Methods and Protocols
in Food Science

Springer Protocols

José Manuel Lorenzo · Rubén Domínguez
Mirian Pateiro · Paulo E. S. Munekata *Editors*

Methods to Assess the Quality of Meat Products

Humana Press

Methods and Protocols in Food Science

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Methods to Assess the Quality of Meat Products

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ISSN 2662-950X ISSN 2662-9518 (electronic)
Methods and Protocols in Food Science
ISBN 978-1-0716-2001-4 ISBN 978-1-0716-2002-1 (eBook)
https://doi.org/10.1007/978-1-0716-2002-1

This Humana imprint is published by the registered company Springer Science+Business Media, LLC, part of Springer Nature.
The registered company address is: 1 New York Plaza, New York, NY 10004, U.S.A.

Preface

The meat industry is a sector with great importance worldwide. However, current consumer trends as well as regulatory changes make essential the determination of the constituents and the additives. The different analytical techniques that can be applied arise from the need to provide correct nutritional information in accordance with the legislation (labeling), as well as to determine the nutritional quality and chemical composition of meat and meat products. On the other hand, the physicochemical and degradative changes that can take place can also be a source of significant economic losses or even lead to the appearance of toxic substances, which would put the health of the consumer at risk. Therefore, the determination of these changes should be also carried out.

This book arises from the collaboration between different institutions and centers at an international level, which are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568). Therefore, the publication of this book is a very useful tool in order to standardize the analytical processes, as well as to serve as a basis for the development and validation of these techniques in those research groups that do not have them implemented.

Several analytical techniques have been developed for each of the determinations described in this book. However, the variations between them as well as the use of different techniques could lead to the results obtained not being reproducible or comparable. With this in mind, the current book examines the techniques used for the analysis of meat and meat products. A complete and comprehensive description of the methods and materials used is made so that each technique can be reproduced by other researchers. In addition, the techniques include explanatory notes and elucidate the possible points to take into account for the correct achievement of the determination. The ultimate goal is to support the scientific community, professionals, laboratories, food companies, and regulatory bodies in their aim to identify and measure meat and meat products composition and properties. The book consists of 14 chapters.

Chapter 1 provides the complete material and methods description for the determination of the different chemical parameters, including moisture, fat, protein, and ash. In addition to being mandatory to include part of this information on the products labeling, these determinations also offer nutritional information on the final product.

Chapter 2 discusses the techniques used for the measurement of pH and color. In this case, color is considered one of the most important parameters that influence consumer acceptance. Thus, the correct determination of physical (CIELab) and also chemical (haem pigments) color is important to control the meat and meat products quality.

Not only color but also texture is important in consumer preferences. With this in mind, Chapter 3 provides different procedures for the correct determination of texture parameters in different meat and meat products.

Chapter 4 includes the complete method for fat extraction, transesterification, and determination of fatty acids with the aim to have complete nutritional information. This is important to quantify saturated fatty acids (necessary for products labeling) and also for nutritional claims (e.g., source of omega-3, low saturated fats, etc.). Additionally, the fatty acid determination also provides information about the lipid stability of the product.

In Chapter 5, a complete description of free amino acids and hydrolyzed amino acids extraction is explained, followed by the derivatization and chromatographic analysis. The

free amino acids content is related to the proteolytic phenomena during meat products manufacturing and processing, while hydrolyzed amino acids indicate the nutritional quality of meat and meat products (e.g., essential amino acids).

Chapter 6 deals with the analysis of total cholesterol in meats. It is well known that cholesterol consumption is related to the development of some human diseases. Thus, the correct determination of this compound provides both the consumer and the manufacturer with important information.

Similarly, the content of several minerals is important to human health. Thus, in Chapter 7, a comprehensive explanation about the sample treatment and mineral composition analysis using IPC-OES is provided.

Chapter 8 focuses on the analytical determination of nitrites and nitrates in meat and meat products, using liquid chromatography. These additives are necessary for several meat products manufacture since they control the growth of some pathogenic microorganisms and stabilize the color. However, they are also related to the development of toxic molecules; thus the residual content of both is regulated.

The release of biogenic amines during processing, mostly in fermented or ripened products, is also frequent. These compounds are related to some allergenic reactions and are harmful for humans. Therefore, Chapter 9 provides a complete description of the materials and methods for their determination using liquid chromatography.

On the other hand, it is well known that oxidative processes are the most important degradative reactions during both the processing and storage of meat and meat products. They produce loss of nutrients, reduce sensory properties, develop dangerous compounds, and decrease their shelf life. With this in mind, the analysis of protein carbonyls using a spectrophotometric technique for the determination of protein oxidation is completely explained in Chapter 10, while comprehensive procedures for the determination of primary and secondary lipid oxidation products are described in Chapter 11.

Volatile compounds are vital for the correct development of the typical aroma of meat and meat products. Additionally, the release of some specific compounds allows the monitoring of degradative processes. Therefore, the analysis of these compounds is important to monitor the sensory quality and the manufacturing process. Chapter 12 provides a complete description of the analysis of volatile compounds using a solvent-free technique, based on solid-phase microextraction with gas chromatography-mass spectrometry determination.

Chapter 13 provides a brief overview of specific issues in the meat industry, which may be resolved using the current proteomics-based methodology. The quantification, analysis, and curation of proteomics data are explained in this chapter.

Finally, the antioxidant activity of meat and meat products could exert a protective effect against oxidative degradation. Therefore, Chapter 14 explains in a comprehensive way the main techniques used for the determination of antioxidant capacity in meat and meat products.

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Series Preface

The *Methods and Protocols in Food Science* series is devoted to the publication of research protocols and methodologies in all fields of food science. The series is unique as it includes protocols developed, validated, and used by food and related scientists as well as theoretical bases are provided for each protocol. Aspects related to improvements, adaptations, and further developments in the protocols may also be approached.

The *Methods and Protocols in Food Science* series aims to bring the most recent developments in research protocols in the field as well as very well-established methods. As such the series targets undergraduate, graduate, and researchers in the field of food science and correlated areas. The protocols documented in the series will be highly useful for scientific inquiries in the field of food sciences, presented in such a way that the reader will be able to reproduce the experiments in a step-by-step style.

Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol is clarified. Then, an in-depth list of materials and reagents required for employing the protocol is presented, followed by comprehensive and step-by-step procedures on how to perform that experiment. The next section brings the dos and don'ts when carrying out the protocol, followed by the main pitfalls faced and how to troubleshoot them. Finally, template results will be presented and their meaning/conclusions addressed.

The *Methods and Protocols in Food Science* series will fill an important gap, addressing a common complaint of food scientists, regarding the difficulties in repeating experiments detailed in scientific papers. With this, the series has a potential to become a reference material in food science laboratories of research centers and universities throughout the world.

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Chapter 1

Chemical Composition

Roberto Bermúdez, Noemí Echegaray, Maria João Fraqueza, Mirian Pateiro, Rubén Domínguez, Paulo E. S. Munekata, José Manuel Lorenzo, and Eva María Santos

Abstract

Meat composition influences the nutritional and sensory quality of meat products, therefore elucidating its composition is essential for the production chain. Moisture, fat, protein, and ash are the main components of these products whose determinations have been widely studied for many years. That is why today traditional methods, new techniques, and a host of smart equipment can be used, which could affect the results obtained and their comparison.

This chapter give a complete analysis guideline of the aforementioned determinations. Moisture determination is carried out by thermogravimetry, according to sample weight loss. Fat and protein contents are determined by Ankom technology and Kjeldahl method, respectively. Ashes are calculated by weight loss once the sample has been calcined in a muffle furnace.

Key words Moisture, Protein, Fat, Ash, Proximate composition, Gravimetric method, Ankom extractor, Hydrolysis, Kjeldahl method, Calcination process

1 Introduction

Chemical composition, also known as proximate composition, is very important in the food industry for the development of new products, quality control, consumer information or regulatory purposes. In this way, it is necessary to know the percentage of moisture, protein, fat, and ash of the products to establish rules that regulate the diet of the people to make it healthier. Therefore, these analyses are very important since they are directly related to the health of consumers, as well as for the food production [1, 2]. All of these parameters could be modified both by changing the diet of the animals or by using new ingredients during processing [3]. The best level of each of them depends on the type of consumer.

Taking into account the aforementioned facts, people have the right to know the nutritional composition of the meat and meat

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_1,

products that they eat. Therefore, Governments ensure this to their citizens through several rules, such as Regulation (EU) 1169/2011 in the European Union (EU) [4] or CFR (21CFR101) of the Food and Drug Administration (FDA) of the United States of America (USA) [5], among others. So, companies have to label their products according to these regulations. They are many similarities between them, only with slight differences. According to Regulation 1169/2011, it is compulsory to label the percentage of fat, protein, carbohydrates, total sugars, sodium chloride, saturated fatty acids and energy (Kcal and KJ), as well as, all allergens presented in products. In addition, other nutrients can also be labeled, such as dietary fiber, mono and polyunsaturated fatty acids, vitamins, etc. In addition, it is necessary to include on the label important information about the origin, expiration date, etc.

All methods, which we are going to describe below, are supported by international organizations such as the International Organization for Standardization (ISO) or the American Oil Chemists Society (AOCS). Firstly, water occurs as the main constituent of meat and meat products. Therefore, the determination of the water content of meat and meat products is very important, since water is an essential constituent and its interaction with other components condition the shelf life and the quality of the product [6]. Moisture analysis is a gravimetric method based on loss of weight by drying. This method has been used in the same way for many years. Similarly, ashes determination is based on a loss of weight due to the calcination of organic matter in a muffle furnace. Secondly, lipids play an important role in sensory quality of meat and meat products, since it contributes to attributes such as texture (juiciness), the formation of the characteristic flavor of the product, and its final appearance. Therefore, fat determination is one of the required analyses in food labeling and quality control [7]. Fat analysis can be performed with or without hydrolysis, depending on the type of the sample. In many samples, fat is not freely accessible to the solvent and cannot be fully extracted. The bonding of fats in starch-lipid complexes or with lipoproteins or phospholipids, prevents direct extraction of the fat. For this reason, hydrolysis with HCl to prepare samples for fat determination is prescribed by law in a wide range of national and international methods. Fat determination with hydrolysis is known as "Total fat determination," while the method without hydrolysis determines crude fat. In addition, the analysis of fat has changed over the years, specially, regarding the equipment used. In this regard, Soxhlet extractor sometimes has limitations, especially when the fat content is very low, which makes it not very reproducible. Below, an alternative to this apparatus is shown.

Proteins are also very important constituents of meat and meat products, since they have a high nutritional value and their structural and physicochemical characteristics are related to their

functional properties [7]. As with fat determination, protein analysis has also changed over the years regarding the equipment used, so numerous methods have been developed. Two steps are necessary, an acid digestion and a titration to obtain the total nitrogen content present in the sample. The application of a correction factor, which is specific for each type of sample, allows to calculate the percentage of protein.

The aim of this chapter is to make a guide for the analysis of the chemical composition of meat and meat products, so that they can compare the results minimizing the error due to the method used.

2 Materials

Prepare all solutions using distilled water and/or analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials (*see* **Note 1**).

2.1 Sample Preparation for Analysis

1. Professional mill KN 295 Knifetec (Foss).
2. Homogeniser HM 294 (Foss).
3. Cutting board.
4. Knife.

2.2 Moisture Determination

1. Drying oven with capacity to dry the samples at 105 ± 5 ° C, mod. UFP 600 (Memmert).
2. Analytical balance, with a resolution of 0.0001 g, mod. ME 614S (Sartorius).
3. Desiccator equipped with an effective dehydrator (Silica Gel with humidity indicator).
4. Stainless steel capsules of 60 mm diameter and 25 mm high.
5. Glass rod with rounded tip, which goes completely into the capsule.
6. Eppendorf Research Micropipette 500–5000 μL.
7. Washed sea sand, fine grain. General purpose grade.
8. Ethanol 96% for analysis, ACS, Reagent grade.

2.3 Fat Determination

1. Filter Bags, mod. XT4 (Ankom).
2. Indelible marker.
3. Filters support (Ankom).
4. Stainless steel capsules of 60 mm diameter and 25 mm high.
5. Analytical balance, with a resolution of 0.0001 g, mod. ME 614S (Sartorius).

6. Thermosealer Impulse Sealer, mod. AIE-200.
7. Drying oven with capacity to dry the samples at 105 ± 5 ° C, mod. UFP 600 (Memmert).
8. Desiccator equipped with an effective dehydrator (Silica Gel with humidity indicator).
9. Extraction Unit, mod. XT10 (Ankom).
10. Ankom hydrolysis unit, mod. HCl (Ankom).
11. Weight to dry the samples after hydrolysis (Blotter), mod. H35 (Ankom).
12. Rapid Dryer HCl Filter RD2 (Ankom).
13. Support for samples.
14. Borosilicate glass graduated cylinder of 250 mL, class A.
15. Solvent recovery glass bottle 500 mL.
16. Petroleum ether with a boiling point range 40–60 °C, reagent grade, ACS, ISO (*see* **Note 2**).
17. Celite Hyflo Super Cel, RE.
18. Hydrochloric acid solution (3 N): Mix 248 mL of hydrochloric acid (37%) with 752 mL of distilled water.

2.4 Protein Determination

1. Analytical balance, with a resolution of 0.0001 g, mod. ME 614S (Sartorius).
2. Precision balance with a resolution of 0.01 g, mod. TE 612 (Sartorius).
3. Temperature Controller, mod. TZ (Gerhardt).
4. Nitrogen Digestor Kjeldatherm, mod. KB20 (Gerhardt).
5. Nitrogen Distiller, mod. VAPODEST 50 carrousel (Gerhardt).
6. Kjeldatherm digestion tubes, 250 mL (Gerhardt).
7. Metal tube rack (Gerhardt).
8. Filter paper 5 × 8 cm approximately.
9. 1000 mL Glass Beaker.
10. 1000 mL volumetric flask, Class A.
11. Liquid dispenser with a resolution of 0.5 mL and capable of dispensing in a range of 2.5–25 mL, mod. Genius 1,605,006 (Walu).
12. 500 mL washing bottle.
13. Distilled water.
14. Sulfuric acid 96% for analysis, ISO (*see* **Note 2**).
15. Universal indicator of pH, solution for volumetric analysis.
16. Kjeldahl catalyst tablets: Kjeltabs 5 g K_2SO_4 + 0.5 g $CuSO_4 \times 5H_2O$ (Gerhardt).

17. Boric acid 2%. Dissolve 20 g of boric acid in 1 L of distilled water.
18. Sodium hydroxide solution 32% for Kjeldahl.
19. Sodium hydroxide pellets, ACS, reagent grade.
20. Sodium hydroxide 15%. Dissolve 180 g of sodium hydroxide pellets in 1.2 L of distilled water. Add several drops of universal pH indicator. It will be valid until the indicator turns from blue to yellow (*see* **Note 3**).
21. Hydrochloric acid 0.1 N PA, quality titrated solution.
22. Buffer solution pH 7.00 ± 0.02.
23. Buffer solution pH 4.00 ± 0.02.

2.5 Ash Determination

1. Muffle furnace, mod. RWF12–13 (Carbolite).
2. Analytical balance, with a resolution of 0.0001 g, mod. ME 614S (Sartorius).
3. Desiccator equipped with an effective dehydrator (Silica Gel with humidity indicator).
4. Socorex ACURA 825 Micropipette 100–1000 μL.
5. Porcelain crucibles of 25 mL, glazed inside and out, except base. Certificate according to DIN 12904. Withstands maximum temperature of 1000 °C.
6. Suitable forceps for handling porcelain crucibles.
7. Heat resistant gloves (*see* **Note 4**).
8. Drying oven with capacity to dry the samples at 105 ± 5 ° C, mod. UFP 600 (Memmert).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Sample Preparation for Analysis

The first step is to chop the sample into small cubes and homogenize it correctly [8]. Both chopping and homogenization are carried out with a professional mill KN 295 Knifetec (Foss) or a Homogeniser HM 294 (Foss) depending on the type/size of the sample. This step is very important in order to achieve good and representative results.

3.2 Moisture Determination

Moisture content is determined by oven drying (Memmert UFP 600, Schwabach, Germany) at 105 °C until constant weight, and is calculated as sample weight loss, according to ISO 1442 (1997) [9]. Figure 1 shows in a simplified and schematic way the steps of the moisture determination.

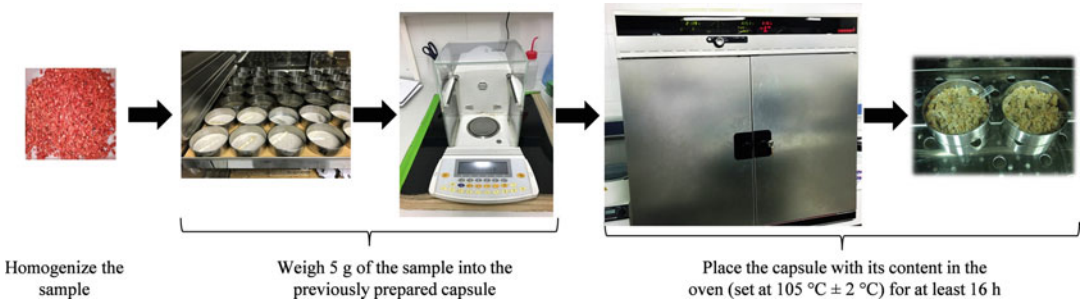

Fig. 1 Schematic and simplified representation of the steps of the moisture determination

1. Firstly, prepare the stainless steel capsules with sea sand and a glass rod (*see* **Note 5**). Then, the set of capsules, sea sand, and glass rod must be dried for at least 1 h in the oven (set at 105 °C ± 2 °C).
2. Before weighing the capsules, sea sand and glass rod must be at room temperature. To do this, they need to be placed in a desiccator (containing an efficient desiccant, such as silica gel) for 1 h.
3. Next, weigh the set (capsule, sea sand, and glass rod) on an analytical balance, with an accuracy of 0.0001 g.
4. Weigh 5 g of minced and homogenized sample into the capsule which has sea sand and a glass rod.
5. Mix the contents of the capsule (sample + sea sand) with the help of the glass rod (*see* **Note 6**).
6. Place the capsule with its content (sea sand + sample + ethanol) in the oven (set at 105 °C ± 2 °C) for at least 16 h (until reaching constant weight).
7. After the necessary time, remove the capsules with its contents from the oven and place them in the desiccator for 1 h (until reaching room temperature).
8. Finally, weigh the capsules and its content on an analytical balance, with an accuracy of 0.0001 g.
9. Expression of results
   (a) Calculate the moisture content, as a percentage by mass, using the following equation:

$$\%\text{Moisture} = \left[\frac{(W_1 - W_2)}{(W_1 - W_0)}\right] \times 100$$

where:

- W_0 is the capsule, glass rod, and sea sand weigh (g).
- W_1 is the capsule containing the sample, glass rod, and sea sand weigh before drying (g).
- W_2 is the capsule containing the sample, glass rod, and sea sand weigh after drying (g).

