distillation and solid-phase extraction for the determination of NDMA, NDEA, NMOR, NPIP, and NPYR in sausages was proposed by Sanches Filho et al.⁵⁰ For this purpose, 150 g of sample was added to 100 mL of water, and the nitrosamines were separated by vacuum steam distillation using a rotary evaporator (65°C for 80 min). To the distillate active carbon powder (100–400 mesh) was added, and the mixture was shaken for 45 min. The sorbent was removed by filtration, and the nitrosamines were eluted from it with acetone and dichloromethane. After concentration under a nitrogen stream, nitrosamine quantitation and identity confirmation were performed by micellar electrokinetic chromatography (MEKC) and GC-MS, respectively. Powdered activated carbon for the clean-up and concentration of NDMA and NDEA from aqueous solutions (water and beer samples) was also employed by Ayügin et al.⁵⁵

Sen et al.⁴⁸ described the use of SPME for the clean-up step in the determination of *N*-nitrosodibutylamine (NDBA) and *N*-nitrosodibenzylamine (NDBZA) in smoked hams. The method consists of the isolation of the volatile nitrosamines by steam distillation. A polyacrylate coated silica fiber was introduced into the headspace of the distillate. Quantitation was conducted by GC-TEA, and the identity confirmation was done by GC-MS. The authors stated that the SPME extraction efficiency, using an extraction time of 60 min at room temperature, was too low for most of the nitrosamines evaluated (NDMA: 0.08%; NDEA: 0.17%; *N*-nitrosodipropylamine [NDPA]: 2.04%; NDBA: 19.3%; NPIP: 0.07%; NPYR: 0.07%; NMOR: 0.02%; NDBZA: 1.9%; and lower than 1% for *N*-nitrosodioctylamine [NDOA]). Using a temperature and a time of extraction of 80°C and 60 min, respectively, and by addition of alkali (3 mol/L KOH) and salt saturation (NaCl), better efficiencies were achieved only for NDBZA and NDBA.

32.6.1.2 Solvent Extraction Followed by Cleanup Using Liquid–Solid Extraction

Solvent extraction has been widely used as the clean-up step of the aqueous distillate obtained by the extraction of volatile nitrosamines from the food matrices. In a few circumstances,

solvent extraction was employed as the extraction step of nonvolatile nitrosamines from meat samples. Nevertheless, Sen et al.⁴⁶ described a solvent extraction procedure for the determination of 2-hydroxymethyl-*N*-nitrosothiazolidine (HMNTHZ) and 2-hydroxymethyl-*N*-nitrosothiazolidine-4-carboxylic acid (HMNTCA) in smoked meats. For this purpose, 10–20 g of the food sample are mixed with sulfuric acid and sulfamic acid, and extracted with 100 mL acetonitrile (for processed meat) or methanol (bacon). After the first filtration, the residue was further extracted with two 60-mL portions of the solvent. The combined filtrates were washed with 80 mL of iso-octane to remove fats and lipids. NH_4OH was added to the remaining extract, and the mixture was evaporated to 10 mL in a rotary evaporator. Water was added to the evaporated residue, the pH adjusted to 2–2.3, and the solution saturated with NaCl before extraction with three portions of 50 mL ethyl acetate. The combined extract was concentrated to 1 mL in a rotary evaporator and cleaned up by LSE on an acidic alumina cartridge.

Another LSE procedure for extraction of nitrosoamino acids *N*-nitrosoproline (NPRO) from bacon was reported by Hansen et al.³⁸ The raw bacon (100 g) was added to water and then homogenized, centrifuged, and stored at 0°C until the fat had been solidified. The supernatant was removed, and the procedure was repeated two more times. The combined supernatants were filtered and cleaned up on an anion-exchange column (Dowex 2X8-100 strongly basic anion-exchange resin). After LSE, the nitrosoamino acid was extracted with dichloromethane from the eluate and concentrated in a K-D flask. NPRO was quantified by reverse-phase HPLC using a photohydrolysis system.

32.6.1.3 Matrix Solid-Phase Dispersion and Liquid–Liquid Extraction

Pensabene et al.⁴³ introduced a rapid method for the determination of NPYR in fried bacon using a dry column of acid—Celite. The ground food sample (10 g) was mixed thoroughly with 25 g anhydrous sodium sulfate and 20 g Celite and added to the chromatographic column containing 10 g Celite previously washed with phosphoric acid. At the top of the column, 30 g of anhydrous sodium sulfate was added. The column was rinsed with 100 mL pentane–dichloromethane (95 + 5 v/v) and 125 mL dichloromethane. Only the last 40 mL of the eluate was collected and concentrated in a K-D flask to a final volume of 1 mL before GC-TEA quantitation. In this sample preparation technique, the solid food sample matrix is dispersed into the adsorbent material (diatomaceous earth), which is subsequently packed into a column from which the nitrosamines are eluted. In this manner, the sample becomes dispersed throughout the column and is part of the overall chromatographic character of the system. Interactions involve the stationary phase, the solid support, the mobile or eluting phase, and all of the sample matrix components as well.

Pensabene and Fiddler⁴⁴ also reported a method using a dual-column chromatographic procedure (Celite + alumina columns) for the determination of NTHZ (nonvolatile nitrosamine) and NPYR (volatile nitrosamine) in fried bacon, and compared this procedure with the MOVD. The extraction procedure reported is as described previously, with the modification that the first 85 mL of dichloromethane eluted from the Celite column was collected and concentrated to a final volume of 6 mL in a K-D flask. The concentrate was added to 2 mL hexane and quantitatively transferred to an alumina column containing anhydrous sodium sulfate at the top. An initial volume of 25 mL hexane was added to the column, and the NTHZ was eluted with 125 mL dichloromethane. The eluate was concentrated, and NTHZ was quantified by GC-TEA. The authors verified that the MOVD extraction procedure introduces artifacts and observed *in situ* nitrosamine formation during this analytical step, which thus requires the addition of nitrosating inhibitors. Sulfamic acid and ascorbic acid were shown to be effective for this purpose, as sulfamic acid reduces the pH and thereby removes any nitrite present in the sample, as well as prevents the bacterial reduction of nitrate.⁵⁶

Raoul et al.⁴⁹ presented a rapid, time- and solvent-sparing MSPD plus SPE method to determine NDMA, *N*-nitrosoethylmethylamine (NEMA), NDEA, NDPA, NMOR, NPYR, NPIP, and NDBA in thermally processed sausages. The food sample (6 g) homogenized in 6 mL 0.1 mol/L NaOH was dispersed in Extrelut (6 g) and packed into a column. The nitrosamines were eluted with 40 mL of hexane:dichloromethane (60:40 v/v), and the eluate was concentrated in a K-D flask. The extract was cleaned up on a commercial Florisil cartridge. In comparison to the vacuum distillation technique, this sample preparation approach requires less food sample and solvents without affecting the detectability of the method, and could also be applied to determine the less volatile nitrosamines NDBZA and NTHZ, which have been found in smoked meat products. This sample preparation procedure, using Extrelut and Florisil, was also employed by Yurchenko and Mölder¹⁹ for the determination of volatile nitrosamines in several meat matrices.

32.6.1.4 *Solid-Phase Microextraction*

SPME was first described by Pawliszyn, and since then this technique has been extensively used for several analytical purposes in substitution of traditional solvent extraction, including the evaluation of the volatile compounds present in the vapor or in the liquid phase of solid and liquid foods. The advantages of the SPME method over other methods of extraction are numerous. SPME can be significantly faster and easier than solvent extraction methods, it is easily automated, and it does not require the use of potentially toxic and expensive solvents.⁵⁷ SPME has gained widespread acceptance in many areas in recent years, and has been applied to a wide spectrum of analytes, including the determination of nitrosamines in food. Commercially available fused-silica fibers coated with polydimethylsiloxane (PDMS), carboxen–polydimethylsiloxane (CAR/PDMS) polyacrylate (PA), divinylbenzene–carboxen–polydimethylsiloxane (DVB/CAR/PDMS), carbowax–divinylbenzene (CW/DVB), and carbowax-templated resin (CW/TPR) are available.⁵⁸

Andrade et al.⁵¹ described a simple method using headspace sampling by SPME with GC-TEA detection (HS-SPME-GC-TEA) for the determination of NDMA, NDEA, NPIP, and NPYR in sausages. Two fused-silica fibers, one coated with PDMS/DVB and another with PA, were evaluated, and the experimental conditions (equilibrium time, salt addition, extraction time, and temperature) were optimized using an experimental design. The PDMS/DVB-coated fiber showed better recoveries for the extraction of NDMA and NDEA in sausages in comparison with the PA-coated fiber, which presented higher efficiency for NPIP and NPYR. The optimum recoveries were obtained with the following experimental conditions: PDMS/DVB (equilibrium time: 10 min; salt addition: 36% w/v NaCl; temperature: 30°C; and extraction time: 30 min) and PA (equilibrium time: 10 min; salt addition: 36% w/v NaCl; temperature: 50°C; and extraction time: 20 min).

The outstanding advantage of HS using the SPME technique in food analysis is the prevention of direct contact of the fiber with the food matrix; therefore, the fiber has a longer lifetime, and the selectivity of the method could be enhanced. On the other hand, HS-SPME is limited to volatile nitrosamines, which present high vapor pressure. The extraction efficiency onto the fiber depends on the polarity and the thickness of the stationary phase, extraction time, and concentration of the nitrosamine in the sample. Extraction efficiency could be improved by agitation, addition of salt, pH, and temperature.⁵⁸

Ventanas et al.⁵⁹ employed SPME coupled to a direct extraction device (DED) for extracting nine volatile nitrosamines (NDMA, *N*-nitrosomethylethylamine [NMEA], NDEA, NPYR, NMOR, NDPA, NPIP, NDBA, and *N*-nitrosodiphenylamine [NDPheA]) from a solid food

model system (gelatin) at refrigeration and at room temperature. The DED enables the introduction of the SPME fiber in the core of the solid matrices, with the advantage of determining volatile compounds from solid foods without deterioration of the product. In a subsequent work, Ventanas and Ruiz⁵² studied the feasibility of using SPME-DED for extraction of nitrosamines from solid matrices mimicking solid foodstuffs, and compared the efficiency of different fiber-coatings for extraction (CAR/PDMS, DVB/CAR/PDMS, and DVB/PDMS). Meat patties spiked with nitrosamines were also analyzed using a PDMS/DVB-coated fused-silica fiber. The authors reported that with the patties instead the gelatin matrix, lower reproducibility and poorer linearity were obtained, and concluded that quantitation of nitrosamines in solid meat samples using SPME-DED was not fully reliable. However, the proposed technique is promising for qualitative assessment.

32.6.1.5 *Supercritical Fluid Extraction*

Supercritical fluids have been successfully used to extract a wide variety of analytes from several matrices, including food, with the advantages of providing fairly clean extracts, minimizing sampling handling, reducing the use of toxic solvents, and expediting sample preparation. Fiddler and Pensabene⁴⁷ reported a method using supercritical extraction (SFE) of NPYR and NDMA from fried bacon. Fried bacon (5 g) was added to 250 mg propyl gallate and 5.0 g Hydromatrix. The homogenized mixture was transferred to the extraction vessel of the SFE system attached to an SPE cartridge (silica). The extraction was carried out at 10,000 psi with a flow rate of expanded CO₂ of 2.8 mL/min for a total of 50 L. The SPE cartridge was washed with pentane–dichloromethane (72:25 v/v), and the nitrosamines were eluted with dichloromethane:ether 70:30 v/v. The quantitation was performed by GC-TEA. The authors compared the SFE method to SPE, mineral oil distillation, and low-temperature vacuum distillation, and concluded that SFE was superior in relation to recovery, repeatability, rapidity of analysis, and lower solvent consumption, and that the method is not susceptible to artifactual nitrosamine formation.

Recently, Sanches Filho et al.⁵³ reported a procedure for the extraction of NDMA, NDEA, NMOR, NPIP, and NPYR from sausages, using CO₂ as extraction fluid. Several parameters were evaluated and optimized such as density, temperature at constant pressure of 200 bar (40°C), dynamic extraction time (20 min), organic modifier, flow rate (3 mL/min), and trap adsorbent (Florisol). The quantitation was done by MEKC. The nitrosamine recoveries from spiked sausages (0.2 g sample) at three concentration levels (0.4, 1.0, and 10 mg/kg) ranged from 20.9 to 81.6%. The authors attributed the low recovery values to the presence of lipids in the matrix and losses during the evaporation and change of solvents. The method, due to instrumental limitations, was developed for the quantitation of nitrosamines at the milligram per kilogram level and needs to be improved in relation to sample amount and concentrations steps to allow the determination of nitrosamines in food at the microgram per kilogram level.

32.6.1.6 *Quantitation Methods*

Several analytical methods have been employed in the past for the semiquantitative and quantitative determination of nitrosamines in food, including thin-layer chromatography,^{60,61} spectrophotometry, colorimetry, and polarography.^{62,63} In general, these methods lack selectivity and do not allow nitrosamine determination at the microgram per kilogram level required for foodstuffs. Only after the development of chromatographic methods with adequate sample preparation

procedures, including clean-up and concentration steps, and the use of selective detector devices, did it become possible to establish reliable methods for the determination of volatile and nonvolatile nitrosamines in food.

32.6.1.6.1 Gas Chromatography

GC has been the method of choice for the determination of volatile nitrosamines around the world. Furthermore, some nonvolatile nitrosamines, such as hydroxylated nitrosamines and nitrosoamino acids, were determined by GC after derivatization by acylation or trimethylsilylation.⁶⁴

Several stationary phases of moderate to strong polarity in packed, megabore, and capillary columns have been employed for the separation of the nitrosamines using GC-TEA, including 15% Carbowax 20 M/terephthalic acid on 100/120 mesh Gaschrom Q,³⁹ glass capillary column coated with UCON 5100,⁴² silica capillary column coated with Supelcowax 10,⁴⁸ 88% methyl, 7% phenyl, 5% cyanopropyl capillary column,⁴⁹ 11% Carbowax 20M on 60/120 Chromosorb W packed column,⁴¹ and HP-INOWAX megabore column.⁵¹ For GC-MS analysis, silica capillary columns coated with DB-5,⁴⁸ 5% phenyl-methyl silicone (HP-5),⁵⁹ 14% cyanopropyl–86% methyl polysiloxane (HP 1701),¹⁹ and HP-1⁵⁰ have been employed.

In the past, flame ionization detectors, thermionic detectors, Coulson electrolytic conductivity detectors, and electron capture detectors were employed for volatile nitrosamines quantitation. A comparison of the performance of those different detection devices was reported by Fine and coworkers.³⁷ Later, the selective TEA became the internationally recognized standard detector for quantitation purposes. Despite the high selectivity characterizing the TEA detector for *N*-nitroso compounds, which allows reduced clean-up procedures in the sample preparation step, identity confirmation by mass spectrometry is mandatory. Basically, the TEA is composed of a catalytic pyrolyzer, a trap, a reaction chamber, and a photomultiplier tube. The principle of operation of the TEA consists of the cleavage of the N–NO bond of the nitrosamine in the catalytic pyrolyzer chamber, forming the nitrosyl radical (NO•). The by-products of the pyrolysis are removed in the trap. The nitrosyl radicals are conducted by vacuum to the reaction chamber, where they are oxidized with ozone, forming electronically excited nitrogen dioxide (NO₂* [“*” electronically excited state]). When the excited molecule decays to its ground state, it emits near infrared radiation (600 nm). At the last stage the radiation is detected by a sensitive photomultiplier tube, where the intensity of the radiation is proportional to the nitrosamine content present in the sample. Detectability is at the picogram level.

Nowadays, the mass spectrometer is the most recommended detector for volatile and nonvolatile nitrosamine determination, due to the fact that the technique allows accurate quantitation, as well as confirmation in one run. In this regard, the use of the GC-MS technique for the determination of volatile nitrosamines in meat products has been reported.^{19,48,50,52} Nonetheless, although GC-MS/MS and LC-MS/MS have become a routinely applicable technique for the quantitation of a large number of toxic compounds in several matrices, no scientific publications were found in the literature for the determination of nitrosamines in meat and meat products using these instruments.

32.6.1.6.2 High-Performance Liquid Chromatography

HPLC coupled with the TEA detector (HPLC-TEA) was first employed by Fine et al.³⁷ Afterward, this technique was employed for the determination of nonvolatile nitrosamines in foods, including hydroxyl nitrosamines and *N*-nitrosoamino acids. Early on, the HPLC technique presented

several drawbacks, such as the incompatibility of the TEA system with components of the mobile phase from the HPLC. Furthermore, *N*-nitrosamines do not show relevant absorption in the UV region of the spectra, and derivatization reactions are required to improve the detectability with a UV detector.

Owing to the different physical and chemical properties of the nonvolatile *N*-nitrosamines, a general sample preparation procedure before HPLC quantitation is not possible, and the extension of the clean-up step is related to the selectivity of the detection device used.

Considering the more polar characteristics of the nonvolatile nitrosamines, normal phase HPLC has been, in general, the method of choice, using silica or cyano stationary phases.^{46,65} Only a few papers report performing the nitrosamine separation on reversed-phase octadecyl columns.³⁸

Among the *N*-nitrosoamino acids, NPRO has been the most studied, the reason being that it could originate from the amino acid proline, which is present in all proteins. Wolfram et al.⁶⁵ reported a method for the determination of NPRO using fluorimetric detection (HPLC-FL). The fluorescent derivative was formed by NPRO denitrosation, followed by the derivatization of proline with 7-chloro-4-nitro-benzo-2-oxa-1,3-diazole. The HPLC conditions comprised a LiChrosorb Si stationary phase and a mobile phase composed of *n*-hexane:ethyl acetate:acetic acid (50:50:0.5 v/v/v).⁶⁵ An HPLC-method was reported for the determination of *N*-nitrosobenzylphenylamine (NBPHA) in cooked bacon, luncheon meat, and dried beef.³⁷ For the chromatographic separation, a μ -Porasil column and acetone:2,2,4-trimethylpentane (5:95 v/v) as column and mobile phase, respectively, were used. Sen et al.⁴⁶ used a LiChrosorb Si 100 column for the determination of HMNTHZ and HMNTCA in meat products. Whereas HMNTHZ did not require derivatization before HPLC-TEA quantitation, the HMNTCA was derivatized with diazomethane. For the identity confirmation, both compounds were derivatized: HMNTCA with heptafluorobutyric anhydride, whereas HMNTHZ was converted to its *O*-methyl ester derivative.

Sen et al.⁴⁵ described a method employing HPLC-TEA for the determination of HMNTHZ in fried bacon, as its *O*-methyl ester derivative, using a Lichrosorb Si 100 column (5 μ m) and a mobile phase composed of acetone and *n*-hexane with linear gradient elution. The detection limit is about 1–2 μ g/kg.

The detectability for the determination of nonvolatile *N*-nitroso compounds can be improved, in relation to precolumn and postcolumn derivatization, by denitrosation of the nitrosamines and derivatization of the liberated secondary amines with fluorescent agents, such as dansyl chloride. In this regard, Cárdenes et al.⁶⁶ described a microwave-assisted method (radiation power 378 W, maximum pressure 1.4 bar, reaction time 5 min) for dansylation of NMOR, NDMA, NPYR, NDEA, and NPIP with subsequent quantitation by HPLC with fluorimetric detection. The denitrosation was achieved using hydrobromic acid–acetic acid. The method was employed to study the recoveries of *N*-nitrosamines from beer.

32.6.1.6.3 Electrophoresis

Although capillary electrophoresis has been increasingly used in the separation of a large variety of compounds in several matrices, only one paper using MEKC reports the determination of volatile nitrosamines (NDMA, NMOR, NPYR, NDEA, NPIP), employing a fused-silica capillary and a diode array detector; sodium dodecyl sulfate was used as the pseudo-stationary phase. The limit of quantitation was between 520 and 820 μ g/L, and the authors pointed out that the method is simple, and has a short analysis time and high efficiency.⁵⁰

32.7 Conclusions

Reliable analytical methods are available for determination of volatile nitrosamines at concentration levels lower than 10 µg/kg in meat and meat products. Although a large number of sample preparation procedures for the volatile nitrosamines are reported in the literature, most of them are time-consuming and labor-intensive, and require large volumes of solvents. Artifactual nitrosamine formation during analysis should be considered in all analytical procedures, and should be evaluated for each sample preparation to allow the acquisition of reliable results. There is a need for development of simple, low-cost, and environmentally friendly sample preparation procedures for the quality control of meat and meat products in relation to the content of nitrosamines to avoid or minimize human exposure to these toxic compounds through consumption of foods.

Undoubtedly, the use of the specific TEA detector coupled to chromatographic systems has simplified sample preparation without minimizing selectivity and detectability, and represents a great contribution to the quantitation of nitroso compounds in food—in particular, volatile nitrosamines in meat products. Nevertheless, this detector lacks versatility in comparison to the mass spectrometer. In addition, in view of the fact that the confirmatory evidence for an analyte is indispensable in the quality control of any toxic compound in food, the use of the mass spectrometer coupled to the chromatographic becomes the technique of choice. As a consequence, the TEA has been replaced in many laboratories dealing with the quantitation of toxic compounds in food.

The newer generation of mass spectrometers, including tandem mass spectrometers, coupled to gas or liquid chromatographic systems, due to their higher detectability and selectivity capacities, as well as simplified sample preparation procedure requirement, have been shown to be a potential technique for the determination of nitrosamines in meat and meat products.

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Chapter 33

Polycyclic Aromatic Hydrocarbons

Peter Šimko

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33.1 Introduction

Meat smoking is one of the oldest food technologies, having been used by mankind for a minimum of 10,000 years. Probably as a protection against canines a man might hang a catch over the fire, and from this smoking came to be widely used, not only for the production of smoked products with a special organoleptic profile, but also for its inactivating effects on enzymes and microorganisms. The techniques of smoking have gradually improved and various procedures have been developed in different regions for treating meat and fish. Currently, the technology is used mainly for enrichment of foods with specific taste, odor, and appearance that are in wide demand on the market. On the other hand, the role of the preservative effects is gradually diminishing in importance as a result of more recent trends in alternative preservation procedures. Today it is supposed that the technology is used, in many forms, to treat 40–60% of the total amount of meat products [1] and 15% of fish [2].

33.1.1 Principles of Smoking

In general, smoke is a polydispersed mixture of liquid and solid components with diameters of 0.08–0.15 μm in gaseous phase of air, carbon oxide, carbon dioxide, water vapor, methane, and other gases. Smoke has a variable composition depending on various conditions including procedure and temperature of smoke generation, origin and composition of wood, water content in wood, etc. [1]. To date, up to 1100 various chemical compounds have been identified and published in the literature [3]. The smoking treatment itself is based on successive deposition of compounds such as phenol derivatives, carbonyls, organic acids and their esters, lactones, pyrazines, pyrroles, and furan derivatives [4] on a food surface and their subsequent migration into the food bulk. Smoke is generated during thermal combustion of wood, consisting roughly of 50% cellulose, 25% hemicellulose, and 25% lignin, with limited access to oxygen. The thermal combustion of hemicelluloses, cellulose, and lignin occurs at 180–300, 260–350, and 300–500°C, respectively. However, the decomposition of the wood components also proceeds at temperatures reaching up to 900°C and, in the presence of an excess of oxygen, even 1200°C. The smoke produced at 650–700°C is richest in components able to impart desirable organoleptic properties to treated products. The temperature of generation of smoke can be decreased by increasing the humidity of the wood [5]. The quantitative composition of smoke depends not only on the kind of wood used, on the temperature of the generation, and the excess of oxygen, but also on cleaning procedures applied immediately after smoke generation [1].

33.1.1.1 Traditional Procedures of Smoking

After generation, smoke is driven into a kiln, during which time its temperature is going down, which is accomplished by partial condensation of smoke components (especially compounds with high boiling point) in pipes, walls, or on foods. The rate of smoke deposition depends on the temperature, humidity, volatility, and velocity of a smoke stream. When the smoke comes into contact with a food surface, there are three modes of smoke treatment procedures, related to the temperature of smoke, as follows:

1. *Cold smoking.* Temperature of the smoke between 15 and 25°C (used for aromatization of uncooked sausage, raw hams, and fermented—not thermally treated—salami)
2. *Warm smoking.* Temperature between 25 and 50°C (used for aromatization and mild pasteurization of frankfurters, sausages, meat pieces, and gammon)
3. *Hot smoking.* Temperature between 50 and 85°C (used for both aromatization and thermal treatment of hams, salami, sausages, etc.)

To achieve a rich, deep brown coloring on the surface and very strong aroma profile formation, the time of smoking must be considerably prolonged. Such products are frequently termed “black-smoked” or “farmhouse-smoked.” These products contain far higher contents of polycyclic aromatic hydrocarbons (PAH) [3,6]. “Wild” smoking occurs under uncontrolled technological conditions and without legislative regulation, which is typical for households and developing countries; this can lead to very high PAH content in smoked foods [7–9].

33.1.1.2 Alternatives to Traditional Smoking Procedures

A Kansas pharmacist named Wright developed and patented the first liquid smoke flavor (LSF) to be prepared from primary smoke condensate in the late nineteenth century. The use of LSF has important advantages: It reduces considerably the time necessary to reach the required organoleptic profile of flavored foods and makes it possible to control more effectively the “addition” of contaminants, including PAH, into aromatized products. Currently, LSF is used in the following forms:

- Liquids for spraying, nebulization, immersion, or showering
- Emulsions incorporated into foods by injection or mixing
- Water-mixable emulsions for showering or curing brine
- Powders such as maltodextrins, salt, saccharides, starch, proteins, and seasonings
- Solutions in vegetable oils [10].