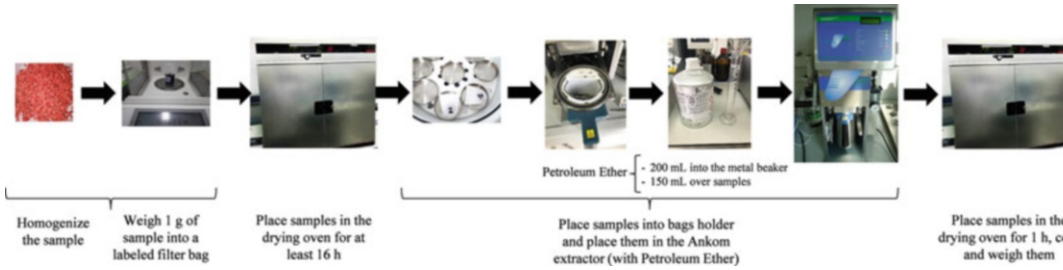

Fig. 2 Schematic and simplified representation of the steps of the fat determination without hydrolysis

3.3 Fat Determination

Fat content is obtained by weight difference. It is carried out with or without hydrolysis, depending on the type of the sample (*see* **Note 7**). We performed the analysis according to AOCS Am5-04 (2005) [10].

3.3.1 Total Fat Extraction Without Hydrolysis

Figure 2 shows in a simplified and schematic way the steps of the fat determination without hydrolysis.

1. Label the filter using a permanent marker. Place the filter holder on the analytical balance and tare. Place the previously labeled filter on the holder so that the filter remains open to allow the sample to enter in it. Weigh the filter on the analytical balance with an accuracy of 0.0001 g, note the value obtained ("Filter weight (g)") on the laboratory sheet and tare again.
2. Weigh 1 g of sample into the filter and record on the laboratory sheet ("Sample weight W_1 (g)"). Take the filter and seal the open part with the heat sealer about 2 mm from the end of the filter (*see* **Note 8**).
3. Place the sealed filter in a stainless steel capsule, recording the number and weight of it previously, with an accuracy of 0.0001 g ("No./Weight Ankom capsule (g)"), on the worksheet (*see* **Note 9**).
4. Bring the samples to the oven for drying at 105 ± 5 °C for at least 16 h. Record on the worksheet. After drying, place the samples in a desiccator for 1 h and then, weigh them on the analytical balance with an accuracy of 0.0001 g ("Dry sample weight 1 W_2 (g)").
5. Then take the samples to the Ankom XT10 extractor and place them in the sample holder (maximum 15 samples). In a fume hood, pour 200 mL of petroleum ether, measured with a measuring cylinder, into the metal beaker of the extractor, place the plastic upper part and the samples inside it and pour 150 mL of solvent over them, and close the equipment and press start. You can see the extraction conditions in Table 1 (*see* **Note 10**).

Table 1
Ankom fat extractor working conditions

Fat extractor working conditions	
Extraction time (min)	60
Solvent recovery time (min)	10
Extraction temperature (°C)	90

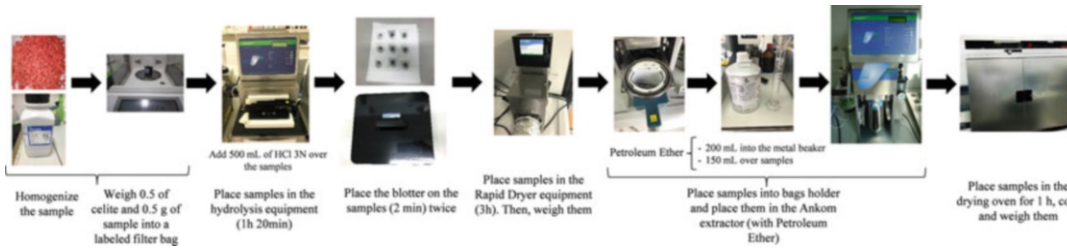

Fig. 3 Schematic and simplified representation of the steps of the fat determination with hydrolysis

6. Finally, remove the samples from the equipment, and place them in the oven under the same conditions as during drying (105 ± 5 °C) for 1 h, to eliminate the residual solvent. After this time, place the samples in a desiccator for 1 h, and weigh them on an analytical balance with an accuracy of 0.0001 g ("Dry sample weight 2 W_3 (g)") (*see* **Note 11**).

3.3.2 Total Fat Extraction with Hydrolysis

Figure 3 shows in a simplified and schematic way the steps of the fat determination with hydrolysis.

1. Label the filter using a permanent marker. Place the filter holder on the analytical balance and tare. Place the previously labeled filter on the holder so that the filter remains open to allow the sample to enter in it. Weigh the filter on the analytical balance with an accuracy of 0.0001 g, note the value obtained ("Filter weight (g)") on the laboratory sheet and tare again.
2. Weigh 0.5 g of celite onto the filter, tare and weigh 0.5 g of sample onto the filter (inside of the celite) with an accuracy of 0.0001 g, and record both on the procedure test sheet ("Sample weight W_1 (g)") (*see* **Note 12**). Take the filter and seal the open part with the heat sealer about 2 mm from the end of the filter (*see* **Notes 8** and **13**).
3. Place the samples in the HCl Hydrolysis System equipment to carry out the hydrolysis process. To do this, put the samples in the corresponding support and the set, samples-support, in the equipment vessel. We fill it with 500 mL of 3 N HCl. You can see the hydrolysis conditions in Table 2 (*see* **Note 10**).

Table 2
Ankom hydrolysis system working conditions

Hydrolysis system working conditions	
Hydrolysis time (min)	60
Hydrolysis temperature (°C)	90
Rinse time (min)	20

4. Once the cycle is finished (1.20 h, approx.), we turn off both the water and the equipment. Remove the samples from the equipment, place them between absorbent paper, and place the blotter on top to dry them for 2 min. We repeat this step one more time.
5. Then, we transfer the samples to the rapid drying equipment (Rapid Dryer), and we keep them in it for 3 h at 105 ± 5 °C. Once finished, place the samples in a desiccator for 1 h and weighed them on an analytical balance with an accuracy of 0.0001 g, recording this value ("Dry sample weight 1 W_2 (g)") on the worksheet.
6. Then, take the samples to the Ankom XT10 extractor and place them in the sample holder (maximum 15 samples). In a fume hood, pour 200 mL of petroleum ether, measured with a measuring cylinder, into the metal beaker of the extractor, place the plastic upper part and the samples inside it and pour 150 mL of solvent over them, and close the equipment and press start. You can see the extraction conditions in Table 1 (*see* **Note 10**).
7. Finally, remove the samples from the equipment, and place them in the oven under the same conditions as during drying (105 ± 5 °C) for 1 h, to eliminate the residual solvent. After this time, place the samples in a desiccator for 1 h, and weigh them on an analytical balance with an accuracy of 0.0001 g ("Dry sample weight 2 W_3 (g)") (*see* **Note 11**).
8. Expression of results
   (a) The results for the samples made without hydrolysis are expressed as a percentage of fat following the formula:

$$\%\text{Fat (without hydrolysis)} = \left[\frac{((W_2 - C) - W_3)}{W_1}\right] \times 100$$

where:

- W_1 is the sample weight (g).
- W_2 is the sample, capsule, and filter weight before drying (g).

- W_3 is the sample and filter weight after extraction (g).
- C is the capsule weight (g).

(b) The results for the samples made with hydrolysis are expressed as a percentage of fat following the formula:

$$\%\text{Fat(with hydrolysis)} = \left[\frac{(W_2 - (W_3 + (B_1 - B_2)))}{W_1}\right] \times 100$$

where:

- W_1 is the sample weight (g).
- W_2 is the dry sample + filter + celite weight after hydrolysis (g).
- W_3 is the dry sample + filter + celite weight after extraction (g).
- B_1 = "Blank" sample weight after hydrolysis (g).
- B_2 = "Blank" sample weight after extraction (g).

3.4 Protein Determination

Protein determination is carried out into two phases according to ISO 937 (1978) [11]. First a digestion of the sample and second a distillation, and subsequent potentiometric titration. Figure 4 shows in a simplified and schematic way the steps of the protein determination.

3.4.1 Digestion

1. Weigh the sample on a filter paper on the analytical balance with a precision of 0.1 mg, and take it to a digestion tube that will be supported by the metal rack designed for this purpose (*see* **Note 14**).
2. Depending on the type of samples, proceed as follows:
   (a) For dehydrated samples: 0.5 g ± 0.1 g of sample must be weighed on filter paper and placed in the digestion tube.
   (b) For fresh samples: 1.0 g ± 0.2 g of sample should be weighed on filter paper and introduced into the digestion tube.

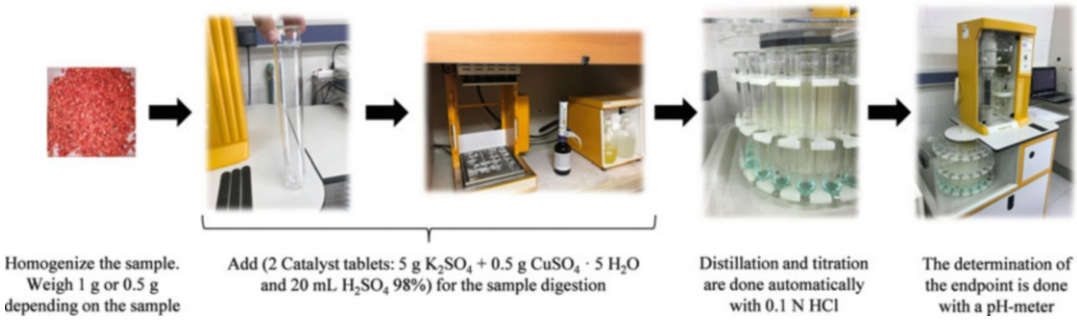

Fig. 4 Schematic and simplified representation of the steps of the protein determination

Table 3
Summary of the digestion programs

Program	Step	Time	Temperature (°C)
1	1 2	15 min 1 h 30 min	0–400 400
2	1 2 3 4	15 min 20 min 15 min 2 h	0–200 200 200–400 400

Table 4
Washing program characteristics of distillation equipment

Step	Value
H_2O addition	120 mL
NaOH addition	0 mL
Distillation time	7 min
Esteam generator	100%
Sample suction	25 s
Titration vessel suction	25 s
H_2BO_3 addition	80 mL

3. Add two catalyst tablets to each tube and 20 mL of 96% sulfuric acid and place the rack with the tubes in the digestor. Then, you must select the digestion program (Table 3; *see* **Note 15**). Once digestion is complete, allow tubes to cool for 30 min.

3.4.2 Distillation and Titration

1. While digestion is taking place, we must wash the distillation equipment. For that, we place three tubes with distillated water (up to half of the tube) in the "Vapodest" and start the washing program in the software (Table 4). Previously, we must calibrate the pH electrode with buffers 4 and 7.
2. Once the digestion is finished, we take the samples to the distillation equipment (*see* **Note 16**) and we place the samples on the carousel in the corresponding order. In this moment, we must record the sample weight with its name in the software. Then, we start the sample program (Table 5; *see* **Note 17**).
3. At the end of the distillation and titration, the equipment gives us the total nitrogen content of the sample. In addition, the software gives us the total protein content, obtained by multiplying the nitrogen percentage by a conversion factor that depends on the type of sample (Table 6) [12].

Table 5
Samples program characteristics of distillation equipment

Step	Value
H_2O addition	100 mL
NaOH addition	80 mL
Distillation time	4 min
Esteam generator	100%
Sample suction	25 s
Titration vessel suction	25 s
H_2BO_3 addition	80 mL
Conversion factor	6.25

Table 6
Nitrogen to protein conversion factors

Product	Conversion factor
Rye, barley, oats	5.83
Flour, whole wheat flour	5.83
Flour, medium or low extraction; wheat pasta	5.70
Rice	5.95
Soy *, seeds	5.71 (6.25) *
Chestnut, coconut	5.30
Milk	6.38
Other foods	6.25

4. Expression of results
   (a) The results for the samples are expressed as a percentage of protein following the formula:

$$\%\text{Nitrogen} = \frac{(1.4007 \times \text{TF} \times [\text{HCl}] \times (\text{Sample(mL)} - \text{Blank(mL)}))}{W_1}$$

$$\%\text{Protein} = (\%\text{Nitrogen} \times \text{CF})$$

where:

- W_1 is the sample weight (g).
- TF is the Titration Factor = 1.
- [HCl] is the HCl concentration = 0.1 N
- Sample (mL) is the HCl consumption in the sample.

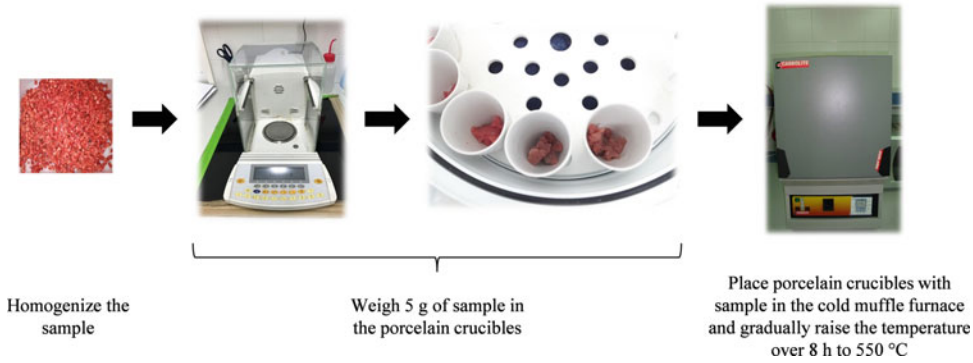

Fig. 5 Schematic and simplified representation of the steps of the ash determination

- Blank (mL) is the HCl consumption in the blank.
- CF is the conversion factor.

3.5 Ash Determination

Ash determination is carried out by weight loss by calcination of organic matter in a muffle furnace at 550 °C, according to ISO 936 (1998) [13]. Figure 5 shows in a simplified and schematic way the steps of the ash determination.

1. Firstly, porcelain crucibles must be dried for at least 1 h in the oven (set at 105 °C ± 2 °C).
2. Then, cool the porcelain crucibles in a desiccator at room temperature and weigh (W_0) on the analytical balance, with an accuracy of 0.0001 g.
3. Weigh 5 g of minced and homogenized sample into the porcelain crucibles (W_1).
4. Place the porcelain crucibles with its contents in the cold muffle furnace and gradually increase the temperature of the muffle furnace over 8 h to 550 ± 25 °C. Continue the incineration at 560 ± 25 °C until the ash has a gray-white appearance (*see* **Note 18**).
5. When the ash has a gray-white appearance, remove the porcelain crucibles with their contents from the muffle furnace and allow to cool in the desiccator to room temperature (1 h).
6. Finally, weigh the porcelain crucibles and their content (ashes) (W_2) on the analytical balance, with an accuracy of 0.0001 g.
7. Expression of results.
   (a) The results for the samples are expressed as a percentage of ash following the formula:

$$\%\text{Ash} = \left[\frac{W_2 - W_0}{W_1 - W_0}\right] \times 100$$

where:

- W_0 is the porcelain crucible weigh (g).
- W_1 is the sample and crucible weigh before calcination (g).
- W_2 is the sample and crucible weigh after calcination (g).

4 Notes

1. All laboratory waste must be eliminated according to its nature following current regulations, differentiating at least acids, bases, organic solvents, halogenated solvents, and non-halogenated solvents.
2. Due to the toxic and/or corrosive nature of the solvents and reagents used in all described determinations, it is necessary for the operator to take the necessary protective measures (gloves, glasses, etc.), as well as to carry out all the operations in laboratory fume hoods.
3. The concentration of this reagent is approximate, since it is only used to neutralize gases in the digestor.
4. Temperatures of over 500 °C are reached in the muffle furnace, so it is necessary to handle the crucibles with gloves and special forceps to avoid burns.
5. In order to prepare the stainless steel capsules, you need to add an amount of sand (dry sea sand) equal to three to four times the mass of the sample portion that you will add to the stainless steel capsules, and place the glass rod inside the capsule and on the sea sand.
6. If you find it difficult to mix the sample with sea sand, you can use as much ethanol as you need (approximately 5 mL). The ethanol will evaporate during drying of the sample in the oven.
7. In samples where the fat is not freely accessible to the solvent such as fats in starch-lipid complexes or to lipoproteins or phospholipids, as in mixed products such as baked goods, hydrolysis must be performed.
8. For proper sealing, the heat sealer must be kept at a power of 6 and keep the filter in it for approximately 5 s after the red light has turned off.
9. At the same time as samples, we will insert an empty filter that will serve as a blank and another one with a reference material in the same way as the rest of the samples.
10. We must be sure that the water is open for cooling the equipment before starting it.
11. The result is expressed as a percentage, grams of fat per 100 g of product.