33.1.2 Polycyclic Aromatic Hydrocarbons

Apart from the compounds mentioned earlier, there are also conditions suitable for formation of other compounds during smoke production. One of the most important groups that are actually harmful to human health are PAH. These are formed during the thermal decomposition of wood, especially under limited oxygen access, in the range of 500–900°C [11]. PAH are characterized by two or more condensed aromatic rings in a molecular structure and have a strong lipophilic character. The temperature of smoke generation plays a decisive role, because the amounts of PAH contained in smoke (which are formed during a pyrolysis) increase linearly with the temperature of smoke generation in the interval of 400–1000°C [12]. Apart from the formation of the compounds, the temperature also affects the structure and number of PAH. The number of PAH present in smoked fish can reach up to 100 different compounds [13] that have various effects on living organisms.

33.1.2.1 Behavior of Polycyclic Aromatic Hydrocarbons in an Organism

According to current knowledge, some PAH are able to interact in organisms with enzymes (such as aryl hydrocarbon hydroxylases) to form PAH dihydrodiol derivatives. These reactive products (so-called “bay region” dihydrodiol epoxides) are believed to be ultimate carcinogens that are able to form covalently bounded adducts with proteins and nucleic acids. In general, deoxyribonucleic acid (DNA) adducts are thought to initiate cell mutation, resulting in a malignancy [11]. A direct mutagenic potential of 14 PAH and PAH, containing fractions isolated from smoked and charcoal-broiled samples, was studied for strains TA 98 and TA 100 using the Ames test. The greatest potential mutagenicity was observed with PAH fractions isolated from smoked fish treated before

smoking with nitrites in an acid solution [14]. To simplify an interpretation of the real risk of PAH to human health, there have been attempts to express objectively the risk using toxic equivalency factors (TEF) [15]. However, this approach does not reflect wider aspects of the potential toxicity of oxidized PAH products due to the effect of ultraviolet (UV) light or other environmental factors [16]. Moreover, PAH content in smoked foods can be affected not only by environmental factors, but also by diffusion processes from plastic packaging materials [17].

33.1.2.2 *Legislative Aspects and International Normalization of Polycyclic Aromatic Hydrocarbons in Smoked Meat and Liquid Smoke Flavor*

With regard to the harmful effects of PAH on living organisms, some European countries have enacted maximum limits for these compounds in smoked meat products. To simplify problems associated with the variability of PAH composition, benzo[*a*]pyrene (BaP) has been accepted as the indicator of total PAH presence in smoked foods, although BaP constitutes only between 1 and 20% of the total carcinogenic PAH [18]. At present, the situation in the European Union (EU) has been resolved by adoption of the European Commission (EC) Regulation 208/2005 limiting BaP content to a level of 5 $\mu\text{g kg}^{-1}$ in smoked meats, smoked meat products, muscle meat of smoked fish, and smoked fish products. The regulation entered into a force as of February 28, 2005, to be applied from April 1, 2005. The EC has also adopted Directive 2005/10/EC, describing sampling methods and methods of analysis for the official control of BaP levels in foodstuffs and the recommendation 2005/108/EC on the further investigation into the levels of PAH in certain foods, such as benzo[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF), benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), benzo[*g,h,i*]perylene (BghiP), chrysene (Chr), BaP, cyclopenta[*c,d*]pyrene (CcdP), dibenzo[*a,h*]anthracene (DahA), dibenzo[*a,e*]pyrene (DaeP), dibenzo[*a,h*]pyrene (DahP), dibenzo[*a,i*]pyrene (DaiP), dibenzo[*a,l*]pyrene (DalP), indeno[*1,2,3-cd*]pyrene (IcdP), and 5-methylchrysene. The Joint Expert Committee on Food Additives (JECFA) of FAO and WHO (JECFA) has defined another compound benzo[*c*]fluorene (BcF), which should also be monitored with regard to its effects on living organisms. Concerning LSF, the EC has adopted Regulation 2065/2003, relating to the production of smoke flavorings intended to be used for food flavoring. This regulation limited the maximum acceptable concentrations of BaP to 10 $\mu\text{g kg}^{-1}$ and BaA to 20 $\mu\text{g kg}^{-1}$ in these products. Finally, the Directive 88/388/EEC limited the maximum residual levels of BaP to 0.03 $\mu\text{g kg}^{-1}$ in foodstuffs flavored by LSF. For international trade purposes, JECFA has adopted a specification that tolerates the concentration in LSF at the levels of 10 $\mu\text{g kg}^{-1}$ for BaP, and 20 $\mu\text{g kg}^{-1}$ for BaA [19].

33.2 Analysis of Polycyclic Aromatic Hydrocarbons

Owing to the fact that PAH are present in food at the micrograms per kilogram levels, analysis usually consists of such steps as extraction/hydrolysis of food matrix, liquid/liquid partition, cleanup procedures, concentration, chromatographic separation, and, of course, determination. Although all steps are very important, chromatographic separation is the most important for correct evaluation of real risk assessment; for example, while BaP is a very strong carcinogenic agent, the carcinogenic activity of its isomer benzo[*e*]pyrene (BeP) is quite low. The methodology of PAH analysis has been strongly affected by levels of development of chromatographic methods. In the middle of the last century, a separation of BaP isomers by paper and column chromatography was

practically impossible [20]. With regard to complex mixtures of PAH, the presence of a variety of interfering substances and the need to assess correctly the concentrations of the most dangerous compounds made it necessary to overcome problems regarding resolution of so-called “benzopyrene fraction,” which consisted of BaP and its isomer BeP, BkF, BbF, and perylene (Per). In 1968, at a joint meeting of Indiana University Cancer Center and the International Agency for Research on Cancer, it had been specified that any acceptable analytical method should be capable of separating at least BaA, BaP, BeP, BghiP, pyrene (Py), BkF, and Cor [21]. Collaborative studies of a method specific for BaP and a general procedure for PAH were conducted under the auspices of the Association of Official Analytical Methods (AOAC) and the International Union of Pure and Applied Chemistry (IUPAC). Procedures consisted of an initial saponification of the sample in ethanolic potassium hydroxide solution, followed by a partition step involving dimethylsulfoxide (DMSO) and an aliphatic solvent, followed by column chromatography on pretreated Florisil. For determination of individual PAH, a cellulose reverse-phase technique in conjunction with cellulose acetate multiphase technique was used. This method was adopted as an AOAC official first action method in 1973 and accepted as a recommended method by IUPAC. Statistical evaluation of the data obtained by interlaboratory tests, in which ham samples were fortified with BaP, BeP, BaA, and BghiP at a level of $10 \mu\text{g kg}^{-1}$ and analyzed by the aforementioned method, showed standard deviation between 7.4 and 12.7%. On this basis, the method has been adopted as official method of the AOAC [22].

33.2.1 Sample Preparation

Smoked meat and LSF represent two different matrices, which have in common the organoleptic profile and compounds to be determined. For this, various procedures for sample pretreatment are taken to reach the highest recoveries of analytes possible.

33.2.1.1 Sample Treatment of Smoked Meat

From an analytical point of view, meat and its products belong to problematic matrices with regard to the presence of various interfering compounds. Moreover, PAH, as lipophile compounds, have a tendency to diffuse not only into the nonpolar part of the sample but also inside tissue cells depending on the existing concentration gradient. For this reason a simple solvent extraction with nonpolar solvent seems to be insufficient to reach high recovery. Grimmer and Böhnke [13] isolated PAH from smoked fish and smoked-dried cobra with boiling methanol prior sample hydrolysis with methanolic KOH. It was found that only about 30% of BaP and other PAH was extractable from the samples, whereas an additional alkaline hydrolysis of meat protein yielded another 60% of PAH. It was concluded that PAH were linked adsorptively to high molecular-weight structures not destroyed with boiling methanol. Although more than 80% of the methanol used could be recovered, this contained only one-third of the PAH contained in sample. As postulated, alkaline hydrolysis with aqueous methanolic KOH is an absolute necessity to isolate PAH quantitatively from such samples. Alkaline hydrolysis usually takes 2–4 h of time, depending on the character of the sample. Lean tissues take less time than adipose and collagen containing tissues. This sample treatment has been adopted in many experimental works [23–26]. On the other hand, in a study by Vassilaros et al. [27], the use of an alcohol is superfluous and contributes to interference problems because of methyl esters formed from fatty acids and methanol, which are than difficult to remove from the PAH fraction. Takatsuki et al. [28] found that during alkaline

hydrolysis BaP may be partially decomposed by the coexistence of alkaline conditions, light oxygen, and peroxides in aged ethyl ether. They proposed to use amber glass, the addition of Na₂S as an antioxidant, distillation with ethyl ether just before use, and prevention of air from contact with adsorbents. To protect PAH from light decomposition, Karl and Leinemann [29] used brown glassware carefully rinsed with acetone before using an alkaline hydrolysis. Some authors also recommended direct extraction with organic solvents. Potthast and Eigner [30] proposed a procedure based on mixing of preground sample with chloroform and anhydrous Na₂SO₄ to remove water from the extract. After adding Celite, the portion became uniformly distributed over the surface of the adsorbent. Although the authors achieved a recovery 95–100% of added BaP at a level of 10 µg, there is an assumption that they recovered only “free” PAH accessible with solvent. This procedure was also used in the work of Alonge [8]. Cejpek et al. [31] tested the efficiency of several organic solvents to obtain fat from meat samples. The most efficient solvent was a mixture of chloroform:methanol (2:1); less effective was chloroform; and the worst yields were achieved with methanol. This confirms observations of Grimmer and Böhnke [13] regarding the inability of methanol to extract quantitatively PAH from meat samples. The chloroform–methanol mixture, called the Folch agent, is widely used in food analysis for the extraction of lipids, while methanol makes possible the extraction of lipids from inside cells by denaturation of the cell wall proteins. Joe et al. [32] digested samples of smoked food with KOH, with PAH extracted with Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane). Chen et al. [33] compared the efficiency of extraction from freeze-dried sample using sonication and Soxhlet procedures. Recovery studies showed that Soxhlet extraction was more suitable than the sonication method. An accelerated procedure of extraction was tested by Wang et al. [34]. Samples were extracted in a Dionex extractor as well as a Soxhlet apparatus. Advanced solvent extraction (ASE) technique was found to be comparable to or even better than the reference Soxhlet method, and significant reductions in time of extraction and solvent consumption were achieved. García-Falcón et al. [35] accelerated extraction of PAH from freeze-dried samples into hexane with microwave treatment and hexane extract, then saponified with ethanolic KOH.

33.2.1.2 *Sample Treatment of Liquid Smoke Flavors*

Sample treatment of LSF matrix is different from the treatment of processed meats due to easy access of organic solvent “inside” a liquid matrix. For this, there is not usually any reason to treat samples by time-consuming hydrolysis under reflux. Other situations could arise when LSF are in solid state (e.g., applied on starch, gelatine, or encapsulated). Despite this, some authors preferred alkaline hydrolysis of liquid LSF under reflux. However, addition of KOH is strongly recommended to transform phenols to polar, nonextractable phenolates prior the PAH extraction with a nonpolar solvent. White et al. [36] alkalinized water-soluble LSF (and also resinous condensates that settled out of LSF after storage) with KOH solution and extracted PAH into isooctane. Silvester [37] extracted PAH from alkalinized liquid SFA with hexane. Radecki et al. [38] alkalinized LSF with ethanolic KOH solution and maintained it at 60°C for 30 min prior to extraction into cyclohexane. After alkalization, a direct extraction of PAH with cyclohexane was used by Šimko et al. [39]. On the other hand, Gomaa et al. [40] saponified liquid LSF with methanolic KOH for 3 h and then extracted PAH into cyclohexane. Laffon Lage et al. [41] used a solid-phase extraction (SPE) technique on Sep Pak C18 for PAH isolation and compared it to the supercritical fluid extraction (SFE) procedure, in which the sample for SFE was mixed with alumina and extracted PAH were concentrated in an octadecylsilane (ODS) trap. In both cases, 91% recoveries of BaP spiked at 15 ng were found and no statistically significant differences were observed. Taking into account

the expensive SFE extractor, they recommended the use of the simple SPE procedure. Guillén et al. [42,43] alkalinized LSF with methanolic KOH and heated under reflux for 3 h, following with extraction of PAH into dichloromethane or cyclohexane.

33.2.2 Preseparation Procedures

At this time, both procedures are more or less equivalent for processed meats and LSF. But sometimes, mainly after adipose tissue hydrolysis, a presence of lipoproteins in nonpolar solvent requires removal prior to preseparation with a one-step liquid–liquid partition between nonpolar and polar solvent (e.g., hexane–water/dimethylformamid [13], methanol/water, or DMSO/water–cyclohexane [26,29]), a two-step liquid–liquid partition (e.g., NaCl/water and dimethylformamide/water [44]), or precipitation of lipoproteins with Na_2WO_4 [6,45–47]. For preseparation, deactivated Florisil [6,26,34,40,43,47–49], silica gel [25,28,48], alumina [44], and Celite [36,37] are used frequently. Only one study [37] reported that elution of BaP from Florisil and silica gel with hexane was impossible, and for this reason alumina was recommended for preseparation of concentrated PAH extracts. Guillén et al. [44] preferred elution of silica with cyclohexane prior to Florisil dichloromethane elution to obtain higher recoveries, with reduced amounts of interfering substances, which were eluted from Florisil with dichloromethane. Another preseparation procedure is gel permeation chromatography (GPC) on Sephadex LH 20 [28] or BioBeads S-X3 [31]. Mottier et al. [48] cleaned concentrated cyclohexane extracts by SPE, using conditioned isolate aminopropyl and C_{18} columns. Also, the use of two different cleaning techniques is possible, with cyclohexane extract first cleaned with GPC on Sephadex LH 20, then cleaned on silica gel [44]. The last procedure can also be carried out in reverse mode [9]. In all cases, removal of organic solvents by vacuum evaporation to concentrate PAH is an unavoidable operation. This may be a critical step, especially if there is a presumption of the presence of light PAH such as fluorene (Flu), anthracene (Ant), or phenanthrene (Phe) in the extracts. In this case, organic solvents should not be evaporated to dryness because these PAH could be lost due their volatility. This cautious manipulation is not necessary if only PAH with boiling points above 370°C are determined [13].

33.2.2.1 Thin-Layer Chromatography

Thin-layer chromatography (TLC) is one of the older analytical methods used for determination of PAH in various matrices. Haenni [50] discussed the development of analytical tools for control of PAH in food additives and in food by the use of UV specification within specific wavelength ranges. Schaad [20] reviewed various chromatographic separation procedures, including TLC. White et al. [36] used two systems for PAH separation. The first consisted of 20% *N,N*-dimethylformamide in ethyl ether as the stationary phase and isooctane as the mobile phase. Fluorescent spots were scraped out from cellulose layer and eluted with hot methanol. After concentration, the sample was developed in the second system, using ethanol–toluene–water (17:4:4) as developer. Fluorescent spots were eluted again from the cellulose acetate layer and a UV spectrum was recorded against isooctane in a reference cell. The observed maxima were compared with those in the spectra of known PAH obtained under the same instrumental conditions. Estimation of the quantity of the identified compounds was made by the baseline technique in conjunction with spectra of these PAH and the identification was confirmed by spectrophotofluorometry. This method has become a base of AOAC Official Method 973.30, adopted in 1974 [22].

33.2.2.2 Gas Chromatography

Currently, gas chromatography (GC) is widely used for determination of PAH in food analysis. The determination of the large number of PAH in samples requires columns with high efficiency. To separate some critical pairs as well as isomers of methyl derivatives of certain PAH, capillary columns (50 m × 0.3–0.5 mm) which can achieve 50,000–70,000 high equivalent theoretical plate (HETP) are especially convenient. However, packed columns used for determination of PAH [13] had lower HETP, ranging between 20,000 and 30,000, and for this reason were not suitable for quantity determination. Two stationary phases, OV-17 and OV-101, were used for separation of BaP from BeP, DajA from DahA, and Phe from Ant. Successful separation of Chr from BaA was achieved using the OV-17 stationary phase, but separation of BbF, BjF, and BkF isomers on packed columns was not possible [13]. Radecki et al. [38] tested various stationary phases (GE SE 30, OV-1, SE-52, OV-7, OV-101, BMBT, BBBT) on Chromosorb W, Chromosorb W HP, Gas Chrom, and Diatomite CQ supports in packed columns to develop a precise GC method for assaying BaP in LSF. Separation of BaP from BeP and Per was not possible using SE 30, OV-1, SE-52, OV-7, or OV-101 stationary phases. Nematic phases gave a good separation of BaP from its isomers, but they were not suitable for analysis due to their poor thermal stability. Detection of PAH is not a serious problem, because the response of a flame ionization detector (FID) is practically equal for all compounds and is linear over a large concentration range (about 1–1.10⁶), according to the carbon content. However, the use of FID is sometimes hampered by the need for very thorough cleanup procedures with the accompanying risk of severe losses and possible misidentification [51]. A mass spectrometry detector (MSD) has also successfully been used for PAH analysis in many cases [52]. In particular, the use of MSD operating in selected ion monitoring mode makes it possible to simplify the time-consuming cleanup procedure [51], and it is recommended especially for quantitative analysis. The ion trap detector (ITD) has some advantages over traditional MSD. The ITD utilizes electric fields to hold ions within the ion storage regions. The ITD is then scanned through the mass range, causing the ions to be ejected from this region sequentially, from low to high mass. The ejected ions are detected by a conventional electron multiplier. Thus, the characteristic of the ITD is that ionization and mass analysis take place in the same space. This contrasts with a conventional MSD, which requires a separated ionization source, focusing lenses and analyzer [53]. Sometimes, separation of isomers is quite a serious problem even when capillary columns are used. Dennis et al. were not able [54] to separate BjF from BkF. Speer et al. [55] were not able to separate Chr from triphenylene (Tph); BbF, BjF, and BkF from each other; or DahA from DacA. Problems associated with separation of Chr from Tph are also reported in works of Guillén et al. [42,43]. Wise et al. [56] discussed difficulties in separating isomers BbF and BkF. On the other hand, Chen and Chen [57] separated BbF and BkF sufficiently on a DB-1 fused silica capillary column. Review of pre-separation procedures as well as GC conditions to be used for determination of PAH in smoked meat products and LSF are summarized in Table 33.1.

33.2.2.3 High-Pressure Liquid Chromatography

In recent years, the high-pressure liquid chromatography (HPLC) method has been used intensively for determination of PAH in food, as reported in review works [11,58,59]. Formerly used stationary phases such as alumina and silica gel were later replaced with chemically bonded phases, particularly reverse phases such as ODS, widely used at the time. For determination of PAH in food, Hunt et al. [60] developed a phthalimidopropylsilane (PPS) stationary phase and compared

Table 33.1 Preseparation Procedures as Well as GC Conditions to Be Used for Determination of PAH in Smoked Meat Products and LSFs

Sample	Sample Treatment and Preseparation	Column/Stationary Phase	Temperature Program	Detection	Reference
Barbecued sausages	Saponification with mixture of ethanol, water, and KOH, extraction with cyclohexane, preseparation by SPE on isolate aminopropyl and C ₁₈ columns	25 m × 0.2 mm capillary column/SPB-5	80°C for 0.5 min → 230°C at 8°C min ⁻¹ → 300°C at 5°C min ⁻¹	MSD	48
Smoked fish	Extraction with pentane, precleaning on silica gel and Sephadex LH-20	25 m × 0.2 mm quartz capillary column/SE-54	100 → 260°C, 3°C min ⁻¹	MSD	9
Smoked fish	Saponification in methanolic KOH, liquid-liquid extraction (methanol-water-cyclohexane and DMF-water-cyclohexane), and GPC on Sephadex LH 20	10 m × 2 mm packed columns/5% OV-101 and OV-17 on sorbent Gas Chrom.	120 → 250°C, 1°C min ⁻¹ 250°C isothermal	FID, MSD	13
Smoked sausages	Saponification in methanolic KOH, liquid-liquid extraction (methanol-water-cyclohexane and DMF-water-cyclohexane), precleaning on silica gel, and GPC on Sephadex LH 20	10 m × 2 mm packed column/5% OV-101 on sorbent Gas Chrom.	260°C isothermal	FID	23
Smoked meat products	Saponification with mixture of methanol, water, and KOH, partition with DMF, precleaning on Kiesel gel 60	25 m × 0.28 mm capillary column/ SE-54	240°C isothermal	MSD	24
Smoked fish and fish products	Saponification in methanolic KOH, liquid-liquid extraction (methanol-water-cyclohexane and DMF-water-cyclohexane), precleaning by CC on silica gel, and GPC on Sephadex LH 20	55 m × 0.3 mm glass capillary column/SE-54	165°C for 6 min, 165 → 255°C, at 4°C min ⁻¹	FID	25
Smoked fish, smoked meat spreads	Saponification with mixture of methanol, water, and KOH, extraction with cyclohexane, cleanup on Florisil, partitioning with DMSO/hexane	30 m × 0.25 mm capillary column/DB-5	25 → 180°C rapidly → 320°C at 8°C min ⁻¹	FID, MSD	26
Smoked fish	Saponification with methanol-water-KOH mixture under reflux, extraction into cyclohexane, extraction of PAHs with caffeine/formic acid, washing with NaCl solution, extraction into cyclohexane, preseparation on silica gel	30 m × 0.25 mm capillary fused silica column/DB-5	110°C isothermal for 1.5 min → 210°C at 30°C min ⁻¹ → 290°C at 3°C min ⁻¹ → 300°C at 10°C min ⁻¹	MSD	29

(Continued)

Table 33.1 (Continued)

Sample	Sample Treatment and Preseparation	Column/Stationary Phase	Temperature Program	Detection	Reference
Smoked salmon, sausages, pork LSF	Direct solvent extraction (ASE), cleanup on Florisil	30 m × 0.25 mm capillary column/cross-linked 5% phenyl methyl siloxane HP-5MS	40°C isothermal for 1 min → 250°C at 12°C/min → 310°C at 5°C/min	MSD	34
LSF	Alkalinization with KOH solution, extraction with cyclohexane, cleanup on silica	25 m × 0.2 mm fused silica capillary column/HP-5 cross linked with 5% henylmethylsilicone	50°C isothermal for 0.5 min → 180°C at 30°C min ⁻¹ → 300°C at 7°C min ⁻¹	MSD	39
LSF	Heating with methanolic KOH under reflux, extraction with cyclohexane, cleanup by SPE technique on Florisil	60 m × 0.25 mm fused silica capillary column/HP-5MS, 5% phenyl methyl siloxane	50°C isothermal for 0.5 min → 130°C at 8°C min ⁻¹ → 290°C at 5°C min ⁻¹	MSD	42
LSF	Heating with methanolic KOH under reflux, extraction with cyclohexane, cleanup by SPE technique on LC silica	60 m × 0.25 mm fused silica capillary column/HP-5MS, 5% phenyl methyl siloxane	50°C isothermal for 0.5 min → 130°C at 8°C/min → 290°C at 5°C/min	MSD	43
Smoked meats	Saponification with methanolic KOH, extraction with cyclohexane, partition with DMF/water, cleanup on silica gel, and with GPC on Bio Beads S-X3	50 capillary column/DB-5	70 → 280°C at 5°C min ⁻¹	MSD	55
Smoked chicken	Extraction with methanol in Soxhlet app., + KOH, extraction into <i>n</i> -hexane, cleanup on Sep-Pak Florisil cartridge	30 m × 0.32 mm/DB-5	70°C isothermal for 1 min → 150°C at 10°C min ⁻¹ → 280°C at 4°C min ⁻¹ hold for 14 min	ITD	63

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it with ODS. The PPS column was able to separate BkF from Per, which was impossible by ODS column. HPLC has some advantages in PAH analysis, as follows [58]:

- Separation of isomers shows very good resolution
- Sufficient sensitivity and specificity of ultraviolet detection (UVD) and fluorescence detection (FLD)
- Molecular sizes of PAH can be estimated on the base of retention time using a reversed-phase (RP) column
- Ability to determine compounds with high molecular weight
- Analysis is usually carried out at ambient temperature; there is no risk of thermal decomposition of analytes

HPLC equipped with MSD is an effective tool for characterization of high molecular-weight, thermally unstable compounds; for example, BaP metabolites were identified and determined by this method in microbore mode [61]. Owing to a high absorption of light in the UV part of spectrum and intensive fluorescence (FL), both types of detectors are able to detect reliable concentrations at the micrograms per kilogram levels. On the other hand, measurements by nonspecific detection systems, particularly optical detectors, though often precise, can be much less accurate due to possible chemical interferences not having been chromatographically resolved or otherwise avoided prior to the measurement. The major impurities in the PAH fractions appear to be alkylated PAH, which have responses in optical detection systems very similar to their unsubstituted analogs [62]. Regarding diode array detector (DAD), confirmation of peak purity and identification is possible, but due to the broad absorption bands in UV spectra it is highly probable that there will be some interference if one particular wavelength is chosen for quantification. In any case, identification must be based on retention time. The FL detector provides very high selectivity and sensitivity, particularly those with excitation and emission wavelengths that can be varied throughout the analysis. However, FL suffers from not being able to provide "broad-spectrum" analyses (i.e., a wide variety of compounds) because of the presence of alkylated PAH compounds. Review of preseparation procedures as well as HPLC conditions to be used for determination of PAH in smoked meat products and SFA are summarized in Table 33.2.