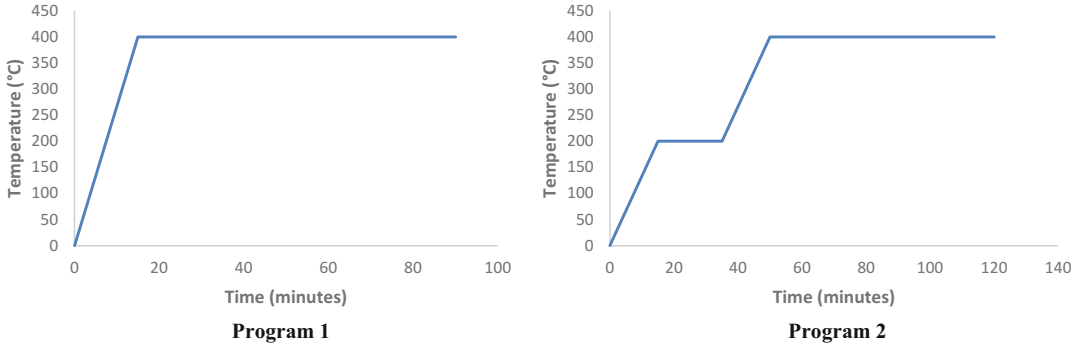

Fig. 6 Digestion programs

12. In order to place the sample inside the celite, a hole must be made with a glass rod.
13. At the same time as samples, we will insert a filter with 0.5 g of celite that will serve as a blank, and another one with a reference material in the same way as the rest of the samples.
14. In each batch of samples, a blank, ammonium sulfate, and a control sample (reference material) will be analyzed. With ammonium sulfate we can check the correct functioning of the digestion, and with the reference material we can check both the digestion and the distillation.
15. You must select a digestion program depending on the type of sample:
    (a) Program 1 (Fig. 6): Fresh samples.
        - *Step 1*: 15 min—400 °C. During 15 min there is a constant rise in temperature until reaching 400 °C.
        - *Step 2*: 1 h 30 min—400 °C. Once 400 °C has been reached, it is kept at this temperature for 1 h and a half.

    (b) Program 2 (Fig. 6): Dehydrated samples.
        - *Step 1*: 15 min—200 °C. During 15 min there is a constant rise in temperature until reaching 200 °C.
        - *Step 2*: 20 min—200 °C. Once 200 °C is reached, it is kept at this temperature for 20 min.
        - *Step 3*: 15 min—400 °C. After 20 min of the previous step, there is another constant rise in temperature until reaching 400 °C.
        - *Step 4*: 2 h—400 °C. Once 400 °C has been reached, it is kept at this temperature for 2 h.
16. The equipment must be turned on 20 min before starting the analysis. At the same time, we must turn on the "Vapodest Manager" software.
17. The sample distillate is collected in a beaker with boric acid (2%) and titrated with hydrochloric acid (0.1 N). The

equipment records the initial pH of the boric acid and ends the analysis when the final pH is equal to the initial one.

18. The muffle furnace is programmed to maintain incineration for 8 h. This time should be sufficient for most samples.

Acknowledgments

This work was supported by Centro Tecnolóxico de la Carne de Galicia. Thanks to GAIN (Axencia Galega de Innovación) for supporting this book chapter (grant number IN607A2019/01). Authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568). Noemí Echegaray acknowledges to Consellería de Cultura, Educación e Ordenación Universitaria (Xunta de Galicia) for granting with a predoctoral scholarship (Grant number IN606A-2018/002).

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Chapter 2

pH and Color

Alfredo Teixeira, Rubén Domínguez, Javier F. Rey, Gonzalo Aleu, Mirian Pateiro, and José Manuel Lorenzo

Abstract

pH measurement and color evaluation are two important parameters for determining meat quality. There are the characteristics that after slaughter until the installation of rigor mortis should be assessed to define the quality of a meat and its ability to be processed into quality products. The aim of this chapter is to give a clear and concise view of the techniques used to assess pH and color both physical (CIELAB) and chemical (heme pigment content) in meat and meat products.

Key words Meat, Carcass, pH, Color, CIELAB, Heme pigments, Spectrometer, Spectrophotometer

1 Introduction

The pH and color are probably the most important physical-chemical characteristics of meat quality evaluation. Both are important in the detection of meat quality deviations once they are related with the post-mortem chemical and biochemical reaction occurring to conversion of muscle to meat [1] and particularly the color is the only characteristic that consumers could assess at the time of purchase and decide this option to buy or not since consumers relate the color of meat with its sensory properties. On the other hand, the discoloration is one of the first indicator of meat spoilage. The pH value of meat affects the color and its quality in terms of water-holding capacity and the sensory quality namely its tenderness and flavor [2]. At slaughter the meat pH is neutral (around 7.0) and after slaughter the muscle under anaerobic metabolism conditions the pH decreases as result the conversion of glycogen to lactate [1] and the pH value at rigor mortis is named the ultimate pH and depending on animal species and muscle type the ultimate pH of 5.3–5.8 is reached at different times [2]. As a result of the effect of the pH in the physicochemical characteristics of the meat, a wrong

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_2,

kinetics of pH during the installation of rigor mortis can affect the suitability of meat to technological processing.

Therefore, pH measurement gives important information on the quality of meat and particularly in the pork the pH value 24 h after slaughter is a fundamental data to detect PSE (pale, soft, exudative) and DFD (dark, firm, dry) meat. Also, the pH measurement is essential to detect DFD beef and sheep meats. So, to monitor the ultimate pH kinetics the method and process of measurement should be defined.

In addition, color deviations in the abnormal course of rigor mortis and meat aging conditions could occur [3]. Color deviations assume a color perceived as normal and in meat quality a normal color is not universally consensual. Anyway, globally is expected that beef and mutton are red, pork, veal, and lamb pink and poultry white meats. Besides white, or red, terms such as shine, pale, or dark are also usually used in meat color characterization. Thus, depending on what is intended to characterize or classify it is important to set the color evaluation procedure method. Color measurement in association with pH is useful for detection of quality deviations such as PSE and DFD in pork or DFD in beef and sheep. Beyond the DFD and PSE meats the National Pork Producers Council (NPPC) [4], based in the pH and color assessment, defines three more categories: RFN (red, firm, non-exudative), RSE (red, soft exudative), and PFN (pale, firm, non-exudative). Color is also used by NPPC to analyze and define the marbling scores corresponding to intramuscular lipid content (Fig. 1). Normal color deviations can result in abnormal kinetics of muscle talk in meat, compromising their quality and adequacy for industry processing or influencing consumer purchase decision. Thus, the color measurement during slaughter and rigor process as well as the values of pH measured at 45–60 min after slaughter and the ultimate pH are essential to assess and define the quality of meat.

Until the middle of twentieth century, pH measurement was made using as pH indicators a wide diversity of color substances in solution or paper strips or subsequently using glass electrodes that incorporate reference electrodes. Nowadays pH was measured using pH-meter, with large portability and easy handling, with different devices and producers. In the recent years, non-invasive hyperspectral methodologies [5] for rapid pH monitoring for meat quality assessment have been developed. The Raman spectroscopy, a portable device shows considerable potential for some applications in the meat sector, including color measurements and pH [6]. In any case, in the slaughterhouse in the system most currently used to measure the pH is the pH-meter directly on carcass or meat using a probe or an electrode provided with a penetration blade.

The color of the meat can be analyzed using visual or instrumental methods. The visual evaluation of the color consists basically in the use of color pattern cards or photographic scales,

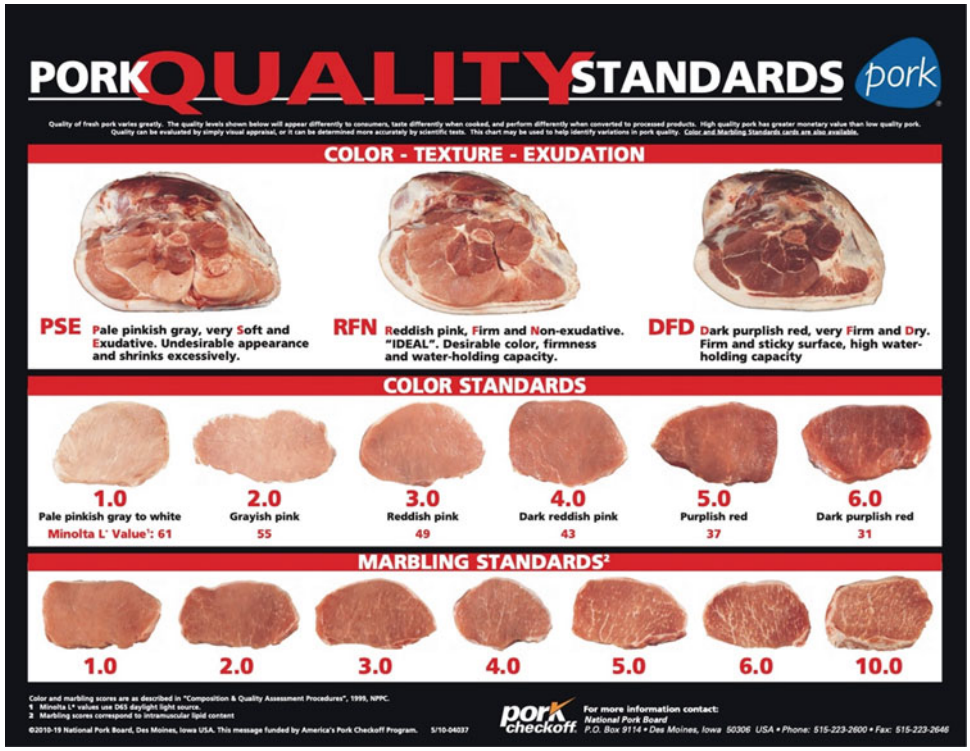

Fig. 1 Pork Quality Standards defined as Color—Texture—Exudation, Color Standards, and Marbling standards as described [4]

resulting in a subjective evaluation regarding the conditions of assessment, lighting, description of the different color tones, training of assessors, and the difficulty to find corrected matching between the patterns and the samples analyzed. Instrumental evaluation using the CIELAB system [7] specifies the color in a space of three coordinates defined as L*, a*, b*. L* is the lightness and varies from 0 (black) to 1 (white). Each series of L *, a*, and b* values correspond a point defined as an exact color in the three-dimensional color sphere (Fig. 2). The a* and b* are chromatic coordinates and measure de red-greenness and yellow-blueness, respectively and are used to calculate saturation or chroma (C*) and hue (h°) [1]. Color coordinates are measured using portable color analyzers. These devices also automatically compute the C* and h°.

Measuring the concentration of heme pigments is another way to analyze the color of meat. Color is determined by the chemical form and the concentration of the heme pigment myoglobin and hemoglobin: oxymyoglobin (MbO_2), deoxymyoglobin (Mb), and metmyoglobin (MMb). The MbO_2 is responsible for the bright red color of fresh meat and the deoxymyoglobin or reduced myoglobin

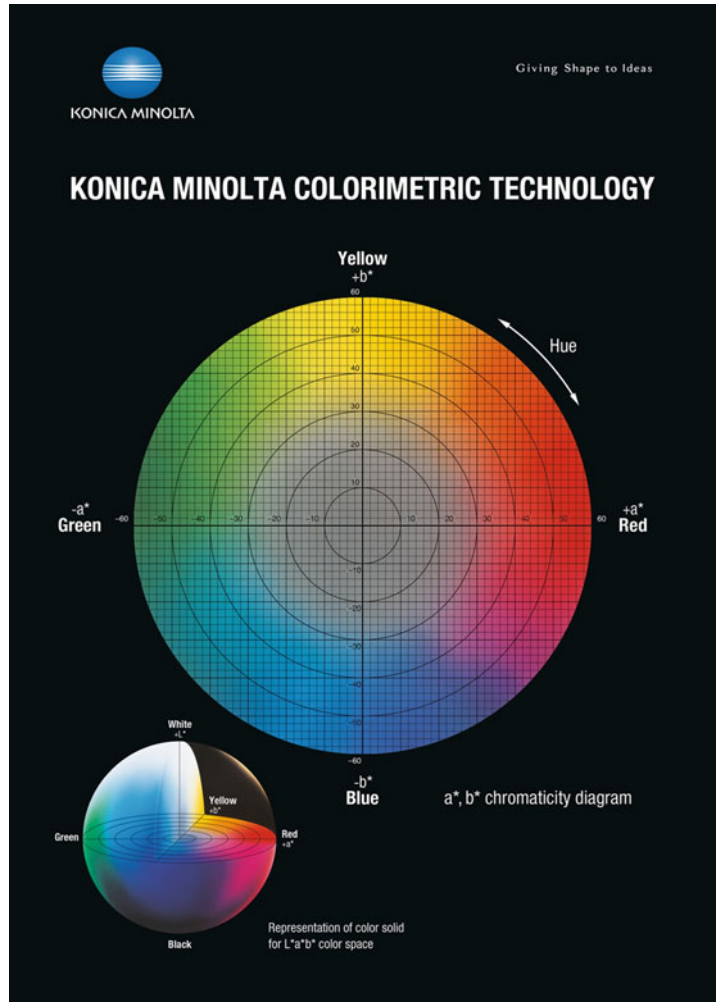

Fig. 2 The CIELAB color space defined by the coordinates L*, a*, and b* and the color attributes C* and h° [8]. Free available from Konica Minolta webpage (https:/www5.konicaminolta.eu/en/measuring-instruments/media-centre/poster-download.html)

(Mb) shows a purple-red meat color while the MMb exhibits a brown color as a result of oxidation of myoglobin pigment [1]. The quantification of total three states of pigment in meat samples could be assessed by reflectance spectrophotometry [9].

Therefore, the present chapter of the book aims to describe the procedures to assess pH and color characteristics as important factors to evaluate meat and meat products quality.

2 Materials

In this section, we will describe the procedures for the determination of pH, color by the CIELAB system, and color measurement of total heme pigment concentration.

2.1 pH Determination

The pH determination can be carried out on the carcass or on meat samples or meat products, depending on what is to be analyzed or evaluated (*see* **Note 1**). Currently, the most common method uses the following material:

1. A portable pH-meter provided with a penetration electrode (*see* **Note 2**).
2. Buffer solutions with pH of 7.0 and 4.0.

2.2 Physical Color (Cielab System)

The model and make of the device used can be varied. The most usual are the chromameters Konica Minolta from the series CR200, CR300, CR400, CR410 (*see* **Note 2**).

2.3 Chemical Color (Heme Pigments)

The chemical color determination needs a meat sample preparation that requires the use of some solutions.

1. Distilled water.
2. Acetone.
3. HCl 35%.
4. Filter paper Ø 150 mm and a maximum of 31 μm of pore.
5. A spectrophotometer (*see* **Note 3**).

For the measurement of pigments deoxymyoglobin (Mb), oxymyoglobin (MbO_2), and metmyoglobin (MMb):

1. PVC film with very low oxygen permeability.
2. 10% of sodium dithionite solution ($Na_2S_2O_4$)
3. 1% ferricyanide and potassium cyanide solution [$K_4Fe(CN)_6$].

3 Methods

3.1 pH Measurement

Before taking a measurement the pH-meter should be calibrated using buffer solutions with a known pH of 4.0 and 7.0. It is very important to stabilize the temperature of the electrode to the temperature of the muscle (*see* **Note 4**). There are metal and glass electrodes, equipped with a piercing tip that allows the measurement of pH directly in the carcass muscles or in pieces of meat or processed meat products (Fig. 3) (*see* **Note 5**). Measurements made directly on the carcass are usually taken at *m. Longissimus thoracis et lumborum* (also known as *longissimus dorsi*) and in

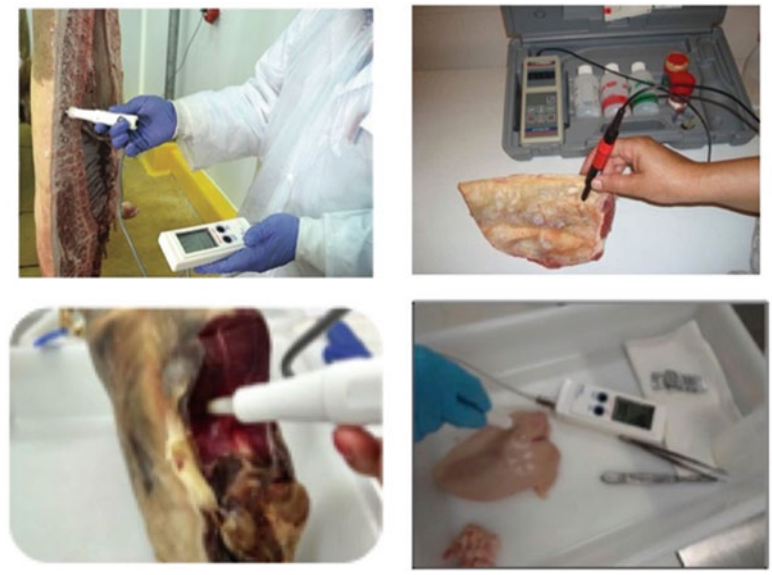

Fig. 3 pH measurement in a carcass, a piece of meat, and a processed meat product

m. Semimembranosus (*see* **Note 6**). The equipment is also able to measure in homogenized meat or in a meat sample homogenized in water.

3.2 Color Measurement (CIELAB System)

The instruments take measurements at 400–700 nm and give readings at intervals of 10 nm, allowing for thorough color data analysis and come with software to automatically compute the h* and C* (Fig. 4). Some practical considerations in relation to its use must be considered:

1. A record of the specifications regarding the animal (species, breed, food, age, sex) conditions of transport and slaughter especially the conditions until reaching the rigor with record of pH (*see* **Note 7**).
2. Time of sampling and location of measurement should be previously defined (*see* **Note 8**).
3. If the samples were stored, the conditions of storage (temperature, moisture, light, and overwrap) should be specified.
4. Fresh muscle samples need at least 15 min of blooming time to the pigments at surface to oxygenate.
5. Three repetitions of each measurement are recommended (*see* **Note 9**).
6. A calibration based on the black standard as L * = 0 and white as L * = 100 provided with each chromometer must be performed.

Fig. 4 Color coordinates L^*, a^*, and b^* assessment by a Minolta chromameter

Finally, whenever color measurements are made on samples of meat or fat, all information on the conditions under which they were carried out, as described here, from 1 to 6 must be provided, including the make and model of the equipment used.