33.2.3 *Comparison of Gas Chromatography and High-Pressure Liquid Chromatography*

In many works, authors studied advantages and drawbacks of both methods, with studies aimed especially at recovery procedures, quality of separation processes, time of analysis, price of equipment, etc. Dennis et al. [54] compared results of analysis of some food (two smoked) obtained by GC and HPLC. Thirty-five pairs of analyses were tested using statistical procedure (student *t*-test). Of these, 25 were not significantly different within the 95% confidence limits employed. But data for BkF/benzofluorantenes and DahA/dibenzoanthracenes were not compared because different analytes were measured. Standard deviations indicated that repeatability of both methods was very good, usually within 10%, and provided comparable data throughout a wide range (0.2–1000 $\mu\text{g kg}^{-1}$). In the conclusion of this study it was stressed that capillary GC possessed a much greater resolving power, in terms of plate number, so that many more PAH can be separated and determined. On the other hand, HPLC was able to separate individual isomers (BbF and BkF; Chr and Tph); that is, it had greater selectivity. Chiu et al. [63] compared separation and

Table 33.2 Preparation Procedures as Well as HPLC Conditions to Be Used for Determination of PAH in Smoked Meat Products and LSFs

Sample	Sample Treatment and Preseparation	Column/Stationary Phase	Mobile Phase	Detection	Reference
LSF, smoked meats	Saponification with ethanolic KOH, extraction into cyclohexane, washing with saturate NaCl solution, cleanup on silica gel	25 cm × 4 mm Lichrosorb RP 18	Acetonitrile/water 8:2, isocratic, 1.5 mL min ⁻¹	FLD Ex: 305, 381 nm Em: 389, 430, 520 nm	7
Smoked meat products	Saponification with mixture of methanol, water, and KOH, extraction with cyclohexane, washing with Na ₂ WO ₄ solution, cleanup on Florisil	30 cm × 3 mm, Separon SGX C ₁₈ RP, 5 μm	Acetonitrile/water 3:1, isocratic, 1.5 mL min ⁻¹	FLD Ex/Em 310/410 nm	6,45–47
Smoked fish, smoked meat spreads	Saponification with mixture of methanol, water, and KOH, extraction with cyclohexane, cleanup on Florisil, partitioning with DMSO/hexane	25 cm × 4.6 mm, RP–18, 5 μm	Acetonitrile/water 7:3, isocratic, 3 mL min ⁻¹	UVD 254 nm FLD Ex/Em 250/370 nm	26
Fish, shellfish	Saponification with methanol–water–KOH mixture under reflux, extraction into <i>n</i> -hexane, cleanup on silica gel	Radial-Pak PAH	Acetonitrile/water 8:2, isocratic, 1 mL min ⁻¹	FLD Ex/Em 370/410 nm	28
Smoked fish	Saponification with methanol–water–KOH mixture under reflux, extraction into cyclohexane, extraction of PAHs with caffeine/formic acid, washing with NaCl solution, extraction into cyclohexane, preseparation on silica gel	ET 15 cm × 4 mm, Nucleosil 5 C ₁₀ PAH	Acetonitrile/water 7:3 for 1 min, then gradient linearly up to 9:1 in 19th min, then to 100% acetonitrile from 20 to 40 min, then isocratic till 55 min	UVD 240, 254, 260 nm FLD Ex/Em 300/408 and 280/395 nm	29
Smoked sausage, smoked meat	Extraction with chloroform/methanol mixture, preseparation by GPC on Bio Beads S-X3	15 cm × 4.6 mm Supelcosil LC PAH, 5 μm	A: methanol/acetonitrile/water 50:25:25 B: acetonitrile; 1 min 100% A, 25th min 100% B	FLD Variable Ex (240–293) Em (340–498) nm	31

Smoked frankfurters, smoked meats	Extraction with methanol in Soxhlet app. + KOH, extraction into <i>n</i> -hexane, cleanup on Pep-Pak Florisil	12.5 cm × 4.6 mm Envirosep-pp C ₁₈ 5 μm	I. Acetonitrile/water 7:3, isocratic, 2 mL min ⁻¹ II. Acetonitrile/water 40:60, gradient to 100% acetonitrile within 25 min III. Acetonitrile/water 55:45, gradient to 100% acetonitrile within 23 min	UVD 230–360 nm FLD Variable Ex (232–302) Em (330–484) nm	33
LSF	Alkalinization with NaOH solution, extraction with hexane, cleanup on alumina	25 cm × 4.6 mm Partisil 10 ODS	Methanol/acetonitrile/water 35:35:30, isocratic	FLD Ex/Em 280/390 nm	37
LSF	Alkalinization with ethanolic and aqueous NaOH, extraction into cyclohexane, partitioning with DMSO/water, extraction into cyclohexane	30 cm × 4 mm, μBondapak C ₁₈ /Corasil	Methanol/water 7:3, 2 mL min ⁻¹	UVD 280 nm	38
LSF, smoked food products	LSF: Saponification with methanolic KOH, extraction into cyclohexane, purification on Florisil Meat products: digestion with KOH solution, extraction with Freon 113, purification on Florisil	25 cm × 4.6 mm, Supelcosil LC-PAH	Acetonitrile/water 60:40 for 5 min, then 100% of acetonitrile in 15 min hold for 15 min, then decrease to 60% over 10 min	FLD Ex/Em 254/375 nm	40
Smoked fish	Direct extraction with chloroform, prepreparation on preparation silica column	Preparation column: 25 cm × 4.6 mm, silica 5 μ Analytical column: 15 × 4.6 mm 5 μ particle, Supelcosil LC-PAH	Preparation column: pentane/5% DCM, 0.8 mL min ⁻¹ Analytical column: water/acetonitrile 6:4 for 5 min, then to 100% acetonitrile over 40 min, 1.5 mL min ⁻¹	FLD Variable nm	63
Smoked fish, ham	Saponification with mixture of methanol, water, and KOH, extraction with cyclohexane, partitioning with DMSO/hexane	Spherisorb ODS 5 μm precolumn and 5 μm VydacODS analytical column	Acetonitrile/water 6:4, linearly to 9:1 over 35 min	FLD Ex/Em 290/430 nm	54

(Continued)

Table 33.2 (Continued)

Sample	Sample Treatment and Preseparation	Column/Stationary Phase	Mobile Phase	Detection	Reference
Smoked meats products	Saponification with methanolic KOH, extraction with <i>n</i> -hexane, preparation by SPE on CN bonded silica	Nucleosil 100–5 C 18 PAK	Acetonitrile/water 8:2, isocratic, 0.5 mL min ⁻¹	FLD Ex/Em 290/430 nm	64
Smoked fish	Saponification with methanol–water–KOH mixture under reflux, extraction into <i>n</i> -hexane, cleanup on silica gel	15 cm × 6 mm, ODS, 5 μm particles, 1 mL min ⁻¹	Acetonitrile/water 8:2, isocratic	FLD Ex/Em 370/410 nm	65
LSF, smoked foods	LSF: Saponification with methanolic KOH, extraction into cyclohexane, purification on Florisil	12.5 cm × 4 mm Lichrosphere 100 RP–18	A: water; B: methanol/ acetonitrile 1:1 I. segment: 1:80–100% B for 20 min II. segment: 100% B for 5 min III. segment: 100–80 B for 5 min	FLD Ex/Em 365/418 nm	66
Smoked meat products	Smoked products: digestion with KOH solution, extraction with Freon 113, purification on Florisil	12.5 cm × 4 mm, Chrompack PAH-Säule	Acetonitrile/water 9:1, isocratic, 0.5 mL min ⁻¹	FLD Ex/Em 290/430 nm	67
Smoked chicken	Saponification with methanolic KOH, extraction into cyclohexane, preparation by SPE on Kiesel gel Extraction with methanol in Soxhlet app. + KOH, extraction into <i>n</i> -hexane, cleanup on Sep-Pak Florisil	12.5 cm × 4.6 mm Envirosep-pp 5 μm C ₁₈	Acetonitrile/water 55:45, gradient to 100% acetonitrile within 23 min 1.2 mL min ⁻¹	FLD Variable nm	68

Note: Ex, Excitation and Em, Emission.

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detection conditions of both methods analyzing smoked chicken. As found, 16 priority PAH pollutants defined by the Environmental Protection Agency (EPA) can be separated simultaneously by HPLC using a gradient solvent system and detection by FLD at variable wavelength settings due to different excitation/FL spectra. The same mixture can also be separated successfully by GC using an appropriate temperature program. The presence of impurities in smoked meat products can interfere with the identification and quantification of PAH by HPLC. With ITD, the PAH can be identified even in the presence of fat- or PAH-like impurities. The retention times by HPLC were shorter than those by GC, while HPLC had better separation for most compounds than GC. Sim et al. [62] compared GC and HPLC methods analyzing 16 PAH pollutants. Chromatographic resolution involves a combination of column capacity, column efficiency, and separation selectivity. GC has a higher column efficiency and thus has an advantage for complex mixture analysis, but HPLC can often have a higher column selectivity, which is more suitable for separation of isomeric compounds. Thus, the two methods should be viewed as complementary in the analysis of PAH, and they are essential for precise and reliable analysis.

33.2.4 Occurrence of Polycyclic Aromatic Hydrocarbons

After gleaning information regarding carcinogenic effect, research workers started to find real situations of PAH content in smoked meat products. These data prove that technologically correct smoking process contaminate meat products with only small levels of PAH content—usually below $1 \mu\text{g kg}^{-1}$. Far more dangerous is the smoking process under uncontrolled conditions, typical of home “wild” smoking in the preparation of heavily smoked “farm” products, as well as smoking being done in developing countries, without any technological knowledge or hygienic control. These products bring a serious real risk to consumer in terms of cancer, especially after a long period of consumption due to BaP content reaching even up to $100 \mu\text{g kg}^{-1}$ [69].

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Chapter 34

Detection of Irradiated Ingredients

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34.1 Introduction to Food Irradiation

Food irradiation is a process by which food is exposed to ionizing radiation in a controlled manner, either using gamma rays (produced mostly from cobalt 60) or by electron beams or x-rays (generated electrically). These are high-energy sources, which act in the same way to bring about changes to the foodstuff. When food is irradiated, energy is absorbed, and it is this absorbed energy that leads to the ionization or excitation of the atoms and molecules of the food, which in turn results in chemical changes. These changes may result from “direct” or “indirect” action. In “direct” action, a sensitive target such as the deoxyribonucleic acid (DNA) of a living organism is damaged directly by an ionizing particle or ray, whereas “indirect” action is caused mostly by the products of water radiolysis, which disappear quickly by reacting with each other or with other food components [1].

The use of ionizing radiation as a preservation method for foodstuffs is not new. In 1896 H. Minsch, Germany, published a proposal to use ionizing radiation for the preservation of food by destroying spoilage microorganisms. Thus, there is a long history of research on the radiation processing of foodstuffs, including extensive safety studies on irradiated food [2]. In 1980, the Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food (JECFI) met in Geneva, and their landmark report published in 1981 concluded that the “irradiation of any food commodity up to an overall average dose of 10 kGy presents no toxicological hazard.” The Committee also concluded that irradiation up to 10 kGy “introduces no special nutritional or microbiological problems” [3].

As a result of the JECFI report [3], in 1983 the Codex Alimentarius Commission (CAC) adopted the Codex General Standard for Irradiated Foods and the Recommended Code of Practice for the Operation of Radiation Facilities Used for the Treatment of Foods. Irradiated food in international trade should therefore conform to the provisions of the Codex General Standard and recommended Code of Practice. In an effort to harmonize the law of the Member States on food irradiation, the European Union (EU) adopted framework Directive 1999/2/EC and implementing Directive 1999/3/EC [4]. The framework directive sets out the general and technical aspects for carrying out food irradiation, labeling of irradiated foods, and the conditions for authorizing the process, whereas the implementing directive established an initial “positive list” specifying food categories that may be irradiated and freely traded in the EU. The list is still under discussion and currently includes only dried aromatic herbs, spices, and vegetable seasonings. Until this list is complete, EU Member States may continue to apply their own existing national authorizations of irradiated foodstuffs not included in the initial “positive list.”

The two main drivers for treating foods with ionizing radiation are the enhancement of food safety and of trade in agricultural products [5]. The process should not be used as a substitute for

good manufacturing practices, but rather as a means of reducing risk. As food poisoning bacteria are highly sensitive to ionizing radiation, food irradiation has a proven efficacy for destroying microorganisms of public health importance, for example, *Escherichia coli* O157:H7 and *Salmonella* spp., as well as controlling parasitic organisms, such as *Trichinella spiralis*. According to Molins et al. [6], irradiation could be a critical control point in ensuring the microbiological safety of raw foods such as poultry, meat, meat products, fish, seafood, fruits, and vegetables.

Food irradiation can be used to extend the shelf life of perishable foods such as fruits, vegetables, meat, and meat products. As an example, spoilage bacteria such as *Pseudomonas putida* found in poultry meat are highly sensitive to irradiation, thus treatment with doses of 2–3 kGy can extend shelf life by as much as 2 weeks when combined with refrigeration.

Another beneficial use of the process is the prevention of food losses by inhibition of sprouting in bulb and tuber crops. Irradiation of potatoes to prevent sprout inhibition is carried out in Japan with approximately 16,000 t of irradiated potato per annum being distributed on the domestic market [7].

Irradiation is a “cold process,” and thus is suitable for reducing the microbial load in herbs, spices, and seasonings. It is an effective alternative to using chemical fumigants such as ethylene oxide, which are now banned for use in Europe and the United States. One of the benefits of using ionizing radiation is that it does not cause any adverse changes to the important quality characteristics of herbs and spices such as color, aroma, or flavor.

Quarantine security is required to protect the ecology and agriculture of importing regions from pests that may be present on imported goods, while facilitating trade between different regions [5]. Research has demonstrated the suitability of ionizing radiation for the disinfection of cereals, grains, and certain fruits, such as mango and papaya [8], thus the process could play a significant role in fulfilling quarantine needs.

The use of ionizing radiation is, however, not suitable for all food products. Its use for the treatment of foods with a high fat content may lead to off-odors and tastes, as ionizing radiation is known to accelerate rancidity, and food with a high amount of protein can have changes in flavor and odor. It is therefore important that the suitability of a foodstuff is rigorously assessed before treatment and the irradiation conditions optimized to ensure a product of highest quality.

34.2 Can Irradiated Foodstuffs Be Identified in the Marketplace?

Irradiated food on sale in the marketplace should be clearly labeled so that consumers can choose whether or not to buy it. Under EU regulations, and those of other countries, irradiated food must be clearly labeled as “irradiated” or “treated with ionizing radiation.” Such labeling should allow consumers to make informed choices about their food purchases. Thus, if a food is being marketed as irradiated or if irradiated goods are being sold without the appropriate labeling, then detection tests should be able to prove the authenticity of the product.

The reasons for the development of detection methods for irradiated foods can be summarized as follows:

- To control any legislative prohibitions regarding irradiation of specific foods, for example, reirradiation
- To control limitations imposed on the irradiation process
- To control the labeling of irradiated foodstuffs

- To enhance consumer confidence in the correct application of the radiation process and its proper control by the inspection authorities
- To protect the consumers' freedom of choice between irradiated and nonirradiated food products [9,10]

Before the 1980s, little progress was made in the development of detection methods for irradiated foods. The lack of emphasis was partly due to the fact that detection methods were considered unnecessary, because it was believed that food products would be irradiated in licensed facilities and that appropriate documentation would accompany the irradiated food throughout the food chain. However, because of the individual efforts of research teams in many countries and the noteworthy international cooperation in this field, between the years 1985 and 1995 considerable progress was made in the development of reliable methods to identify irradiated foods. The European Community (EC), through its Community Bureau of Reference (BCR), set up a collaborative program to develop methods to identify irradiated food while, on a worldwide basis, the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture set up a co-ordination program on Analytical Detection Methods in Irradiation Treatment of food (ADMIT), which promoted cooperation in this area.

Although it would have been ideal to have developed one method to detect all irradiated foodstuffs, this was not feasible, mainly due to differences in the nature of the foodstuffs being irradiated and the diverse range of changes produced in foods by ionizing radiation. The development of these methods also proved difficult due to the fact that the radiolytic changes that occur in food upon irradiation are minimal and often similar to those produced by other food-processing technologies, such as cooking. The methods that were developed are in fact based on particular physical, chemical, biological, and microbiological changes induced in foods during the irradiation process.

Under EU legislation it also states that Member States shall ensure that the analytical methods used to detect irradiated foods are validated or standardized. In 1993, the European Commission (EC) gave a mandate to the European Committee for Standardization (CEN) to standardize these methods. Consequently, CEN created within its Technical Committee 275 "Food Analysis—Horizontal Methods" (CEN/TC 275) Working Group 8 "Irradiated Foodstuffs" (CEN/TC275/WG8), which had its first meeting in November 1993. As a result of the efforts of this Working Group, 10 European Standards are now available from national standardization institutes [11]. These European Standards have also been adopted by the CAC as General Methods and are referred to in the Codex General Standard for Irradiated Foods in Section 6.4 on "Postirradiation Verification." Table 34.1 lists the 10 methods that are now available and used worldwide for the detection of irradiated foodstuffs. The rest of this chapter will outline these methods and demonstrate how they have been used to detect irradiated foodstuffs on sale in the marketplace and not labeled correctly.

34.2.1 Gas Chromatographic Analysis of Hydrocarbons (EN1784)

European Standard EN1784 was developed for the identification of irradiated food containing fat. As for all the standard methods, EN1784 was validated by a series of interlaboratory trials as a reliable test for the detection of irradiated products such as chicken meat, pork, beef, camembert, papaya, and mango [12]. It is based on the gas chromatography (GC) detection of radiation-induced hydrocarbons.

Table 34.1 European Standards for the Detection of Irradiated Foodstuffs [11]

EN1784:2003	Foodstuffs—detection of irradiated food containing fat—gas chromatographic analysis of hydrocarbons <i>Validated with raw chicken, pork, liquid whole egg, salmon, Camembert</i>
EN1785:2003	Foodstuffs—detection of irradiated food containing fat—gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones <i>Validated with raw meat, Camembert, fresh avocado, papaya, mango</i>
EN1786:1996	Foodstuffs—detection of irradiated food containing bone—method by ESR spectroscopy <i>Validated with beef bones, trout bones, chicken bones—expected that method can be applied to all meat and fish species containing bone</i>
EN1787:2000	Foodstuffs—detection of irradiated food containing cellulose, method by ESR spectroscopy <i>Validated with pistachio nut shells, paprika powder, fresh strawberries</i>
EN1788:2001	Foodstuffs—detection of irradiated food from which silicate minerals can be isolated, method by thermoluminescence <i>Validated with herbs and spices as well as their mixtures, shellfish including shrimps and prawns, both fresh and dehydrated fruits and vegetables, potatoes</i>
EN13708:2001	Foodstuffs—detection of irradiated food containing crystalline sugar by ESR spectroscopy <i>Validated with dried figs, dried mangoes, dried papayas, raisins</i>
EN13751:2002	Detection of irradiated food using photostimulated luminescence <i>Validated with shellfish, herbs, spices, seasonings</i>
EN13783:2001	Detection of irradiated food using Direct Epifluorescent Filter Technique/Aerobic Plate Count (DEFT/APC)—Screening method <i>Validated with herbs and spices</i>
EN13784:2001	DNA comet assay for the detection of irradiated foodstuffs - Screening method <i>Validated with chicken bone marrow, chicken muscle, pork muscle, almonds, figs, lentils, linseed, rosé pepper, sesame seeds, soyabeans, sunflower seeds</i>
EN14569:2004	Microbiological screening for irradiated foodstuffs—Screening method (LAL/GNB) <i>Validated for chilled or frozen chicken fillets (boneless) with or without skin</i>

Source: European Commission, Food irradiation—analytical methods. http://ec.europa.eu/food/food/biosafety/irradiation/anal_methods_en.htm.

As most of the volatile products formed in food by irradiation originate from the fat or lipid content, in 1988 Nawar [13] proposed that measurement of radiolytic products from food lipids could form the basis for a method to identify irradiated foods. Research showed that both the quantitative and qualitative patterns of the radiolytic products depend largely on the fatty acid composition of the fat. Thus, if the fatty acid composition of the fat is known, the composition of the products formed by irradiation of a fat, or fat-containing food, can be predicted to a certain degree [14].

Upon irradiation of foods containing fat, two hydrocarbons are formed in relatively large quantities [15]. In the fatty acid moieties of triglycerides, breaks in chemical bonds occur mainly in the alpha and beta positions with respect to the carbonyl groups. Thus, one hydrocarbon has a carbon atom less than the parent fatty acid, resulting from cleavage at the carbon-carbon bond alpha to the carbonyl group (C_{n-1}), whereas the other has two carbons less and one extra double bond resulting from cleavage beta to the carbonyl ($C_{n-2;1}$).

In 1970, Nawar and Balboni [15] reported on the feasibility of detecting irradiation in pork meat at doses between 1 and 60 kGy by analysis of the six “key hydrocarbons.” Tetradecene ($C_{14:1}$) and pentadecane ($C_{15:0}$) are produced from palmitic acid ($C_{16:0}$) upon irradiation, hexadecene ($C_{16:1}$) and heptadecane ($C_{17:0}$) from stearic acid ($C_{18:0}$), whereas hexadecadiene ($C_{16:2}$) and heptadecene ($C_{17:1}$) are typically produced from oleic acid ($C_{18:1}$). Nawar and Balboni [15] demonstrated a linear relationship between irradiation dose and each of these compounds, with neither of them, nor water, having a significant effect on the quantitative pattern. Work on irradiated chicken reported by Nawar et al. [16] in 1990 considered tetradecene, hexadecadiene, and heptadecene to be the most promising hydrocarbons for reliable detection of irradiation treatment in meat, because they were found in the highest concentrations and were absent or present at a low level in nonirradiated samples.

For detection of irradiated hydrocarbons, the fat is isolated from the sample by melting it out or by solvent extraction. The hydrocarbon fraction is obtained by adsorption chromatography before separation using GC and detection with a flame ionization detector or a mass spectrometer (MS) [12].

Alternatively, the hydrocarbons may be detected using liquid chromatography-GC (LC-GC) coupling [17]. Horvatovich et al. [18] showed how supercritical carbon dioxide can be used to carry out a selective and fast extraction (30 min) of volatile hydrocarbons and 2-alkylcyclobutanones contained in irradiated foods. The supercritical fluid extraction (SFE) method was successfully applied to freeze-dried samples (1 g or less) of cheese, chicken, avocados, and various ingredients (chocolate, liquid whole eggs) included in nonirradiated cookies. The method proved to be 4–5 h faster than the standardized hydrocarbon (EN1784) [12] and 2-alkylcyclobutanone (EN1785) [19] methods, which take 1.5 days each to determine if a food has been irradiated. In addition, the minimal dose detectable by this method was slightly lower than those of the standardized methods.

34.2.2 Gas Chromatography: Mass Spectrometric Analysis of 2-Alkylcyclobutanones (EN1785)

European Standard EN1785, along with EN1784, can be used for the identification of irradiated food containing fat. This method is based on the mass spectrometric detection of 2-alkylcyclobutanones after gas chromatographic separation [19]. It has been proposed that the formation of the 2-alkylcyclobutanones in irradiated foods results from cleavage at the acyl–oxygen bond in triglycerides, with the pathway involving a six-membered ring intermediate. The cyclobutanones so formed contain the same number of carbon atoms as the parent fatty acid, and the alkyl group is located in ring position 2 [10,14]. To date, the cyclobutanones are the only cyclic compounds reported in the radiolytic products of saturated triglycerides. As for the hydrocarbons, if the fatty acid composition of a lipid is known, then the products formed upon irradiation can be predicted to a certain degree. Thus, for example, if the fatty acids palmitic, stearic, oleic, and linoleic acid are exposed to ionizing radiation, then the respective 2-dodecyl-, 2-tetradecyl-, 2-tetradecenyl-, and 2-tetradecadienyl-cyclobutanones will be formed [20].

The method is based on the detection of 2-dodecylcyclobutanone (2-DCB) and 2-tetradecylcyclobutanone (2-TCB), these being the two markers most commonly used for identification purposes. These cyclobutanones have been identified in irradiated foods treated with irradiation doses as low as 0.1 kGy, and to date have not been detected in nonirradiated foods or microbiologically spoiled products. The specificity of the compounds as irradiation markers has been demonstrated in extensive experimental work, which has shown that they are not produced by cooking, by packaging in air, vacuum, or carbon dioxide, or during storage [21].

The 2-alkylcyclobutanones are extracted from the sample using either hexane or pentane along with the fat. The extract is then fractionated using adsorption chromatography before separation by GC and detection using a mass spectrometer [19]. As most foods contain some fat, the method is applicable to a wide range of products, and interlaboratory trials have successfully validated EN1785 for the identification of irradiated raw chicken, pork, liquid whole egg, salmon, and camembert. 2-DCB and 2-TCB have been detected postcooking in such products as irradiated meat, poultry, and egg [22,23]. Detection of irradiated ingredients such as irradiated liquid whole egg in cakes is also possible [10,22,23].

2-Tetradecenylcyclobutanone (2-TDCB) has been detected in irradiated chicken meat, papaya, and mango [24,25]. However, as this cyclobutanone is more difficult to detect and quantify in comparison with 2-DCB and 2-TCB, it is not used routinely for detection of irradiation treatment.

Since the initial development of the 2-alkylcyclobutanone method, alternative procedures have been developed for the extraction and purification of these radiation markers. Studies published by Stewart et al. [23], Gadgil et al. [26], and Horvatovich et al. [27] demonstrated that SFE could be used for the selective and rapid extraction of the cyclobutanones from irradiated foodstuffs without prior extraction of the fat. Obana et al. [22] used an accelerated solvent extraction (ASE) system for extraction of the cyclobutanones. Work by Ndiaye et al. [28] showed that inclusion of a purification step by silver ion chromatography in the EN1785 protocol considerably improved the quality of the chromatograms obtained, thereby allowing the detection of food samples irradiated at doses as low as 0.1 kGy. In addition, Horvatovich et al. [29] used a column containing 60 g silica gel for cleanup and the use of isobutane as a reactant for chemical ionization–mass spectrometric analysis of saturated and monounsaturated alkyl side-chains of 2-alkylcyclobutanones to improve both the sensitivity and selectivity of the method. However, it should be noted that these procedures have not been validated by interlaboratory trials.