3.3 Color Measurement (Heme Pigments Content)

The chemical analysis consists of measuring the amount of pigments present in the muscle. The Hornsey method [10] is a simple and rapid indirect method that allows the determination of the myoglobin hierarchy and hemoglobin. As the residual hemoglobin content is very low, the estimation of the myoglobin obtained in this way is very close to the real value. The procedure consists in (Fig. 5):

1. Take 5 g of meat sample of *m. Longissimus thoracis et lumborum* (*longissimus dorsi*) (*see* **Note 10**).
2. Mince the sample in meat grinder type Moulinex.
3. A duplicate of each sample will be performed with an extraction volume of 25 mL.
4. In a pyrex tube with screw cap, place 1 mL of distilled water and 5 g of ground beef, 20 mL of acetone. Shake with a glass rod until the meat is a uniform whitish color. Then add 0.5 mL of HCl (35%) (*see* **Note 11**).
5. The solution should stand for 24 h in a refrigerator in the dark.
6. The solution is filtered with double filter paper (Ø 150 mm and a maximum of 31 μm of pore).
7. After pigment extraction, measure optical density (OD) with a spectrophotometer at 512 or 640 nm (*see* **Note 12**).
8. The results are expressed in percent (*see* **Note 13**):
   - At 640 nm: OD × 17.75 = mg myoglobin/g fresh muscle.
   - At 512 nm: OD × 8.82 = mg myoglobin/g fresh muscle.

The pigment percentages of the meat, deoxymyoglobin (Mb), oxymyoglobin (MbO_2), and metmyoglobin (MMb) could be

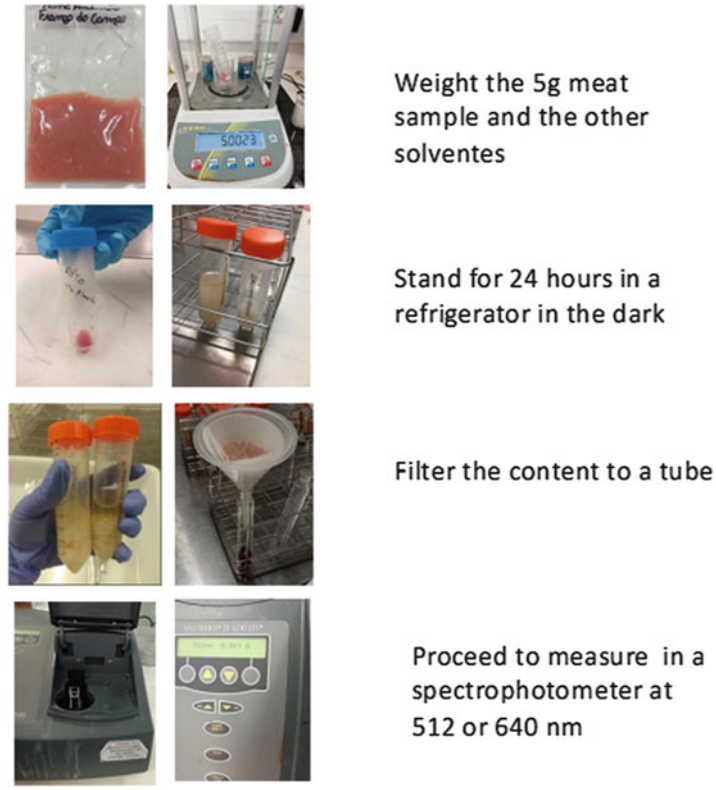

Fig. 5 Determination of meat pigment content [10]

obtained by means of the reflectance on the surface of the meat by the method developed by Hunt et al. [11] (*see* **Note 14**).

1. The reflectance spectrum obtained for a sample of meat (3 × 3 × 2 cm) without visible fat or connective tissue after cutting is 100% of Mb.
2. After the time of blooming, the meat sample should remain in contact with the air in a tray cover with a film permeable to the oxygen. The reflectance spectrum is 100% MbO_2.
3. Finally, the meat piece becomes 100% (MMb) when the meat is introduced in a 0.5% ferricyanide and potassium cyanide solution.
4. To quantify the amounts of myoglobin redox forms we must have the reflectance values of each one of the pigments (Mb, MMb, MbO_2). The process involves the use of surface reflectance to calculate the ratio between K (absorbance coefficient) and S (scattering coefficient) at isobestic wavelengths of each myoglobin redox form, 474, 525, 572, and 610 nm.
5. Once the myoglobin is converted to 100%, the procedure is as follows:

- Deoxymyoglobin: Place the samples in a 10% sodium dithionite (Na_2S2O_4) for 1 min. Remove and clean with absorbent paper. Pack the samples in a vacuum and leave them for 2 h at room temperature. At the end, take the measurements immediately after opening the package.
- Metmyoglobin: Place the samples in a 1% ferricyanide and potassium cyanide solution [$K_4Fe(CN)_6$] for 1 min. Remove and clean with absorbent paper. Pack the samples in a vacuum and leave them for 12 h at 2 °C refrigeration. At the end, take the measurements immediately after opening the package.
- Oxymyoglobin: Place the samples in an atmosphere with a high proportion of oxygen with a temperature between 0 and 2 °C. Oxygenation can be carried out by means of a flow of 100% oxygen for 10 min. After performing the measurements, place the samples in an.

Having obtained the measurements of the K/S ratios, calculate the percentages of the pigments using the formulas [11] (*see* **Note 15**):

$$\%\text{Deoxymyoglobin} = \frac{\frac{K/S\,474}{K/S\,525}\text{ for }100\%\text{of MMb} - \frac{K/S\,474}{K/S\,525}\text{ for sample}}{\frac{K/S\,474}{K/S\,525}\text{ for }100\%\text{of MMb} - \frac{K/S\,474}{K/S\,525}\text{ for sample}} \times 100$$

$$\%\text{Metmyoglobin} = \frac{\frac{K/S\,572}{K/S\,525}\text{ for }100\%\text{of MMb} - \frac{K/S\,572}{K/S\,525}\text{ for sample}}{\frac{K/S\,572}{K/S\,525}\text{ for }100\%\text{of MMb} - \frac{K/S\,572}{K/S\,525}\text{ for sample}} \times 100$$

$$\%\text{Oxymyoglobin} = \frac{\frac{K/S\,610}{K/S\,525}\text{ for }100\%\text{of MMb} - \frac{K/S\,610}{K/S\,525}\text{ for sample}}{\frac{K/S\,610}{K/S\,525}\text{ for }100\%\text{of MMb} - \frac{K/S\,610}{K/S\,525}\text{ for }100\%\text{of MMb}} \times 100$$

4 Notes

1. Although the procedure is the same, the determination of the pH in the carcasses fundamentally depends on the animal species, the time that elapses after slaughter until installation of rigor mortis, the anatomical point, and muscle of the carcass in which we intend to measure.

2. There are different devices of different equipment producers. Before the pH measurement, the pH-meter should be calibrated using buffer solutions.
3. There are several devices from different brands. In meat science, the spectrophotometer commonly used are the Hunter-Lab series Miniscan and Ultra scan, some of them are portable; Konica Minolta series CM-3600A, CM-3700A, CM-36dg.
4. The pH-meter is provided by an automatic system to adjust the temperature to the temperature of the carcass or muscle to the temperature of the carcass and muscle.
5. A scalpel blade or a knife can be used to make a little incision into the muscle to enable the contact of the electrode with the muscle tissue. After each measurement, the electrode must be washed with distilled water. Over time, they may suffer deterioration, delay in response time or with instability in reading. For better conservation, the electrodes should be stored in a 3 M KCl solution.
6. As there are differences in the pH measured in different muscles, the definition of the anatomical location is of great importance, to provide a series of works with great repeatability and that provide comparisons between different studies and laboratories.
7. Due to the close relationship between color and pH, it is recommendable in carcasses to record the pH at the beginning of chilling and the ultimate pH.
8. At least 24 h post-mortem, the measurement must be made. The anatomical point on the carcass must be defined. Usually, color is assessed in a cross section of *m. Longissimus thoracis et lumborum* (Longissimus dorsi) and a minimum of 1–3 cm of thickness is recommended to prevent light passing though it.
9. Different colors in the same muscle can occur and muscle infiltrations of marbling and connective tissue can generate some color variability.
10. The sample must be homogeneous, without fascia and fat, blood vessels, nerves, and tendons. If the meat sample is frozen, the liquid exudated during defrosting should be recovered.
11. A meatless solution (white reading) must be prepared, which will be used for the calibration of the spectrophotometer between sample readings.
12. The points where the absorbances or reflectances are equal for the three forms of myoglobin pigment is an isobestic points (512 or 640 nm).

13. The factors 8.82 and 17.75 correspond to the calculation of the final concentration of pigments calculated by the formula [10]:

$$\text{Factor} = \frac{\text{volume of extract} \times 652 \times 1.000\text{g/kg}}{\varepsilon_{\lambda}^{\text{mM}} \times 10^3\,\text{g of sample}} \times \text{OD} \quad \text{and} \quad \varepsilon_{\lambda}^{\text{mM}}$$

$$= 4.8 \quad \text{at } 640\text{ nm} \quad \text{or} = 9.52 \quad \text{at } 512\text{ nm}.$$

14. The K/S coefficients used are:
    (a) K/S474/K/S525 to estimation of Mb.
    (b) K/S572/K/S525 to estimation of MMb.
    (c) K/S610/K/S525 to estimation of MbO_2.
15. Once the myoglobin is converted to 100% and the calculations of % Metmyoglobin and % Deoxymyoglobin were processed, the % Oxymyoglobin could easily be calculated as:

$$\%\text{Oxymyoglobin} = 100 - (\%\text{Deoxymyoglobin} + \%\text{Metmyoglobin}).$$

Acknowledgments

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support by national funds FCT/MCTES to CIMO (UIDB/00690/2020) and to Laboratory of Carcass and Meat Quality of Agriculture School of Polytechnic Institute of Bragança "Cantinho do Alfredo." The authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568).

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Chapter 3

Texture Analysis

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Abstract

One of the most important quality parameters of the meat and meat products are their texture, which may be influenced by breed, sex, production system, *post-mortem* factors and/or production process. Several years ago, meat industries tested their food products with a panel of expert tasters (sensory analysis). This method alone can have problems because evaluations of the tasters could vary depending on their mood, the ambient temperature, their traditions and culture, among others. In short, this method is good but very subjective. Currently, companies are using both expert tasters and texture analyzers because they are complementary, and instrumental measurement provides more objective values. In this way, it is possible to know if a product satisfies (to a greater or lesser degree) the preferences of most consumers.

This chapter provides a comprehensive guide to instrumental texture analysis in meat and meat products. Warner-Bratzler test (WB) and Texture Profile Analysis (TPA) methods will be detailed step by step carefully.

Key words Warner-Bratzler, TPA, Shear Force, Hardness, Adhesiveness, Springiness, Cohesiveness, Gumminess, Chewiness

1 Introduction

Texture is a very important characteristic of meat and meat products since they give a lot of information about the product that consumers are going to eat or that they are eating. This fact is important, because the texture perception begins in our mind when we see the food. First of all, texture is defined as "all the mechanical, geometrical and surface attributes of a product perceptible by means of mechanical, tactile and, where appropriate, visual and auditory receptors" [1]. Texture greatly influences the perception that the consumer has and feels when eating a product. Consumers can identify several information through it, such as the type of product, its freshness, whether the product is raw or cooked,

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_3,

overcooked or undercooked, and also, it influences taste perception. Therefore, it is a key parameter that should take into account when formulating or improving a meat product [2].

There are a lot of factors (*ante-* and *post-mortem*) that can affect the texture of meat and meat products, such as animal handling [3], the type of muscle, the type of product (fresh and aged meat [4, 5], dry-cured meat products [6] and meat paste as pâté [7]), and how people usually eat it (according to their culture and tradition). In addition, many strategies to make meat products healthier could affect the texture of the products, since reducing the amount of salt, fat, or sugar, it will not only affect the taste but also the texture that consumer expects to find in this product. Therefore, it is important to note that we must analyze the sample in the same way that this product should be consumed, so that the results are a reflection of what the consumer will experience.

Among the textural characteristics, the most commonly used are hardness, cohesiveness, and juiciness. Three types of methods are applied to evaluate texture: sensory, instrumental (known as objective, physical, or mechanical), and indirect methods (collagen content, dry matter, among others) [8]. Instrumental methods are generally based on mechanical tests, which evaluate the resistance of the meat product to forces greater than gravity acting on it. Warner-Bratzler test (WB) and texture profile analysis (TPA) are the most common ways to evaluate meat tenderness. These methods simulate the conditions that the meat or meat product is exposed to in the mouth [8]. Texture analyzers are used for this purpose, usually equipped with interchangeable load cells, WB blade, and cylindrical probes. WB test consists of cutting the sample with WB blade to simulate chewing with the incisor teeth, and maximum shear force, shear firmness, and total necessary work are obtained after selecting parameters such as test speed, probe height, or contact force. The first parameter shown by the peak higher of the force-time curve, represents the maximum resistance of the sample to the cut. Shear firmness is represented by the slope from the beginning of the cut up to the highest point of the force-time curve and total work by the area under the curve [9]. TPA consists of two-cycle compression and simulates chewing with the molars. Hardness, cohesiveness, springiness, gumminess, and chewiness were obtained. Hardness represents the maximal force of the first compression of the product. Cohesiveness is represented by the ratio of work done between the second and the first deformation, whereas springiness is measured at the down stroke of the second compression. Finally, gumminess and chewiness are calculated as Hardness × Cohesiveness and Gumminess × Springiness, respectively [10].

The aim of this chapter is to be a guide for instrumental texture analysis of meat and meat products, from giving the parameters to set up the texture analyzer to the methodology to perform both

test, WB and TPA. So, this chapter will help to standardize the methodologies used, and in this way, it will facilitate the comparison of results.

2 Materials

All tests will be performed with a TA-XT Plus (Stable Micro Systems, Godalming, UK) texture analyzer (*see* **Note 1**) and its corresponding accessories (*see* **Note 2**). In addition, texture analyzer is connected to a computer equipped with a texture analysis software package "Exponent" (*see* **Note 3**).

2.1 Sample Preparation for Analysis

1. Vernier caliper (0–150 mm).
2. Cutting board.
3. Knife.

2.2 Warner-Bratzler Test

The Warner-Bratzler set (Fig. 1) consists of:

1. Warner-Bratzler Blade Set with "V" slot blade for USDA Standard (HDP/WBV).
2. Slotted blade insert.
3. Blade holder.

2.3 Texture Profile Analysis (Compression Test)

The texture profile analysis set (Fig. 2) consists of:

1. Cylinder probe of 50 mm of diameter (P/50).
2. Flat platform.
3. Probe insert.

2.4 Texture Profile Analysis (Penetration Test)

The texture profile analysis set (Fig. 3) consists of:

1. Cylinder probe of 6 mm of diameter (P/6).
2. Flat platform.
3. Probe insert.

3 Methods

All procedures will be carried out at room temperature unless otherwise specified.

3.1 Sample Preparation for Analysis

First of all, we need to prepare the sample. This step is very important because the results depend on it. How to prepare samples for one test or another is different and depends on the type of sample. As a general rule, the sample is analyzed in the same way as it is

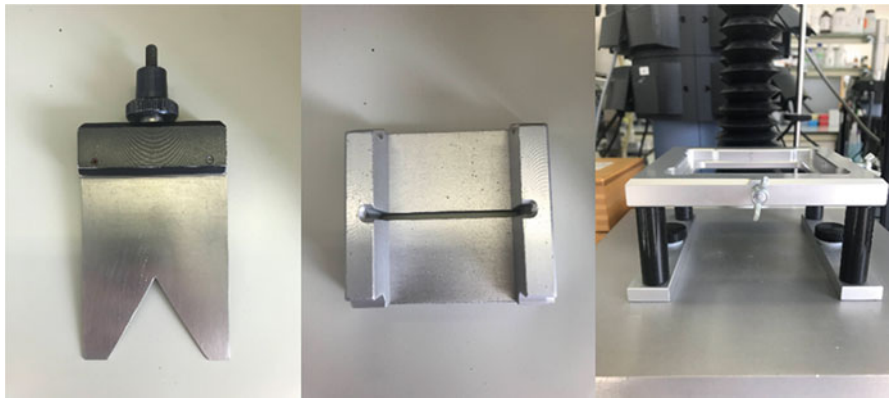

Fig. 1 Warner-Bratzler set

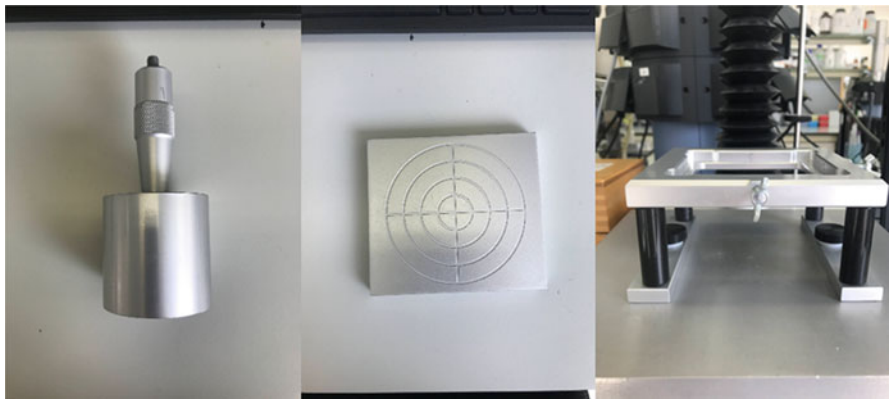

Fig. 2 TPA set (compression test)

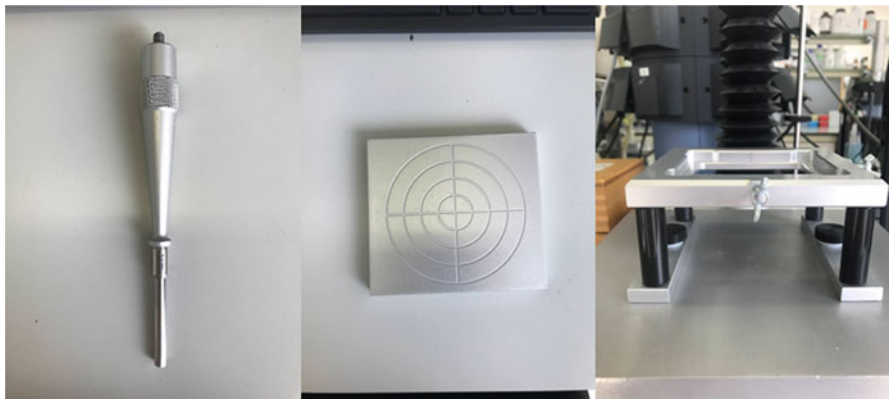

Fig. 3 TPA set (penetration test)

usually eaten, so raw meat should be cooked before. Therefore, in some samples the water holding capacity is also determined at the same time.