34.2.3 *Electron Spin Resonance Spectroscopy*

Three of the European Standards for detection of irradiated foodstuffs use the technique of electron spin resonance (ESR) spectroscopy, also known as electron paramagnetic resonance (EPR) spectroscopy. ESR spectroscopy is a physical technique that detects species with unpaired electrons. Electrons are almost invariably paired. However, some molecules do contain an odd number of electrons, and the one that is unpaired is referred to as a free radical. Free radicals are highly reactive and consequently are short-lived. Some do exist in a stable state for some time, and it is these that are examined by ESR spectroscopy. Ionizing radiation produces free radicals in food, and because ESR spectroscopy detects free radicals, it can be used to determine whether certain foods have been irradiated. In foodstuffs with a relatively high moisture content, such as vegetables and meat, the induced radicals disappear rapidly. On the other hand, if food contains components with a relatively large proportion of dry matter, such as bones, seeds, or shells, the radicals may be trapped and be sufficiently stable to be detected by ESR [30]. The three ESR methods standardized by CEN are used for the detection of irradiated food containing bone (EN1786) [31], cellulose (EN1787) [32], and crystalline sugar (EN13708) [33].

34.2.3.1 *Detection of Irradiated Food Containing Bone by Electron Spin Resonance Spectroscopy (EN1786)*

When bone is subjected to ionizing radiation, free radicals are trapped in the crystal lattice of the bone, and these can be detected by ESR spectroscopy. The use of ESR to detect the presence



Figure 34.1 ESR spectra derived from irradiated (top spectrum) and nonirradiated (bottom spectrum) bone from frog legs.

of radiation-induced free radicals in bone dates back to the mid-1950s, being used to date archaeological specimens, and also as an *in vivo* dosimeter for human to assess their exposure to radiation [34]. Nonirradiated bone gives a weak, broad ESR signal that increases in magnitude if the bone is ground into a powder. The signal derived from irradiated bone (Figure 34.1) is a large axially asymmetrical singlet, and can easily be distinguished from the endogenous signal [10,34]. Two prevailing types of paramagnetic species have been observed after the irradiation of bone tissue. One species is derived from bone collagen, and the other is attributed to the mineral constituent of bone, the hydroxyapatite. It is surmised that the characteristic signal produced on irradiation of the bone is due to either the CO^{2-} or the CO_3^{3-} radical trapped in the hydroxyapatite matrix.

Significant work has been carried out on chicken bone [30,35,36], with the bones from duck, turkey, goose, beef, pork, lamb, and frog legs also being studied to a more limited extent [37–40]. The signal produced from all sources of bone is essentially the same, thus it is evident that ESR can be used for the qualitative detection of irradiation in a wide range of meats containing bone. Interlaboratory trials have validated the method for beef bones, trout bones, and chicken bones [31].

Gray and Stevenson [41] also demonstrated that the method could be used for the identification of irradiated mechanically recovered meat (MRM), a secondary food product from which small bone fragments can be extracted. It has also been shown by Stevenson et al. [42] that ESR could be used to detect irradiated MRM as an ingredient in a food product, for example, burgers, at inclusion levels as low as 3 g/100 g. Work published by Marchioni et al. [43,44] also proved that ESR can be used for the detection of irradiated mechanically recovered poultry meat at very low inclusion levels in tertiary food products such as poultry quenelles and pre-cooked meals.

An ESR signal similar to that of bone has also been derived from irradiated eggshell, as demonstrated by Onori and Pantaloni [45]. When tested by an interlaboratory trial [46], samples of irradiated eggshell were identified with a 100% success rate, even when treated at doses as low as 0.3 kGy.

34.2.3.2 *Detection of Irradiated Food Containing Cellulose by Electron Spin Resonance Spectroscopy (EN1787)*

European Standard EN1787 specifies a method for the detection of foods containing cellulose that have been treated with ionizing radiation [32]. It was Raffi [47] who first examined the ESR signal derived from the seeds of strawberries and derived a multicomponent signal that is typical of that from foodstuffs containing cellulose. A central single line is present in both irradiated and nonirradiated samples (Figure 34.2) that is thought to arise from a semiquinone radical. This single line increases with increasing irradiation dose, but will vary to a large extent with the water content of the sample. For irradiated samples (Figure 34.2), a pair of outlying lines occurs to the left and right of the central signal, the left one of which is most easily detected. It was proposed that these lines originate from cellulose and, as they are not present in nonirradiated samples, they can be used to detect irradiation treatment.

The method has been validated by interlaboratory trials for pistachio nut shells, paprika powder, and fresh strawberries [32].

This method could be used for a wide range of fruits, and has been employed for the detection of irradiated nuts, some aromatic herbs and spices, and for certain packaging materials, containing a high percentage of cellulose [48–50].

34.2.3.3 *Detection of Irradiated Food Containing Crystalline Sugar by Electron Spin Resonance Spectroscopy (EN13708)*

EN13708 [33] uses ESR spectroscopy for the detection of irradiated food containing crystalline sugar. A multicomponent ESR signal is derived from irradiated dried fruits such as dates, grapes, mango, papaya, and pineapple, being easily distinguishable from the single line obtained from nonirradiated samples (Figure 34.3). It was proposed that the complex signal induced by ionizing radiation arises from sugar radicals [51], as the overall sugar content of fruits is high, varying from

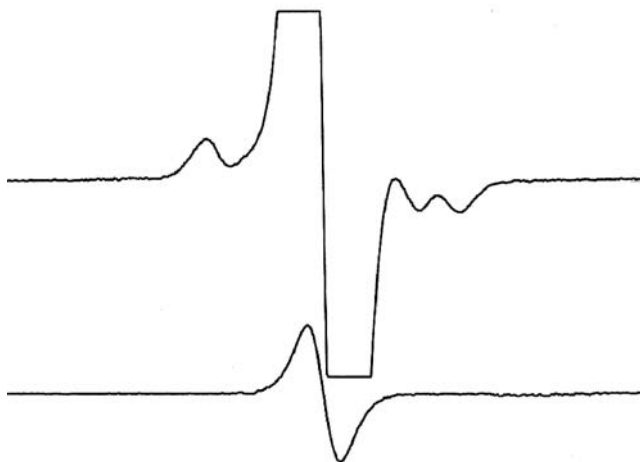


Figure 34.2 ESR spectra derived from irradiated (top spectrum) and nonirradiated (bottom spectrum) paper containing cellulose.

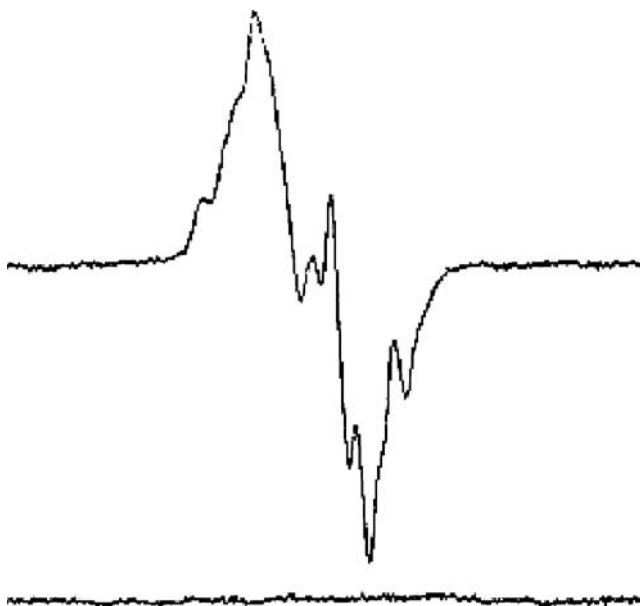


Figure 34.3 ESR spectra derived from irradiated (top spectrum) and nonirradiated (bottom spectrum) samples of dried fruits containing crystalline sugars.

60 to 75%, the main components being D-fructose, D-glucose, and D-saccharose. These radiation-induced signals are, in general, sufficiently stable for the identification of irradiated samples, even when they are stored for several months.

Interlaboratory trials have successfully demonstrated that the method can be used to identify irradiated dried figs, dried mangoes, dried papayas, and raisins [33]. The lower detection limit will mainly depend on the crystallinity of the sugar in the sample. The presence of sufficient amounts of crystalline sugar in the sample at all stages of handling between irradiation and testing will determine the applicability of the method.

34.2.4 Luminescence Methods: Detection of Irradiated Food from Which Silicate Minerals Can Be Isolated

The luminescence methods are probably the most sensitive means by which irradiated products such as herbs, spices, and seasonings can be identified. The methods involve either the thermoluminescence (TL) or photostimulated luminescence (PSL) analysis of contaminating silicate minerals. Mineral debris, typically silicates or bioinorganic materials such as calcite that originate from shells or exoskeletons, or hydroxyapatite from bones or teeth, can be found on most foods [52]. These materials store energy in charge carriers trapped at structural, interstitial, or impurity sites, when exposed to ionizing radiation. Luminescence is the emission of light when this trapped energy is liberated by the addition of either heat (TL) or light (PSL). Two European Standards have been developed based on the use of TL (EN1788) and PSL (EN13751) for the detection of irradiated foodstuffs containing silicate minerals.

34.2.4.1 *Thermoluminescence Detection of Irradiated Food from Which Silicate Minerals Can Be Isolated (EN1788)*

European Standard EN1788 is applicable to those foodstuffs from which silicate minerals can be isolated [53]. The energy stored within the silicate minerals is released by controlled heating of isolated silicate minerals so that light is emitted, the intensity of the emitted light being measured as a function of temperature, resulting in a so-called glow curve.

It was first thought that the TL arose from the organic component of the samples, but research [54,55] has clearly shown that the signals from herbs and spices actually originated from adhering mineral grains, although they accounted for less than 1% of the sample weight. In this method, the silicate minerals are separated from the food matrix, mostly by a density separation step. The isolated minerals should be as free from organic constituents as possible, so as not to obscure the TL. A first glow of the separated mineral extracts is recorded (glow 1). However, as various amounts and types of minerals exhibit variable integrated TL intensities, a second glow (glow 2) of the sample is measured after exposure to a fixed dose of ionizing radiation. The latter step is necessary to normalize the TL response. Thus, a ratio of glow 1 to glow 2 is obtained and used to indicate irradiation treatment of the food, as irradiated samples normally yield higher TL glow ratios than nonirradiated samples. Glow shape parameters can also be used as additional evidence for the identification of irradiated foods. As the method relies solely on the separated silicate minerals, it is not on principle influenced by the kind of food product.

Interlaboratory trials have validated the TL method for a wide range of herbs and spices as well as their mixtures, shellfish including shrimps and prawns, fresh fruits and vegetables (strawberries, avocados, mushrooms, papayas, mangoes, potatoes), dehydrated fruits and vegetables (sliced apples, carrots, leeks, onions, powdered asparagus). In the case of shrimps and prawns, the mineral grains present in the intestinal gut are isolated and analyzed [53].

34.2.4.2 *Detection of Irradiated Food Using Photostimulated Luminescence (EN13751)*

The PSL standard method (EN13751) uses excitation spectroscopy for optical stimulation of minerals to release stored energy [56]. It has been shown that the same spectra can be obtained from whole herbs and spices and other foods using photostimulation. PSL measurements do not destroy the sample, thus whole samples, or other mixtures of organic and inorganic material, can be measured repeatedly. The PSL signals obtained do, however, decrease if the same sample is measured repeatedly.

The method has overcome the need for full mineral separation, and a low-cost instrument is now commercially available for high-sensitivity PSL measurements from food samples using the highly radiation-specific ultraviolet–visible (UV–Vis) luminescence signals, which can be stimulated using infrared sources [57,58]. The SURRC pulsed photostimulated luminescence system (SURRC Pulsed PSL System) was designed and developed at the Scottish Universities Research and Reactor Centre (SURRC). The system is commercially available from the Scottish Universities Environmental Research Centre (SUERC), and has been supplied to more than 80 laboratories in the United Kingdom, Europe, and United States for routine commercial quality testing, and in support of labeling requirements. Originally developed for rapid screening of irradiated herbs, spices, and seasonings, it has been validated for a wider range of foodstuffs, and is finding other scientific applications in assessment of fire-damaged structures and in environmental dosimetry [59].

Two modes of operation can be employed; the screening mode, where the luminescence intensity detected from the samples is used for preliminary classification into negative, intermediate, or positive bands, and calibrated PSL (CalPSL), which can distinguish between low- and high-sensitivity samples, thus resolving ambiguous or low-sensitivity cases. It is necessary to confirm a positive screening result using CalPSL or another standardized method such as EN1788.

The method has been validated by interlaboratory trials [56] for shellfish, herbs, spices, and seasonings. For shellfish, the signals from intestinally trapped silicates can be stimulated through the membranes of dissected guts, and in some cases through the whole body of the creature. From the results of other studies, it has been concluded that PSL is applicable to a large variety of foods [60,61].