3.1.1 Water Holding Capacity (WHC)

The water holding capacity (WHC) of meat is determined by cooking losses according to the method proposed by Honikel (1997) [11].

1. Use steaks approximately 2.5 cm thick.
2. Once weighed, vacuum packed (80%) each fillet in a plastic bag and cooked in a water bath at 80 °C until it reaches an internal temperature of 70 °C (controlled with thermocouples) (*see* **Note 4**).
3. Then, the bags are removed from the bath and placed on a tray until they reach room temperature (approximately 30 min).
4. Finally, cooked steaks are removed from the bags and carefully dried to remove any remaining liquid on the surface. Cooking losses are expressed as the percentage of weight lost with respect to the initial one.
5. Expression of results

   Calculate the cooking loss as a percentage, using the following equation:

$$\%\text{Cooking loss} = \left[\frac{(W_0 - W_1)}{W_0}\right] \times 100$$

   where:

   - W_0 is the steak weigh before cooking (g).
   - W_1 is the steak weigh after cooking (g).

3.1.2 Sample Preparation for WB Test

Once the steak is cooked, we must cut it for carry out the WB test.

1. Firstly, remove the edges of the steak (*see* **Note 5**).
2. Next, we cut six pieces of the steak (*see* **Note 6**). Using vernier caliper, we must cut pieces with a size of 1 cm × 1 cm × 2.5 cm (high × width × length).
3. Finally, take the samples to the texture analyzer.

3.1.3 Sample Preparation for TPA (Compression Test)

In this test we must divide the samples in fresh or dry-cured sausages, and dry-cured meat products such as ham (*see* **Note 7**).

1. Fresh and dry-cured sausages.
   (a) Firstly, fresh sausages must be cooked before in the same way that fresh meat and next, remove the skin from the sausage where it was stuffed.
   (b) Then, we cut four slices of 2 cm wide using a vernier caliper in both fresh and dry-cured sausages. The skin must be also removed in dry-cured sausages.
   (c) Finally, take the samples to the texture analyzer.

2. Other dry-cured meat products

In the case of dry-cured meat products such as dry-cured ham, we must cut a representative slice from the muscle that interests us (usually in dry-cured ham *semimembranosus* or *biceps femoris* muscle).

(a) Firstly, remove the edges of the slice.

(b) Next, we cut six pieces of the steak. Using vernier caliper, we must cut pieces with a size of 1 cm × 1 cm × 1 cm (high × width × length).

(c) Finally, take the samples to the texture analyzer.

3.1.4 Sample Preparation for TPA (Penetration Test)

Usually, we carry out this analysis in spreadable paste of meat like to pâté. These products are ready for analysis, you just need open the can or glass bottle and do the analysis.

3.2 Warner-Bratzler Test

Usually, this test is used in fresh and aged meat. Warner-Bratzler test is carried out according to Honikel (1997) [11] with slight modifications. Figure 4 shows in a simplified and schematic way the steps of the WB test. Once the samples are ready for analysis:

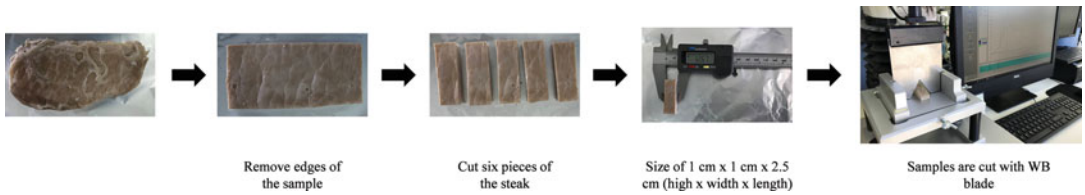

Fig. 4 Schematic and simplified representation of the steps of the WB test

Table 1
Analysis parameters to perform WB test

WB parameters	
Load cell (Kg)	30
Calibration weight (kg)	5
Return distance (mm)	50
Return speed (mm/s)	10
Contact force (g)	2
Pretest speed (mm/s)	3.33
Test speed (mm/s)	3.33
Posttest speed (mm/s)	15
Distance (mm)	30

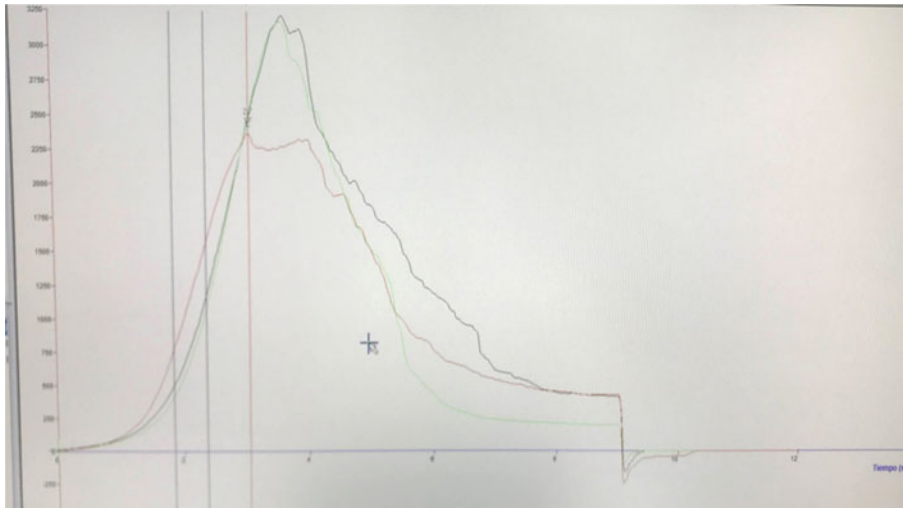

Fig. 5 Typical force deformation curve of the WB test

1. We must set up the parameters of analysis in the "Exponent" software (*see* **Note 8**). You can see the WB parameters for fresh and aged meat in Table 1.
2. Then, samples which have been prepared for the WB test can be cut with the WB blade. In order to achieve a representative result, six pieces must be tested.
3. In Fig. 5, you can see a typical force deformation curve of the WB test.
4. We analyze the curve with "Exponent" software for obtaining the following parameters: Firmness (N/s), Work (N*mm), and Shear Force (N/cm^2).
5. Finally, the result is calculated as the average of the six pieces.

3.3 Texture Profile Analysis (Compression Test)

As we explained above, this test could be carried out on fresh and dry-cured sausages, and other dry-cured meat products. Texture profile analysis is performed according to Honikel (1997) [11] and Bourne et al. (1978) [12] with slight modifications. Figure 6 shows in a simplified and schematic way the steps of the TPA (Compression test). Once the samples are ready for analysis:

1. We must set up the parameters of analysis in the "Exponent" software (*see* **Note 8**). You can see the TPA parameters for fresh and dry-cured sausages in Table 2. In Table 3 you can see the parameters for other dry-cured meat products (*see* **Note 9**).
2. Then, samples which have been prepared for the TPA can be compressed with the cylinder probe of 50 mm. In order to achieve a representative result, six pieces must be tested.
3. In Fig. 7, you can see a typical force deformation curve of the TPA test.

4. We analyze the curve with "Exponent" software to obtain the following parameters: Hardness (N), Adhesiveness (N*s), Springiness (mm), Cohesiveness, Gumminess (N), and Chewiness (N*mm).
5. Finally, the result is calculated as the average of the six pieces.

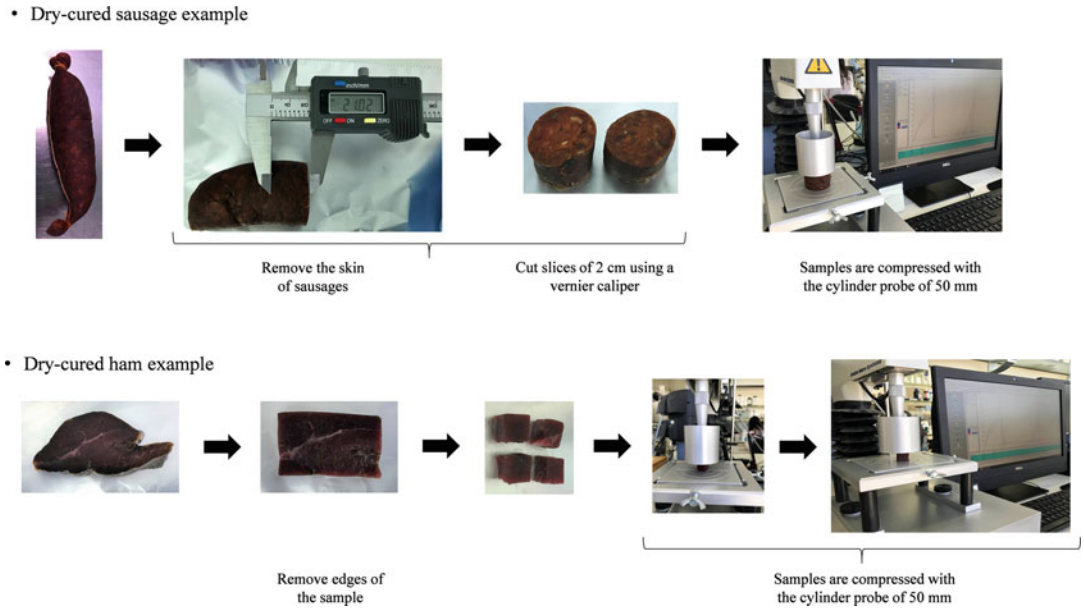

Fig. 6 Schematic and simplified representation of the steps of the TPA (compression test)

Table 2
Analysis parameters to perform TPA (Compression test) on fresh and dry-cured sausages

TPA parameters (Compression test)	
Load cell (Kg)	30–50
Calibration weight (kg)	5
Return distance (mm)	30
Return speed (mm/s)	20
Contact force (g)	2
Pretest speed (mm/s)	10
Test speed (mm/s)	1
Posttest speed (mm/s)	10
Deformation (%)	50

Table 3
Analysis parameters to perform TPA (Compression test) on other dry-cured meat products

TPA parameters (Compression test)	
Load cell (Kg)	30
Calibration weight (kg)	5
Return distance (mm)	20
Return speed (mm/s)	20
Contact force (g)	2
Pretest speed (mm/s)	3.33
Test speed (mm/s)	3.33
Posttest speed (mm/s)	3.33
Deformation (%)	60

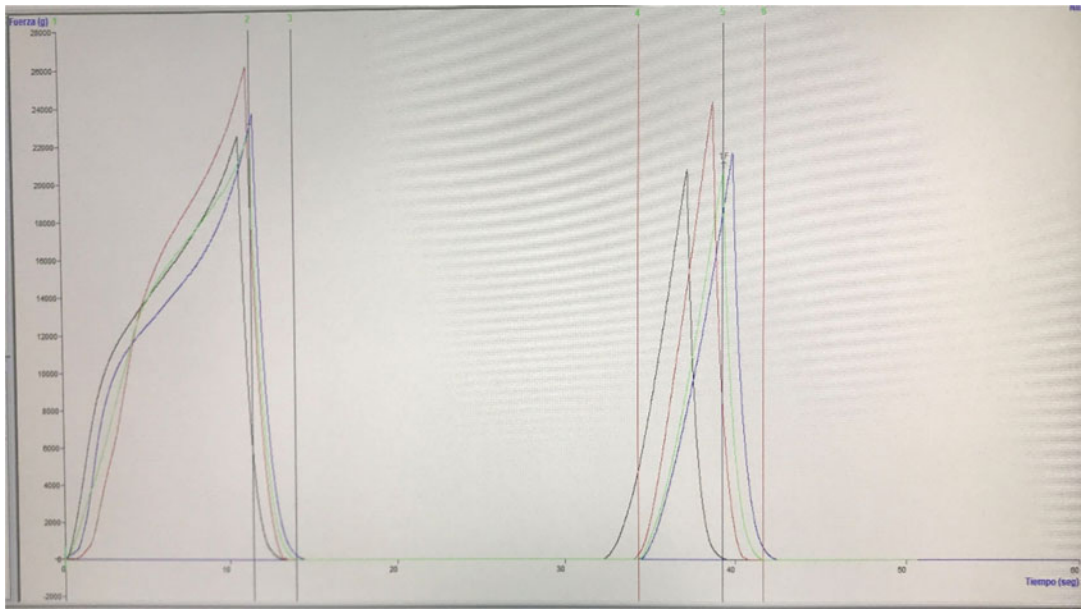

Fig. 7 Typical force deformation curve of the TPA (compression test)

3.4 Texture Profile Analysis (Penetration Test)

Usually, we carry out this analysis in products that are made from a meat paste, as in the case of pâtés. Texture profile analysis is performed according to Vargas-Ramella et al. (2020) [13]. We test this meat product directly in the commercial packaging. Figure 8 shows in a simplified and schematic way the steps of the TPA (Penetration test).

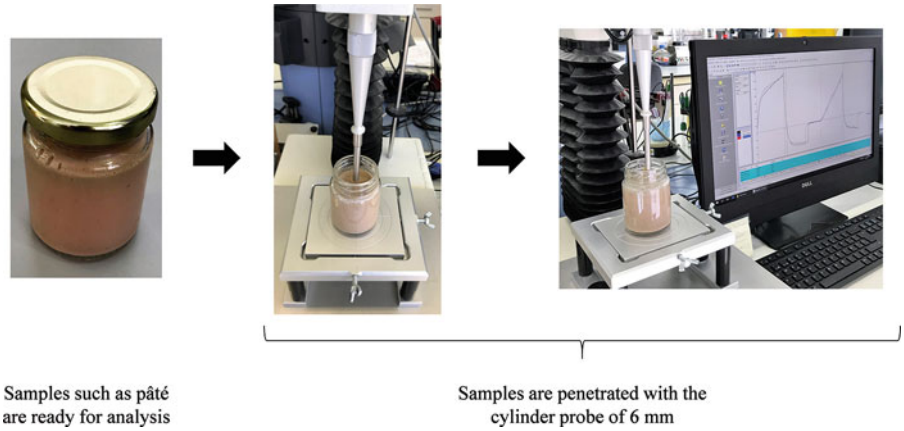

Fig. 8 Schematic and simplified representation of the steps of the TPA (penetration test)

Table 4
Analysis parameters to perform TPA (Penetration test)

TPA parameters (Penetration test)	
Load cell (Kg)	5
Calibration weight (kg)	2
Return distance (mm)	50
Return speed (mm/s)	20
Contact force (g)	2
Pretest speed (mm/s)	3.33
Test speed (mm/s)	0.80
Posttest speed (mm/s)	3.33
Distance (mm)	8

1. We must set up the parameters of analysis in the "Exponent" software (*see* **Note 8**). You can see the TPA parameters for fresh and dry-cured sausages in Table 4.
2. Then, once the container has been opened, the sample can be penetrated with the cylinder probe of 6 mm. In order to achieve a representative result, the test is performed six times in different areas.
3. In Fig. 9, you can see a typical force deformation curve from the TPA penetration test.
4. We analyze the curve with "Exponent" software to obtain the following parameters: Hardness (N), Adhesiveness (N*s), Springiness (mm), Cohesiveness and Gumminess (N).
5. Finally, the result is calculated as the average of the six pieces.

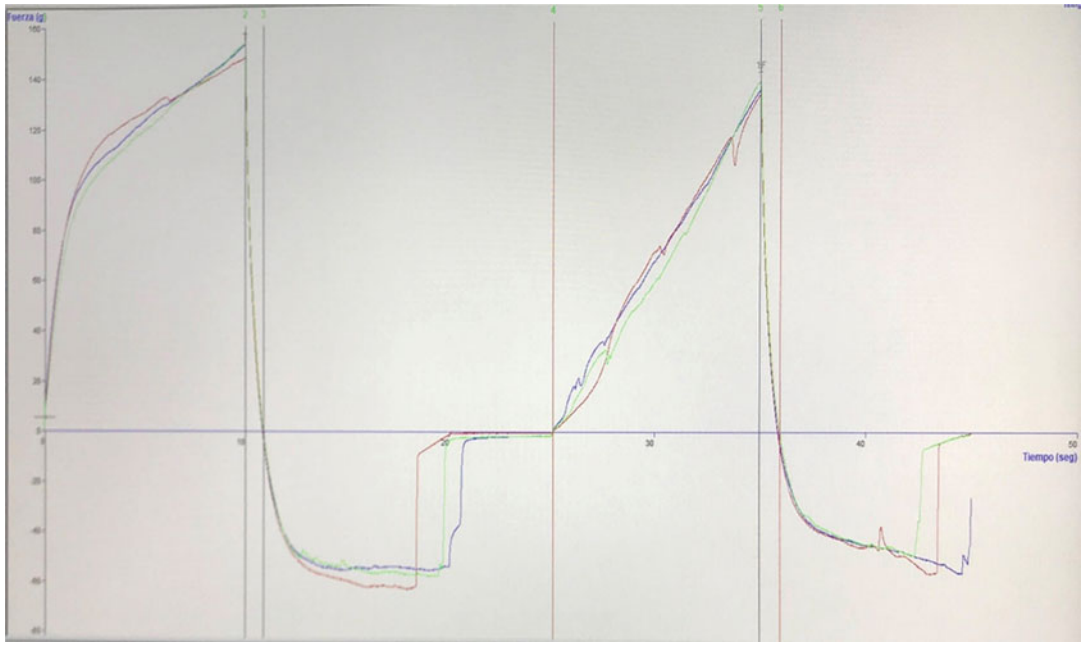

Fig. 9 Typical force deformation curve of the TPA (penetration test)

4 Notes

1. TA-XT Plus has 500 N of force capacity, 0.1 g of force resolution, a speed range between 0.01 and 40 mm/s, a maximum aperture of 370 mm, a distance resolution of 0.001 mm, and a data acquisition rate of 2000 pps.
2. The accessories are:
   (a) On the one hand, two interchangeable load cells of 30 kg and 50 kg. One or the other will be used depending on the expected hardness of the sample. Usually, a 30 kg load cell is used with fresh meat and a 50 kg load cell with drier samples such as dry-cured meat products.
   (b) On the other hand, three different probes. A Warner-Bratzler probe and two cylindrical TPA probes of 50 mm and 6 mm of diameter.
3. "Exponent" software allows us to take control of the texturometer, testing programming, automated curve analysis, statistical analysis, presentation of results, etc.
4. The temperature probe must be placed carefully on the fillet so as not to damage the sample to be used for texture analysis and to correctly control its internal temperature.
5. The edges of the steak are usually very irregular, have some fat and are dried. These facts could affect the results.

6. The pieces of steak should be cut parallel to the fibers.
7. These kinds of sample (dry-cured meat products) should not be cooked because, as a general rule, they are eaten raw. But in the case of fresh sausages, you have to cook them.
8. Texture analyzer should be turned on at least 30 min before analysis. In addition, the equipment must be calibrated. The calibration weight should be 5 kg for 30 kg and 50 kg load cells.
9. Note that the parameters of sausages are different from other dry-cured meat products.

Acknowledgments

This work was supported by Centro Tecnolóxico de la Carne de Galicia. Thanks to GAIN (Axencia Galega de Innovación) for supporting this book chapter (grant number IN607A2019/01). Authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568). Noemí Echegaray acknowledges to Consellería de Cultura, Educación e Ordenación Universitaria (Xunta de Galicia) for granting with a predoctoral scholarship (Grant number IN606A-2018/002).

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Chapter 4

Fatty Acids

Rubén Domínguez, Laura Purriños, Mirian Pateiro, Paulo C. B. Campagnol, Jorge Felipe Reyes, Paulo E. S. Munekata, and José Manuel Lorenzo

Abstract

The development of a common technique for the determination of fatty acids is very important due to the role they play in the nutritional, sensorial, and technological quality of meat and meat products. However, and despite being one of the most studied determinations, there are several techniques for fat extraction, for the methylation, and for identification/quantification of fatty acids. With this in mind, it should be mentioned that each of the procedures has certain limitations.

Therefore, and taking into account the above, this chapter gives a clear, comprehensive and complete vision of a procedure for the fatty acid analysis of meat and meat products, including the fat extraction, fatty acids methylation, and subsequent analysis by gas chromatography with FID detection. All stages are described in detail so that the conditions proposed by us can be reproduced by other researchers. Finally, it should be mentioned that the proposed procedure includes a fast methylation phase at room temperature, combining acidic and basic methylation to ensure complete methylation of fatty acids in a simple way and minimizing the formation of artifacts.

Key words Lipid fraction, Omega-3, Omega-6, Saturated fatty acids, Monounsaturated fatty acids, Polyunsaturated fatty acids, Gas chromatography, Basic-acid methylation, Transesterification

1 Introduction

The fat content as well as the fatty acid content of meat and meat products is of vital importance due to its implications on human health and on the sensory quality of meat products [1]. Due to this, during the last decades, multiple strategies have been proposed by both researchers and the meat industry with the aim of producing healthier products [2]. These strategies range from modifying the animals' diet, which affects the content and profile of fatty acids in fresh meat, to the reformulation of multiple meat products, substituting animal fat (with high values of saturated fatty acids) by vegetable or marine origin oils [3]. However, there are numerous technological limitations, because the consistency of animal fat

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_4,

(saturated) is solid, while oils have a liquid consistency. To overcome this drawback, the immobilization of these oils in gels has been proposed, both using water-based (hydrogels) and lipid-based gels (oleogels) [2]. Furthermore, it must be taken into account that unsaturated fatty acids are also very susceptible to lipid oxidation processes [4], so it is important to control all these aspects when designing strategies for the reformulation of meat and meat products.

With this in mind, it seems clear that a correct determination of the content of fatty acids in meat and meat products is very important. The lipid fraction of fresh meat is mostly made up of neutral lipids or triglycerides (located in adipocytes) and polar lipids or phospholipids (which are constituents of cell membranes). However, in many meat products, mainly those that have a drying-ripening process in their manufacture, there is a significant release of fatty acids, so the fraction of free fatty acids in these products can also be important [5]. In these fractions, about 20 fatty acids, with a chain length ranging between C12 and C22, account for more than 85% of fatty acids, but the number of minor fatty acids is much larger [6]. Currently, there is a great diversity of methodologies for the extraction of total lipids in terms of the combination and proportions of solvents. Some of them use a single solvent (such official method proposed by AOAC, which use petroleum ether), but a combination of various solvents ensure adequate polarity to extract both polar and nonpolar lipids [7]. The two most used methods for fat extraction in meat and meat products are Folch et al. [8] and Bligh and Dyer [9]. In both cases, a mixture of methanol and chloroform are used, but differ in the proportion of chloroform:methanol and solvent:sample ratios [7]. Similarly to fat extraction, several procedures were proposed for the fatty acid esterification, a recommended step for gas chromatography analysis, as ester derivatives are more volatile than their corresponding underivatized fatty acids. In this sense, both, acid and base methylation (or transesterification) were used in different meat and meat products. It is well known that the acid methylation is suitable for a complete transesterification of free fatty acids and O-acyl lipids, while base methylation is preferable for the formation of FAME from neutral and polar lipids [6]. Moreover, the use of some acids could form allylic methoxy ether artifacts. Thus, the use of sodium methoxide to preserve the steric configuration of double bounds and to reduce artifact formation was proposed, but it did not methylate free fatty acids. In addition, several methylation procedures use high temperatures, which promote oxidative reactions [4] and could change the fatty acids composition. Therefore, the methodology proposed in the present chapter used combined methylation, acid and base, at room temperature, in order to ensure the complete transesterification of fatty acids from all fractions and overcome the problems that other procedures have. Finally, various

chromatographic techniques, including gas (GC) and liquid chromatography (HPLC) were used in the fatty acids separation and quantification. Among them, GC is the most used by far. In this regard, capillary columns with polar stationary phases had higher resolution capacity than apolar stationary phase columns [6].

Within the several methods used for fatty acids quantification, the most common methods imply three main steps: (a) a solvent extraction, followed by (b) methylation and (c) gas chromatography (GC) identification. With all the aforementioned, the present book chapter aims to fully and comprehensively describe a procedure for determining fatty acids in meat and meat products. In this case, these three stages are specified, being the extraction of the fat with a mixture of solvents, followed by combined methylation (base and acid transesterification) at room temperature and finally the chromatographic analysis by gas chromatography.

2 Materials

Prepare all solutions using distilled water and/or analytical grade reagents. Use GC-grade hexane. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials (*see* **Note 1**).

2.1 Fat Extraction Step

1. NaCl (1%): Dissolve 10 g of sodium chloride in 1 L of distilled water.
2. Chloroform with 0.1% BHT: Dissolve 1 g of Butylated hydroxytoluene (BHT) in 1 L of chloroform.

2.2 Methylation Step

1. Sodium methoxide (0.5 N): Dissolve 11.5 g of extra pure sodium metal (≥99%) in 1 L of methanol (*see* **Note 2**).
2. Sulfuric acid-methanol solution (10%): Mix 100 mL of sulfuric acid (≥98%) with 900 mL of methanol (*see* **Note 3**).
3. Sodium bicarbonate (saturated solution): Dissolve 100 g of sodium bicarbonate (85%) in 1 L of distilled water.
4. Nonadecanoic acid (C19:0; 5 mg/mL) (*Internal Standard for sample methylation step*): Dissolve 0.25 g of nonadecanoic acid (≥98%) in 50 mL of hexane (*see* **Note 4**).

2.3 Calibration and FAME Identification

All fatty acid methyl ester solutions must contain 10 mg/mL. In this case, the authors indicate the references to the specific standards used for fatty acid determination; however, others with the same characteristics may be used. All standards should be analytical grade and preferably GC grade analysis. All fatty acid methyl esters standards should be stored at −20 °C.

Table 1
Volume (μL) of the fatty acid methyl ester standards and hexane for the elaboration of the different points of the calibration curve (final volume 500 μL)

Fatty acid standard	STD1	STD2	STD3	STD4	STD5
FAME mix 37 comp. (10 mg/mL)	25	50	100	200	400
11 t-C18:1 (TVA; 10 mg/mL)	1.25	2.5	5	10	20
C18:1n-7 (CVA; 10 mg/mL)	1.25	2.5	5	10	20
C19:0 ME (**IS**; 10 mg/mL)	15	15	15	15	15
C22:5n-3 (DPA; 10 mg/mL)	1.25	2.5	5	10	20
9c, 11t-C18:2 (CLA; 10 mg/mL)	1.25	2.5	5	10	20
Hexane (GC grade)	455	425	365	245	5

1. Fatty acid mixture: 37 component FAME Mix (Supelco; ref.: CRM47885). Ampule of 1 mL with 10 mg/mL of total fatty acid methyl esters (in dichloromethane). The amount of each individual fatty acid ranged between 200 and 600 μg/mL.
2. Trans-11-vaccenic methyl ester (11t-C18:1; TVA; 10 mg/mL): (Supelco; ref.: CRM46905). Ampule of 1 mL with 10 mg/mL TVA in heptane.
3. Cis-11-vaccenic methyl ester (C18:1n-7; CVA; 10 mg/mL): (Supelco; ref.: CRM46904). Ampule of 1 mL with 10 mg/mL CVA in heptane.
4. Cis-9, trans-11-octadecadienoic methyl ester (9c,11t-C18:2; CLA; 10 mg/mL): (Matreya LLC; ref.: 1255). Vial with 25 mg CLA (≥98%). In order to obtain a 10 mg/mL solution, dissolve 25 mg in 2.5 mL of hexane.
5. Cis-7, 10, 13, 16, 19-docosapentaenoic methyl ester (C22:5n-3; DPA; 10 mg/mL): (Supelco; ref.: 17269). Ampule of 1 mL with 10 mg/mL DPA in heptane.
6. Nonadecanoic acid methyl ester (10 mg/mL) (*Internal Standard for calibration curve*): Dissolve 0.5 g of nonadecanoic acid methyl ester (C19:0 ME; ≥98%) in 50 mL of hexane.
7. Fatty acid methyl esters standards for calibration curve: In order to obtain a five-point calibration curve, different volumes (μL) of the previously described standards (37 FAME Mix, TVA, CVA, CLA, DPA, and C19:0 ME) were mixed with hexane (Table 1). The final concentrations (ppm) and elution order of each fatty acid methyl ester are those listed in Table 2 (*see* **Note 5**).

2.4 Gas Chromatograph

Separation and quantification of FAMEs are carried out using a gas chromatograph Agilent mod. 7890B (Agilent Technologies)

Table 2
Fatty acid methyl esters (in order of elution) and final concentration (ppm) of each point of the calibration curve

N° #	Fatty acid	STD1	STD2	STD3	STD4	STD5
1	C4:0	20	40	80	160	320
2	C6:0	20	40	80	160	320
3	C8:0	20	40	80	160	320
4	C10:0	20	40	80	160	320
5	C11:0	10	20	40	80	160
6	C12:0	20	40	80	160	320
7	C13:0	10	20	40	80	160
8	C14:0	20	40	80	160	320
9	C14:1n-5	10	20	40	80	160
10	C15:0	10	20	40	80	160
11	C15:1n-5	10	20	40	80	160
12	C16:0	30	60	120	240	480
13	C16:1n-7	10	20	40	80	160
14	C17:0	10	20	40	80	160
15	C17:1n-7	10	20	40	80	160
16	C18:0	20	40	80	160	320
17	9t-C18:1	10	20	40	80	160
18	11t-C18:1 (TVA)	25	50	100	200	400
19	C18:1n-9	20	40	80	160	320
20	C18:1n-7 (CVA)	25	50	100	200	400
21	9t,11t-C18:2	10	20	40	80	160
22	C18:2n-6	10	20	40	80	160
23	*C19:0 (IS)*	*300*	*300*	*300*	*300*	*300*
24	C18:3n-6	10	20	40	80	160
25	C18:3n-3	10	20	40	80	160
26	9c, 11t-C18:2(CLA)	25	50	100	200	400
27	C20:0	20	40	80	160	320
28	C20:1n-9	10	20	40	80	160
29	C20:2n-6	10	20	40	80	160
30	C21:0	10	20	40	80	160
31	C20:3n-6	10	20	40	80	160
32	C20:4n-6	10	20	40	80	160
33	C20:3n-3	10	20	40	80	160

(continued)

Table 2
(continued)

N° #	Fatty acid	STD1	STD2	STD3	STD4	STD5
34	C22:0	20	40	80	160	320
35	C20:5n-3 (EPA)	10	20	40	80	160
36	C22:1n-9	10	20	40	80	160
37	C22:2n-6	10	20	40	80	160
38	C23:0	10	20	40	80	160
39	C24:0	20	40	80	160	320
40	C22:5n-3 (DPA)	25	50	100	200	400
41	C24:1n-9	10	20	40	80	160
42	C22:6n-3 (DHA)	10	20	40	80	160

equipped with a flame ionization detector (FID) and PAL RTC-120 autosampler with liquid injection tool (Pal System). For the separation of FAMEs, a DB-23 fused silica capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies) is used.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Fat Extraction

For fatty acid analysis, about 20 mg of fat should be extracted from 10 g of sample following the procedure described by Bligh and Dyer [9], with modifications.

1. Weigh 10 g of the sample in a centrifuge tube of 80 mL and add *Vol-1* of NaCl (1%) to achieve moisture of 80% (Table 3) (*see* **Note 6**).
2. Add 2 mL of chloroform with 0.1% of BHT, 20 mL of methanol, and 8 mL of chloroform. Homogenize in UltraTurrax during 30 s (at 12,000 rpm). Add 10 mL of chloroform and homogenize (10s). Add 10 mL of NaCl (1%) and homogenize (10s). Centrifuge samples at 3100 × *g* for 10 min. Remove the upper phase (aqueous phase: water and methanol) using a plastic Pasteur pipette. Remove the "meat phase" using a spatula and/or laboratory tweezers (*see* **Note 7**).
3. Transfer *Vol-2* (Table 3; *see* **Note 6**) of lower phase (organic phase; chloroform and fat) towards a test tube (previously weighted). Evaporate to dryness under N_2 on a TurboVap

Table 3
Volume of NaCl (1%) that must be added to the sample depending on sample moisture and volume of chloroform that must be taken to evaporate depending on sample fat content

Sample moisture (%)	Vol-1 NaCl (1%) (mL)	Sample fat (%)	Vol-2 chloroform (mL)
≥80	0.00	≤2	13.0
70	1.00	5	5.40
60	2.00	10	2.70
50	3.00	15	1.80
40	4.00	20	1.35
30	5.00	30	0.90
20	6.00	40	0.675
10	7.00	50	0.540

evaporator (*see* **Note 8**). After cooling the tube to room temperature, the test tube is weighed again and the amount of fat is calculated by difference (*see* **Note 9**).

3.2 Fatty Acids Methylation

The fatty acids were transesterified according to the procedure previously described by Barros et al. [10], with some modifications. Figure 1 shows in a simplified and schematic way the steps of the fat extraction and methylation phases.

1. For the fatty acids transesterification, 20 ± 5 mg of extracted fat is mixed with 120 μL of C19:0 (5 mg/mL; nonadecanoic acid; internal standard) and 880 μL of toluene. The mixture must be vortexed to dissolve the fat in the toluene. Then, add 2 mL of a sodium methoxide (0.5 N) solution, vortex during 10 s and allow standing for 15 min at room temperature.
2. Add 4 mL of a sulfuric acid-methanol solution (10% of H_2SO_4 in methanol) and vortex for a few seconds. Add 2 mL of saturated sodium bicarbonate solution and vortex again (*see* **Note 10**). For the extraction of fatty acid methyl esters, add 1 mL of hexane, vortex for 10 s and allow standing for phase separation. Transfer the organic phase (upper phase) to an appropriate vial.

3.3 Fatty Acid Identification and Quantification (GC)

Separation and quantification of FAMEs are carried out using a gas chromatograph, following the next conditions (Table 4):

1. One microliter of the sample (or the standard) is injected in split mode (50:1) (*see* **Note 11**). The injector is maintained at 250 °C and 64.2 mL/min of total flow. A DB-23 fused silica capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies) is used for FAMEs separation. This

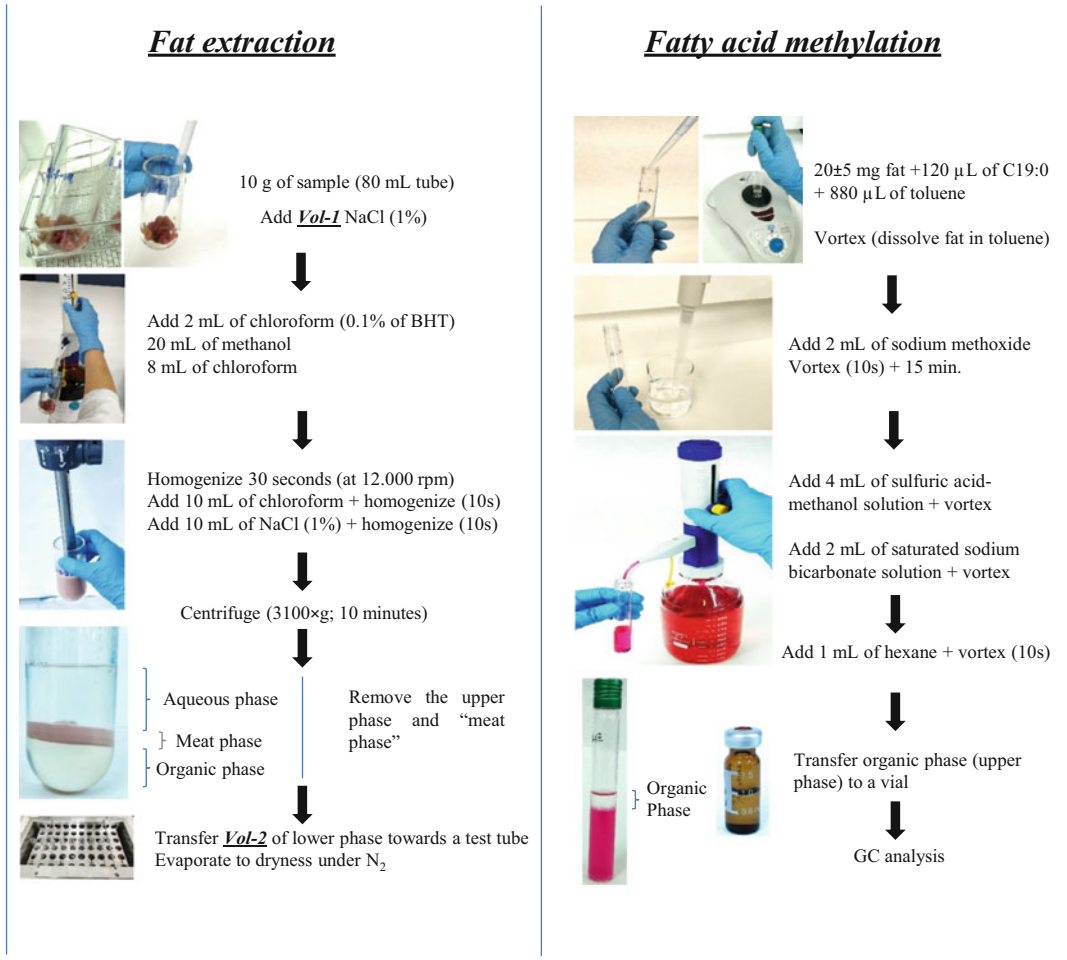

Fig. 1 Schematic and simplified representation of the steps of the fat extraction and methylation phases

column has excellent resolution and a wide operating temperature range, and is well suited for procedures using cis- and trans- FAMEs isomers.