34.2.5 DNA Comet Assay

The DNA Comet Assay EN13784 [62] is a rapid and inexpensive screening test to identify irradiated food [63]. As the DNA molecule is an easy target for ionizing radiation, it was logical to investigate whether radiation damage to DNA in food could be used as a means of detecting irradiation treatment. The irradiation of DNA has been shown to induce three major classes of lesions—double-strand breaks, single-strand breaks, and base damage [64]. A sensitive technique to detect this fragmentation is microgel electrophoresis. The technique analyzes the leakage of DNA from single cells or nuclei extracted from food material and embedded in agarose gel on microscopic slides. In irradiated samples (Figure 34.4), the fragmented DNA leaks from the nuclei during electrophoresis, forming a tail in the direction of the anode and giving the appearance of a “comet” when the gel is stained with a fluorescent dye and viewed with a microscope. The head of the comet is formed by the remaining nucleus, whereas the tail is dominated by the fragments. The extension of the tail is closely related to the damage intensity. Cells from nonirradiated samples will appear as nuclei with no or only slight tails (Figure 34.4). The method is restricted to foods that have not been subjected to heat or other treatments, which would induce DNA fragmentation, resulting in comets similar to those of samples treated with ionizing radiation [65]. It is also necessary to establish background DNA damage in nonirradiated samples for each new type of food under investigation.

As the DNA Comet Assay is not radiation-specific, positive results must be confirmed using specific standardized methods such as EN1784 or EN1785. The method has been validated by interlaboratory trials for identification of irradiated chicken bone marrow, chicken, and pork muscle tissue given irradiation doses of 1, 3, or 5 kGy and plant foods (almonds, figs, lentils, linseed, rosé pepper, sesame seeds, soybeans, and sunflower seeds) given 0.2, 1, or 5 kGy [62].



Figure 34.4 Typical DNA comets from (a) irradiated (at 7.5 kGy) and (b) nonirradiated tissues. (Haine, H., Cerda, H., and Jones, L., *Food Sci. Technol. Today*, 9(3), 139, 1995. Copyright IFST.)

Research has shown that the method can be applied to a wide range of products, but the limitations outlined previously apply [63,66–68], with further development of the method also being reported to allow for more rapid detection and dose estimation [69].

34.2.6 Measurement of Microbiological Changes

Any kind of processing will destroy the microbial flora in food or change the flora present so that the vegetative cells are killed off, whereas the bacterial spores survive. Such microbial reduction and change is to be expected in all kinds of food processing, including irradiation. Thus, it was presumed that simple detection tests for foods could be developed comparing the microbiological quality of nonirradiated and irradiated foods to determine if irradiation treatment has been applied [70]. Consequently, two screening methods were successfully developed, validated, and standardized for the identification of irradiated foods based on modification of the microbiological flora of samples.

34.2.6.1 Direct Epifluorescent Filter Technique/Aerobic Plate Count (DEFT/APC) (EN13783)

One microbiological method that has been developed, validated, and standardized as a screening method for irradiated foods is the DEFT/APC test (EN13783) [71]. The DEFT/APC method can be used for the detection of irradiation treatment of herbs and spices, using the combined direct epifluorescent filter technique (DEFT) and aerobic plate count (APC). The method is based on comparison of the APC with the count obtained using the DEFT. The APC gives the number of viable microorganisms in the sample after irradiation, whereas the DEFT count determines the total number of microorganisms present in the sample, including cells rendered nonviable by irradiation. For a nonirradiated sample, the counts by DEFT are in close agreement with those by APC, because nearly all the cells present are alive. However, when the APC of an irradiated sample is compared with the DEFT count on the same sample, the APC is found to be considerably less than that obtained by DEFT, and the difference indicates that the samples could have been irradiated [72].

The difference between the DEFT and the APC counts in spices treated with doses of 5–10 kGy is generally about or above 3–4 log units. Similar differences between DEFT and APC counts can be induced by other treatments of the foods that lead to death of microorganisms, for example, heat or fumigation treatment. Thus, as the method is not radiation-specific, positive results should be confirmed by another suitable standardized method, such as TL (EN1788) or PSL (EN13751). It has been shown that some spices such as cloves, cinnamon, garlic, and mustards can contain inhibitory components with antimicrobial activity, which may lead to decreasing APC, thereby giving false-positive results.

The DEFT/APC method has been successfully validated for herbs and spices (including whole allspice, whole and powdered black pepper, whole white pepper, paprika powder, cut basil, cut marjoram, and crushed cardamom) by interlaboratory trials [71].

34.2.6.2 *Limulus Amebocyte Lysate/Gram-Negative Bacteria Test (EN14569)*

The *Limulus* amebocyte lysate/Gram-negative bacteria (LAL/GNB) test, European Standard EN14569 [73], is another microbiological screening method comprising two procedures carried

out in parallel to detect an abnormal microbiological profile of foods typically contaminated with predominantly Gram-negative bacteria. It is based on the principle that relatively low doses of irradiation can render large numbers of bacteria nonviable.

The two procedures to be carried out are (i) enumeration of total resuscitated GNB in the test samples and (ii) determination of lipopolysaccharide (bacterial endotoxin) concentration in the test sample using the LAL test. The level of endotoxin (measured in endotoxin units) is directly related to the number of GNB, although it is not species-specific. Thus the test determines the number of viable GNB present in a sample, and the concentration of bacterial endotoxin serves as a measure for the estimation of the amount of total GNB, both viable and dead. If a high LAL value is obtained in the absence of significant numbers of viable GNB, this indicates the presence of a large population of dead bacteria. In the absence of any visible processing of the sample, for example cooking, this profile is indicative of some other processing, such as treatment with ionizing radiation [73,74].

This method is not radiation-specific, as a high amount of dead bacteria in comparison with numbers of viable microorganisms can be due to other reasons, such as cooking or some form of chemical preservation. Freezing after irradiation can also influence the ratio of GNB to endotoxin units due to loss of the viability of microorganisms. On the other hand, regrowth of bacterial flora can occur in irradiated samples that are stored unfrozen.

This screening method was validated by interlaboratory trials [73,74] using boneless chicken breasts with skin and boneless chicken breast fillets. The method is generally applicable to whole parts of poultry, such as breast, legs, and wings of fresh, chilled, or frozen carcasses with or without skin. In addition, it can also provide useful information about the microbiological quality of a product before irradiation.

34.2.7 Other Methods Explored

The methods presented up to this point are those that have been validated and standardized. However, it is worthy of note that other methods have been explored, but for one reason or another have not been standardized. For example, the use of ESR spectroscopy was investigated for the identification of irradiated crustacea. It was found that the ESR signal derived from the shell of prawns or shrimp is species-dependent, with the geographical origin also being shown to influence ESR signal shape. Thus, while detection of irradiation treatment is possible, it is not without its problems, as demonstrated by a number of interlaboratory blind trials [75,76], where the identification rate of certain species was extremely poor. More research would certainly need to be undertaken before the method could be standardized. ESR can also be employed to detect irradiation treatment of shellfish such as mussels, oysters, and scallops [77] and other crustaceans such as crab [78].

Other physical methods investigated included measurement of changes in the viscosity of products, such as suspensions of herbs, spices, and seasonings [79,80], and the electrical impedance of potatoes [81,82]. Studies on chemical methods also explored the potential use of orthotyrosine, formed from phenylalanine, as a radiation marker [83,84]. However, studies showed that this compound can also be found in nonirradiated products, thus it is not radiation-specific. But it was concluded that if the difference in the amounts present in nonirradiated and irradiated samples was sufficiently large, the compound could still have potential as a radiation marker. Significant work on using gas evolution to detect irradiated foods was undertaken by workers such as Furuta et al. [85], Delincée [86], and Hitchcock [87]. The method was based on the detection of evolved gases such as carbon monoxide, hydrogen, hydrogen sulfide, and ammonia.

The use of agarose electrophoresis of mitochondrial DNA (mtDNA) for identification of irradiated foods was studied by Marchioni et al. [88,89]. This method is potentially applicable to foods, particularly meat products, treated with ionizing radiation at doses of 1 kGy or greater, as long as mtDNA can be extracted. The use of immunoassays for the detection of irradiated products has also been explored. Work published by Tyreman et al. [90] described the development of a competitive enzyme-linked immunoassay (ELISA) to detect irradiated prawns. The ELISA described uses a monoclonal antibody against dihydrothymidine, a modified DNA base. It has been successfully applied for the detection of irradiated North Atlantic prawn (*Pandalus borealis*) and Tiger prawn (*Penaeus monodon*), having a working range of 0.5–2 kGy, with detection of irradiation treatment being possible for prawns stored up to 12 months at -20°C . Potentially this method could be applied to a range of foodstuffs, as most food contains DNA, and it is also simple and inexpensive to carry out.

The half-embryo test to measure inhibition of seed germination was also studied as a simple detection method for products such as irradiated apples, cherries, grapefruits, lemons, and oranges [91–93]. The embryos are taken out of the seed shells for germination so that irradiation treatment can be detected within 2–4 days at dose levels as low as 0.15 kGy. The test is simple and inexpensive to perform, not requiring any specialized equipment.

34.2.8 Application of Detection Methods in the Marketplace

Currently with the EU, 10 Member States have facilities approved in accordance with Article 7(2) of Directive 1999/2/EC for the irradiation of food. In 2005, as only eight Member States forwarded to the Commission the results of checks carried out in irradiation facilities, the precise amount of foodstuffs irradiated in the Union could not be determined [94]. During 2005 the main products treated by ionizing radiation within the EU were dried herbs and spices, frog legs, poultry, and dried vegetables.

Within the EU, to ensure that current labeling regulations are being complied with, analytical checks are carried out on foods placed on the market. In 2005 a total of 16 Member States reported checks on foods placed on the market, with a total of 7011 food samples being tested. About 4% of products tested from the marketplace were found to be illegally irradiated or not labeled [94]. Table 34.2 is a summary of the numbers of samples analyzed and the results obtained for the EU as a whole in 2005.

It was found that the infringements were unevenly distributed over product categories. Products from Asia, especially Asian-type noodles and food supplements, represented a significant proportion of the samples that were irradiated and not labeled as such. Only six of the 287 samples found to be irradiated complied with the regulations. It was noted that in 2005, there were no irradiation facilities in Asia approved by the EC. Such incorrectly labeled Asian products were found in Germany, the Republic of Ireland, and the United Kingdom. Incorrectly labeled food supplements were also detected in the same countries as well as in Finland and the Netherlands. In Germany, 47 samples out of 96 soups and sauces tested were found to be treated with ionizing radiation, with irradiation being unauthorized or samples not being correctly labeled. Other products found to be irradiated within the EU and not labeled correctly included dried herbs, spices, vegetable seasonings, fish and fisheries products, frogs legs, dried mushrooms, and tea and tealike products. TL (EN1788) and PSL (EN13751) were the most commonly used methods within the Member States for detection purposes, with PSL being used for screening purposes, and confirmation of positive results being undertaken using TL. The results of these tests within the EU is indicative of the successful detection of irradiated products using standardized analytical methods.

Table 34.2 Summary of Samples Analyzed for Irradiation Treatment and Results Obtained for the EU as a Whole in 2005

<i>Member State</i>	<i>No. of Samples Nonirradiated</i>	<i>No. of Samples Irradiated</i>	<i>Percentage of Samples Irradiated, Not Labeled Correctly</i>
Austria	115	0	0
Belgium	148	0	0
Cyprus	NAC	NAC	NAC
Czech Republic	70	8	10
Germany	3798	143 ^a	3.6
Denmark	NAC	NAC	NAC
Estonia	NAC	NAC	NAC
Greece	54	0	0
Spain	NI	NI	NI
Finland	264	13	5
France	80	6	7
Hungary	134	7 ^a	2
Ireland (Republic)	439	20	4
Italy	107	5	5
Latvia	NAC	NAC	NAC
Lithuania	12	0	0
Luxembourg	40	0	0
Malta	NAC	NAC	NAC
The Netherlands	761	31	4
Poland	116	6	4
Portugal	NAC	NAC	NAC
Sweden	6	0	0
Slovakia	56	0	0
Slovenia	10	0	0
The United Kingdom	514 ^b	42	6
Total	6724	281	4.0

^a Germany and Hungary found respectively 2 and 4 samples that were legally irradiated and correctly labeled.

^b The United Kingdom classified 101 samples as inconclusive.

Note: NI = no information forwarded by the Member State, NAC = no analytical checks performed in 2005.

Source: European Union, *Off. J. Eur. Union*, 2007/C122/03, 2 June 2007.

34.3 Conclusions

This chapter has briefly summarized the main methods currently available for the detection of irradiated foodstuffs, whether they are whole products or ingredients within a foodstuff. As noted, the methods have been successfully applied for the detection of irradiated foodstuffs in the marketplace, thereby giving assurance to retailers and consumers alike that irradiated foods on sale and incorrectly labeled can be identified. The availability and regular use of these methods could

even help to facilitate international trade in irradiated food [95]. A number of reviews have been written on methods for the detection of irradiated foods; for further reference the author suggests reading McMurray et al. [96], which contains the proceedings of an International Meeting on Analytical Detection Methods for Irradiation Treatments of Foods held in June 1994, as well as reviews by Delincée [95,97], Stewart [10], and Marchioni [98].

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