2. The oven conditions are as follows: initial oven temperature of 50 °C (hold for 1 min), first ramp at 25 °C/min to 175 °C, second ramp at 3 °C/min to 230 °C (hold for 3 min), and third ramp at 2 °C/min to a final temperature of 235 °C (hold for 3 min). Helium is used as a carrier gas at a constant flow rate of 1.2 mL/min, with the column head pressure set at 22.9 psi.
3. The FID detector is maintained at 280 °C, while the operational flows are set as 40 mL/min of H_2, 450 mL/min of air, and 30 mL/min of makeup flow (He). The detector signal is recorded at 10 Hz data rate. The total time for chromatographic analysis is 32.83 min.

Table 4
Summary of the chromatographic conditions used for the FAMEs analysis

Inlet	*Temperature (°C)*	*Pressure (psi)*	*Total flow (mL/min)*	*Split ratio*
Split mode	250	22.916	64.2	50:1
Oven	*Rate (°C/min)*	*Temperature (°C)*	*Hold time (min)*	*Total time (min)*
Initial	–	50	1	1
Ramp 1	25	175	0	6
Ramp 2	3	230	3	24.33
Ramp 3	2	235	3	32.83
Column	*Characteristics*	*Temperature (°C)*	*Pressure (psi)*	*Flow (mL/min)*
DB-23	60 m × 250 μm × 0.25 μm	50 (initial)	22.916	1.2
Detector	*Temperature (°C)*	*H_2 flow (mL/min)*	*Air flow (mL/min)*	*He flow (mL/min)*
FID	280	40	450	30

Data acquisition, equipment control, and data analysis are carried out using the GC software (in our case MassHunter GC/MS; Agilent Technologies). Individual FAMEs are identified by comparing their retention times with those of authenticated standards (37 FAME Mix, TVA, CVA, CLA, DPA, and C19:0 ME) (*see* **Note 12**). Figure 2 shows an example of FAME chromatograms of standard (a) and sample (b). The results could be expressed as mg/100 g of meat, g/100 g of fat, and g/100 g of total fatty acids.

4 Notes

1. Due to the toxic and/or corrosive nature of the solvents and reagents used in the extraction and methylation of fatty acids, it is necessary for the operator to take the necessary protective measures (gloves, glasses, etc.) as well as to carry out all the operations in laboratory fume hoods.
2. Exothermic reaction. Make cubes of sodium (metal) of approximately 2 cm and add them to 500 mL of methanol in a 1 L bottle (constant stirring). Finally, wait for the mixture to cool down and add the remaining 500 mL.
3. Extremely exothermic reaction. This solution must be carried out in an ice bath, with constant stirring (magnetic). In a 2 L bottle, add 900 mL of methanol and slowly add concentrated sulfuric acid, letting it run off the wall of the bottle. If bubbles appear (dangerous point of the reaction), wait a few minutes for the temperature to drop and then continue. In the end, add a spatula tip of methyl red so that the solution has a reddish

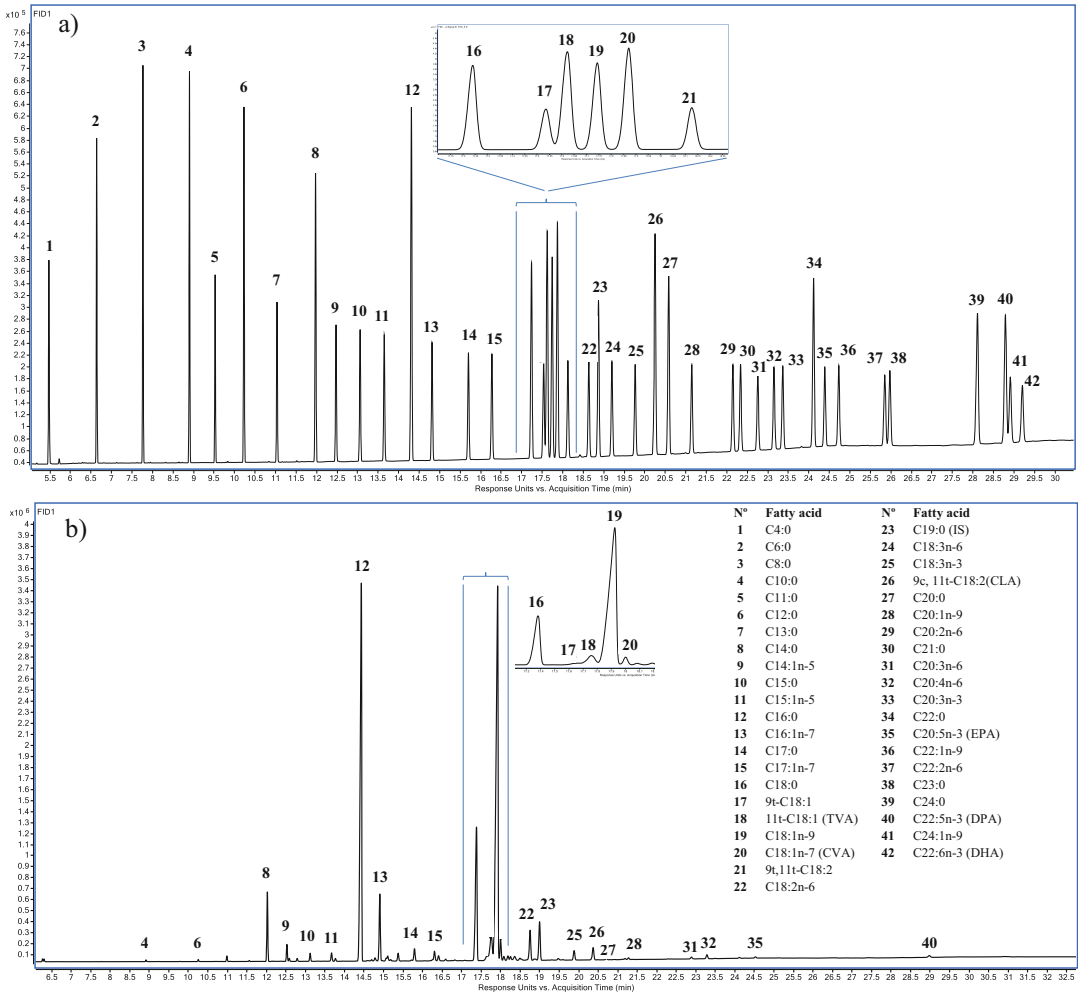

Fig. 2 Fatty acid chromatograms of a standard (**a**) and a fresh meat sample (**b**)

color, and favor the visualization of the two phases (aqueous = red/organic = colorless) after the methylation process.

4. The internal standard (C19:0) should be stored at −20 °C. At this temperature C19:0 precipitates. Therefore, the standard internal needs to be warmed prior to use.
5. Due to the extremely small quantities (few μL), the pipetting process must be scrupulously careful. The tip of the micropipettes should be moistened with the solvent (or standard) to ensure that the correct amount of each standard is taken.
6. The volume of NaCl (1%) [Vol-1] to be added to the samples, as well as the volume of chloroform (which contains the fat) [Vol-2] that must be evaporated to obtain a final content of approximately 20 mg of fat, depending on the initial moisture and fat content of the samples, respectively. A summary of the

volumes that must be added to samples with different moisture and fat contents is shown in Table 3.

7. In order to ensure the complete dehydration of the organic phase, after removing the meat phase, a spatula tip of anhydrous sodium sulfate can be added. After this step, the organic phase is filtered through a filter paper, eliminating the anhydrous sodium sulfate as well as the meat that may remain in the organic phase.
8. The evaporator should be turned on a few minutes before evaporating the solvent from the samples to allow the water bath to have the correct temperature. The conditions of Tubo-Vap used for evaporation are: 1.2 bar nitrogen pressure at 50 °C (water bath temperature) during minimum 30 min. The evaporation time can be extended if the solvent is not evaporated.
9. An analytical balance with 0.1 mg resolution or higher should be used to weigh the tubes. The amount of fat obtained is calculated by difference (tube with fat after evaporating—empty tube). In case the amount of fat obtained is greater than 25 mg, the excess can be removed with a spatula.
10. After vortexing the samples when the sulfuric acid-methanol solution was added, the pressure in the tube should be relieved (slowly opening the cap). The bicarbonate must be added slowly as it causes a reaction that can cause the sample and reagents to jump out of the tube. Once again, after vortexing the sample with the bicarbonate, the tube pressure should be relieved by slowly uncapping the tube (release pressure, CO_2 is generated).
11. Standards should be injected first, for identification and to create calibration curves for each fatty acid. Identification/calibration standards must be prepared each time a calibration is performed. During the analysis of the samples, a blank (hexane) should be injected every 15 samples, and a standard (FAME) should be injected in order to verify the retention times every 40 samples. Calibrations should be performed at least once a month, or when any chromatograph maintenance is performed that may affect the resolution of the equipment or the detector signal.
12. Standards prepared for calibrations can be stored frozen (−20 °C) and used to check retention times (injection every 40 samples). However, these standards cannot be used for calibration, only to verify retention times. Calibration curve standards must be prepared each time a new calibration is performed.

Acknowledgments

This work was supported by Centro Tecnolóxico de la Carne de Galicia. Thanks to GAIN (Axencia Galega de Innovación) for supporting this book chapter (grant number IN607A2019/01). Authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568).

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Chapter 5

Amino Acids (Free and Hydrolyzed)

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Abstract

Amino acids are an important compound since they form part of the proteins and are intermediates in the metabolism. Some amino acids are synthesized by the organism, however, in other cases, they should be ingested in the diet (essential amino acids). Because of the high content of amino acids in meat and meat products and their influence on the nutritional and sensorial quality, it is necessary the validation of new techniques to be more sensitive, faster, and versatile for the determination of both free and hydrolyzed amino acids. Despite that there are published several techniques for amino acids extraction, derivatization and for identification/quantification, it should be mentioned that some of them present certain limitations.

The present chapter gives a clear and complete vision of a procedure for the free and hydrolyzed amino acids analysis in meat and meat products. Free amino acid extraction includes the following steps: homogenization and extraction from the meat matrix, deproteinization, derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and subsequent analysis by liquid chromatography with fluorescence detection (HPLC-FL). Hydrolyzed amino acids are determinate in the following manner: first, the hydrolysis step during 24 h at 110 °C and then the derivatization step with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate prior to chromatographic analysis by HPLC-FL. All steps are described in detail so that the conditions proposed by us can be reproduced by other researchers.

Key words Food analysis, Free amino acids, Hydrolyzed amino acids, Derivatization, Liquid chromatography

1 Introduction

Amino acids are considered important compounds not only because they are part of structural units of proteins but also are intermediates in metabolism [1–3]. Some of them serve as precursors for the biosynthesis of neurotransmitters, porphyrins, polyamines, and nitric oxide [4]. They are widely existing in all kinds of food and beverages [3, 5]. In addition to the nutritional aspects, their presence affects the quality of food products with respect to taste, aroma, and color [2, 5].

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_5,

Amino acids are organic compounds formed by both an amino group and a carboxyl group bound to a specific side chain [1, 6]. In function of the number of carboxylic and amino groups, they are divided into three groups: neutral (e.g., serine), acidic (e.g., glutamic acid), and basic (e.g., arginine) [6]. A total of twenty amino acids form part of proteins, of these there are nine amino acids that are not synthesized by the body and therefore must be introduced into the body through diet. These amino acids are called essential amino acids [1].

In meat and meat products, its quantification is important because these compounds play an important role in the nutritional quality, sensorial characteristics, and acceptability of meat products. Thus, several factors such as the food handling processes, the storage conditions, and the maturation grade affect the proteolytic phenomena, which can change its structure and content producing changes in the sensory of the food [3, 7]. Some amino acids affect the flavor [8] and influence its palatability [9, 10], and also contribute to the formation of amines and volatile compounds [9, 11]. For this, the development of reliable, rapid, and accurate methods of extraction and determination of amino acids is interesting to evaluating the nutritional quality of foods [4, 5]. The extraction of amino acids from the food matrix is the first step to their determination and quantification. Free amino acid extraction includes an extraction step and deproteinization previously to analysis. The extraction consists in the homogenization of the sample in an appropriate solvent, normally hot water, hydrochloric acid 0.01–0.1 N or diluted phosphate buffers [1, 9]. Once homogenized, then the sample is centrifuged at refrigeration and the supernatant is filtered through glass wool [12, 13] or nylon membrane [14] to retain any fat material. Then, the deproteinization process takes place where a portion of the sample is mixed with an organic solvent (methanol, ethanol, or acetonitrile) to precipitate the proteins by denaturation [1]. On the other hand, the extraction of hydrolyzed amino acids consists of a protein hydrolysis step prior to analysis. The hydrolysis of samples can be acid, alkaline, or enzymatic although the more common is the acid hydrolysis normally with hydrochloric acid 6 N at 110 °C during between 20 and 96 h [1].

The first analysis of amino acids had been carried out by ion-exchange chromatography followed by post-column derivatization with ninhydrin and ultraviolet detection. However, this technique is not very selective since amino acids absorb at wavelengths between 190 and 210 nm the same as the majority of solvents and other components of the samples [5]. Due to these, the technique has gradually been supplanted by faster, more sensitive, and versatile methodologies [9], like gas chromatography (GC) [7, 9] and high-performance liquid chromatography (HPLC) [3, 15–17]. Nowadays, in food, the most used method

to determine amino acids is reverse phase HPLC [9]. However, to use these new techniques it is necessary a previously derivatization prior to analysis to convert amino acids into more detectable forms. The principal derivation agents are ninhydrin, dansyl chloride, 1-fluoro-2,4-dinitrobenzene, phenylisothiocyanate, ortho-phthalaldehyde, 9H-fluoren-9-ylmethyl chloroformate, diethyl 2 (ethoxymethylidene)propanedioate, and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [5].

With this in mind, the purpose of this book chapter is to thoroughly describe an analytical procedure for determining free and hydrolyzed amino acids in meat and meat products. Four stages are detailed, one for the extraction of free amino acids, another for hydrolyzed amino acids, a stage common to both extractions in which the derivation process is detailed and finally the chromatographic analysis by liquid chromatography.

2 Materials

Prepare all solutions using milli-Q water and/or analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials (*see* **Note 1**).

2.1 Hydrolyzed Amino Acids Extraction Step

1. Hydrochloric acid solution (6 N): To prepare 1 L, measure 497 mL of HCL (37%) and bring it to 1 L with milli-Q water (*see* **Note 2**).

2.2 Free Amino Acids Extraction Step

1. Hydrochloric acid solution (0.1 N): To prepare 1 L, measure 8.28 mL of HCL (37%) and bring it to 1 L with milli-Q water (*see* **Note 2**).

2.3 Calibration, Amino Acids Derivatization and Identification

All amino acids standards should be analytical grade (preferably HPLC grade analysis) and should be stored at −20 °C.

1. Amino acid standard mixture: Amino acid Standard H (Thermo Scientific, ref.: 20088). Ampule of 1 mL that contain eighteen amino acids at a concentration of 2.5 ± 0.1 μmol/mL in hydrochloric acid 0.1 N each one, except cystine with a concentration of 1.25 ± 0.1 μmol/mL.
2. Taurine (2-Aminoethanesulfonic acid) ≥99.0% from (Sigma-Aldrich; ref.: T0625). In order to obtain a 2.5 μmol/mL standard solution, dissolve 16.4 mg in 50 mL of 0.1 N HCl.
3. L-4-Hydroxyproline ≥99.0% from (Fluka; ref.: 56250). In order to obtain a 2.5 μmol/mL standard solution, dissolve 15.6 mg in 50 mL of 0.1 N HCl.
4. In order to obtain a six-point calibration curve, different volumes (μL) of the previously described standards [Amino

Table 1
Volume (μL) of the amino acid standards and milli-Q water for the elaboration of the different points of the calibration curve (final volume 1 mL)

Amino acids standard	STD0	STD1	STD2	STD3	STD4	STD5
Amino acids standard (2.5 μmol/mL)	1.25	2.5	5	10	20	40
Hydroxyproline (2.5 μmol/mL)	1.25	2.5	5	10	20	40
Taurine (2.5 μmol/mL)	1.25	2.5	5	10	20	40
Milli-Q water	996.25	992.5	985	970	940	880

Table 2
Amino acids (in order of elution) and final concentration (ng/mL) of each point of the calibration curve

N° #	Amino acid	STD0	STD1	STD2	STD3	STD4	STD5
1	Hydroxyproline	40.98	81.96	163.91	327.83	655.65	1311.30
2	Aspartic acid	41.59	83.19	166.38	332.75	665.50	1331.00
3	Serine	32.84	65.68	131.36	262.73	525.45	1050.90
4	Glutamic acid	45.98	91.96	183.91	367.83	735.65	1471.30
5	Glycine	23.46	46.92	93.84	187.68	375.35	750.70
6	Histidine	48.48	96.97	193.94	387.88	775.75	1551.50
7	Taurine	39.11	78.22	156.44	312.88	625.75	1251.50
8	Arginine	54.44	108.88	217.75	435.50	871.00	1742.00
9	Threonine	37.23	74.45	148.90	297.80	595.60	1191.20
10	Alanine	27.84	55.68	111.36	222.73	445.45	890.90
11	Proline	35.98	71.96	143.91	287.83	575.65	1151.30
12	Cysteine	37.86	75.73	151.45	302.90	605.80	1211.60
13	Tyrosine	56.62	113.24	226.49	452.98	905.95	1811.90
14	Valine	36.61	73.22	146.44	292.88	585.75	1171.50
15	Methionine	46.63	93.26	186.51	373.03	746.05	1492.10
16	Lysine	45.68	91.37	182.74	365.48	730.95	1461.90
17	Isoleucine	40.99	81.98	163.96	327.93	655.85	1311.70
18	Leucine	40.99	81.98	163.96	327.93	655.85	1311.70
19	Phenylalanine	51.62	103.24	206.49	412.98	825.95	1651.90

acid Standard H, Taurine (2.5 μmol/mL) and L-4-Hydroxyproline (2.5 μmol/mL)] were mixed with milli-Q water (Table 1). The final concentrations (ng/mL) and elution order of each amino acid are those listed in Table 2 (*see* **Note 3**).

5. For derivatization of amino acids, the commercial derivatization kit (based on derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate—AQC) supplied by Waters is used (AccQ-Tag Ultra Derivatization Kit; ref. 186003836). This kit includes AccQ-Fluor borate buffer, Reagent powder, and reagent diluent.
6. Mobile phase was prepared from commercial Waters AccQ-Tag Eluent A concentrate (ref. WAT052890). According to the instructions, in order to obtain the correct concentration of mobile phase A, add 200 mL of commercial Eluent A concentrate to 2 L to milli-Q water, and filter the mixture through 0.45 μm membrane prior the HPLC analysis.

2.4 Liquid Chromatograph

Separation and quantification of amino acids are carried out using high-performance liquid chromatography (Alliance 2695 model, Waters, Milford, MA, USA) equipped with a scanning fluorescence detector (model 2475, Waters, Milford, MA, USA). For the separation of amino acids, a Waters AccQ-Tag column (3.9 × 150 mm, with a particle size of 3 μm) is used.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Hydrolyzed Amino Acids Extraction

Hydrolyzed amino acids extraction is done according to the protocol described by Domínguez et al. [15].

1. Weigh 100 mg of samples in glass ampoules and add 5 mL of 6 N hydrochloric acid solution [HCl (6 N)] (*see* **Note 4**).
2. Seal the ampoule with fire and kept at 110 °C for 24 h until hydrolysis of proteins has been completed (*see* **Note 5**).
3. After the time, let the vials cool to room temperature (*see* **Note 6**).
4. Dilute 625 μL of the hydrolysate with 25 mL of milli-Q water, mix and then filter through a 0.45 μm filter to an Eppendorf (PP syringe filter).

3.2 Free Amino Acids Extraction

Free amino acids analysis is determinate according to the procedure described by Lorenzo et al. [16].

1. Weigh 5 g of sample in a beaker.
2. Add 25 mL of hydrochloric acid 0.1 N [HCl (0.1 N)].
3. Homogenize the samples with Ika Ultra-Turrax for 8 min while cooled by submerging the extract in ice.
4. Centrifuge the samples at 10,000 × *g* for 20 min at 4 °C.

5. Filter the supernatant material through glass wool (*see* **Note 7**).
6. Take 200 μL of this extract to an Eppendorf and deproteinize adding 800 μL of acetonitrile (ACN).
7. Vortex and put in fridge during 30 min.
8. Centrifuge the Eppendorfs at 10,000 × g for 5 min at 4 °C.
9. Filter with 0.45 μm filter (PP syringe filter).

3.3 Amino Acid Derivatization

The derivatization process is necessary for standards, free and hydrolyzed amino acids. Figure 1 shows in a simplified and schematic way the steps of the free and hydrolyzed amino acid extraction and derivatization. The derivatization is performed as follows:

1. Reconstitute the commercial reagent powder for derivatization (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate—AQC) with the reagent diluent (Acetonitrile). For this, take 1 mL of reagent diluent and transfer to reagent powder vial and then shake vigorously with a vortex to the complete dissolution. Finally, incubate exactly 10 min at 55 °C in a heater (*see* **Notes 8** and **9**).
2. Add 10 μL of sample or standard to a total recovery vial of 2 mL and 70 μL of AccQ-Fluor borate buffer to obtain a pH of 8.8 and vortex.
3. Add 20 μL of Reagent solution in each sample or standard vial. Then cap the vial and vortex to ensure complete derivatization (*see* **Note 10**).
4. Let stand the vials for 1 min at room temperature (RT).
5. Heat the samples or standards vials in an oven for exactly 10 min at 55 °C to complete the derivatization (*see* **Note 8**).
6. Vortex the vials before the injection (*see* **Note 11**).

3.4 Amino Acid Identification and Quantification (HPLC-FL)

Separation and quantification of amino acids are carried out using a liquid chromatograph coupled with a fluorescence detector following the next conditions (Table 3):

1. Ten microliters of the sample (or the standard) is injected (*see* **Note 12**). A Waters AccQ-Tag column (3.9 × 150 mm, with a particle size of 3 μm) is used for amino acid separation. The temperature of the column oven is adjusted at 37 °C.
2. The mobile phase composition and the gradient were defined according to the validated and patented method of Waters Corporation (AccQ-Tag Amino acid analysis protocol), with minor changes. Three solvents make up the mobile phase: (A) AccQ Tag Eluent A solution for amino acids analysis (Waters, Milford, MA, USA), (B) acetonitrile (HPLC grade), and (C) ultra-pure water (Milli-Q). The flow rate is 1.0 mL/min and the solvent gradient was set as follows: 0.0–0.5 min

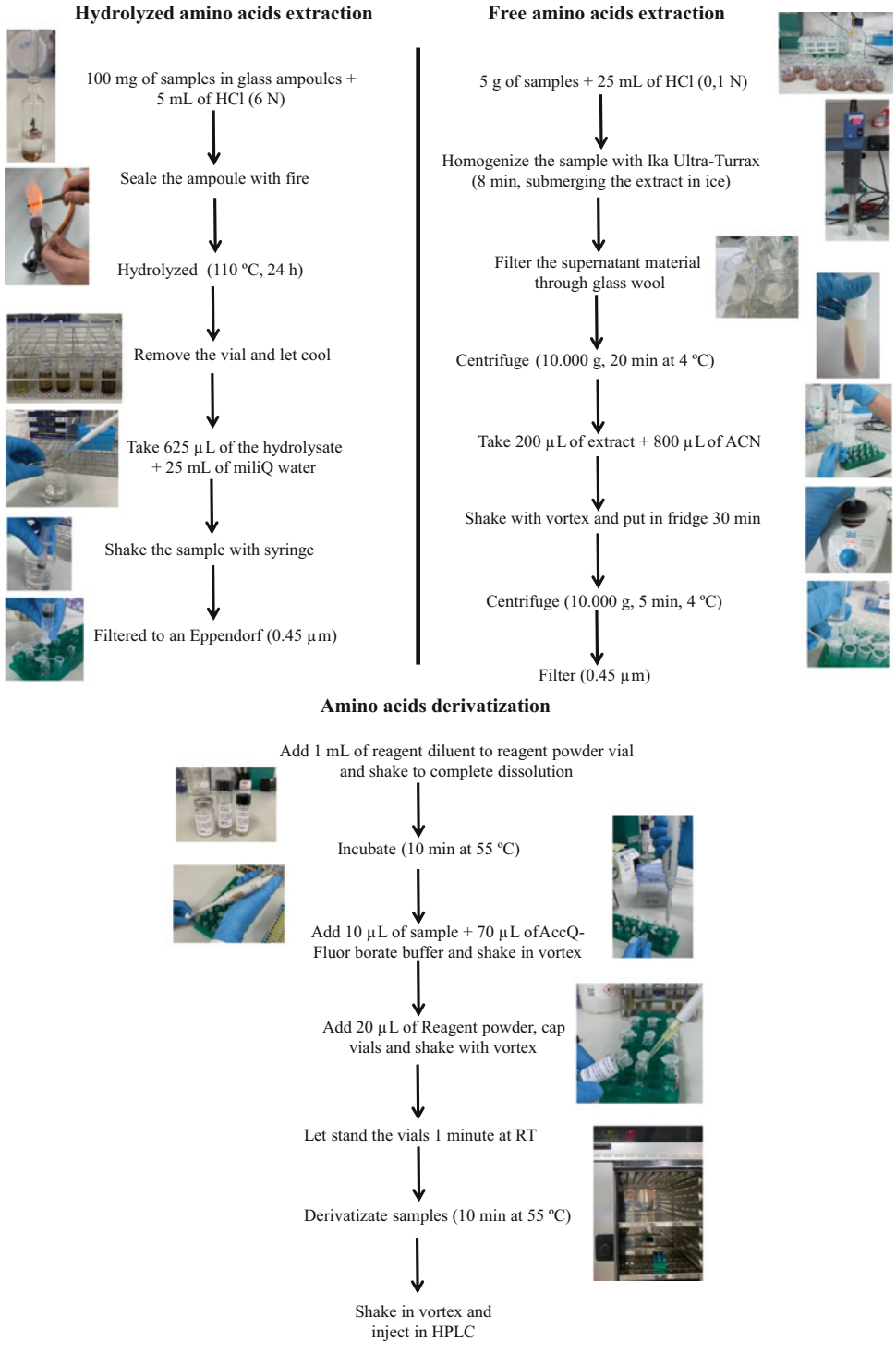

Fig. 1 Schematic and simplified representation of the steps of the amino acids (free and hydrolyzed) extraction and derivatization phases

Table 3
Summary of the chromatographic conditions used for the amino acids analysis

Parameters	Values			
Injection volume	10 μL			
Flow rate	1 mL/min			
Column	AccQ-tag column (3.9 × 150 mm, with a particle size of 3 μm), waters			
Oven temperature	37 °C			
Mobile phase	AccQ tag eluent A solution for amino acids analysis (waters, Milford, MA, USA), Acetonitrile (HPLC grade) and ultra-pure water.			
Gradient elution	Time (min)	AccQ tag eluent (%)	Acetonitrile (%)	Water (%)
	0.0	99	1	0
	0.5	99	1	0
	17	96	4	0
	22	95	5	0
	24	91	9	0
	31.5	83	17	0
	36	83	17	0
	42	0	60	40
	44	99	1	0
Detector	Fluorescence detector			
Excitation wavelength	250 nm			
Emission wavelength	395 nm			
Retention time	Compound		Retention time (min)	
	Hydroxyproline		10.04	
	Aspartic acid		12.05	
	Serine		13.71	
	Glutamic acid		14.84	
	Glycine		16.17	
	Histidine		17.33	
	Taurine		21.45	
	Arginine		22.55	
	Threonine		23.08	
	Alanine		25.07	
	Proline		27.13	
	Cysteine		29.94	
	Tyrosine		30.01	
	Valine		30.97	
	Methionine		31.37	
	Lysine		33.31	
	Isoleucine		34.05	
	Leucine		34.55	
	Phenylalanine		35.65	
Run time	45 min			

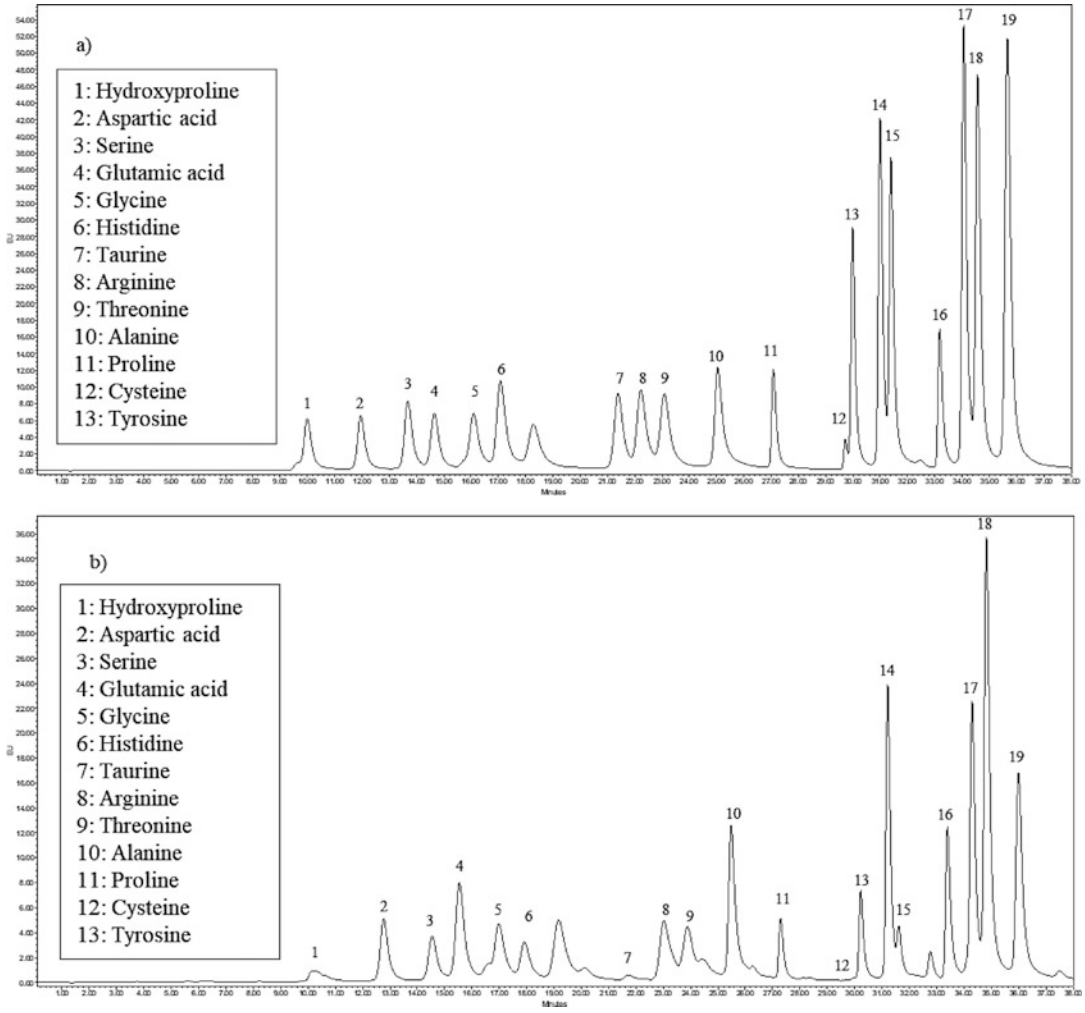

Fig. 2 Amino acids chromatograms of a standard (**a**) and a fresh meat sample (**b**)

99% A and 1% B; 0.5–17.0 min 99% A and 1% B; 17.0–22.0 min 96% A and 4% B; 22.0–24.0 min 95% A and 5% B; 24.0–31.5 min 91% A and 9% B; 31.5–36.0 min 83% A and 17% B; 36.0–42.0 min 83% A and 17% B; 42.0–44.0 min 60% B and 40% C; 42.0–45.0 min 99% A and 1% B.

3. The detection of amino acids is carried out using a fluorescence detector (FL). The wavelengths for excitation and emission are set at 250 and 395 nm, respectively. The total time for chromatographic analysis is 45 min.

Data acquisition, equipment control, and data analysis are carried out using the HPLC software (in our case Empower 3TM; Waters, Milford, MA, USA). The amino acids in meat products are identified by comparing their retention time with the authenticated standards. Figure 2 shows an example of chromatograms of

standard (a) and meat sample (b). The results are expressed as mg/100 g of sample.

4 Notes

1. Due to the toxic and/or corrosive nature of the solvents and reagents used in the extraction and methylation of fatty acids, it is necessary for the operator to take the necessary protective measures (gloves, glasses, etc.) as well as to carry out all the operations in laboratory fume hoods.
2. Add the hydrochloric acid slowly to the water, shake, and finally make the volumetric flask up to the mark with water (use a hood to make these solutions).
3. The pipetting process must be careful due to the small quantities to take (few μL). The tip of the micropipettes should be moistened with the solvent (or standard) to ensure that the correct amount of each standard is taken.
4. An analytical balance with 0.1 mg resolution or higher should be used to weigh the tubes.
5. Preheat an oven at 110 °C.
6. Wait a reasonable time to avoid burning yourself when handling the vials. Use gloves or suitable utensils to open the vials safely, and avoid possible cuts.
7. The filtered extract can be saved at −20 °C until use.
8. Preheat an oven at 55 °C.
9. It is important to dilute completely the reagent powder for a correct derivatization.
10. Make sure that there are no bubbles in the vial insert to ensure the correct derivatization process.
11. Shake vigorously to avoid bubbles in the vial insert that may interfere with the injection.
12. Standards should be injected first, for identification and to create calibration curves for each amino acid. During sample analysis, a blank (acetonitrile) should be injected every 10 samples. Calibrations should be performed each time the reagent powder for derivatization is prepared or when performing any chromatograph maintenance that may affect the resolution of the equipment or the detector signal.

Acknowledgments

This work was supported by Centro Tecnolóxico de la Carne de Galicia. Thanks to GAIN (Axencia Galega de Innovación) for supporting this book chapter (grant number IN607A2019/01). Authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568).

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Chapter 6

Cholesterol

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Abstract

The cholesterol determination in meat and meat products is very important because these products are highly demanded. The intake of high levels of cholesterol is associated with several diseases. Therefore, the development of analytical techniques is necessary to inform the consumer about the meat and meat products nutritional quality.

Cholesterol determination in meat and meat product was studied for decades. The first published studies were based on a previous lipid extraction followed by saponification before its quantification. Nowadays, new methods have been published that simplify the process by making direct saponification with KOH and ethanol. These methods allow a faster determination of cholesterol with less consumption of solvents. The most common chromatographic techniques for determining and quantified cholesterol in meat and meat products are gas chromatography and high-performance liquid chromatography.

This chapter gives a clear and complete vision of a rapid and precise methodology for the cholesterol analysis of meat and meat products, including all the extraction steps and the subsequent analysis by high-performance liquid chromatography with Photodiode Array detection (HPLC-DAD) using a normal phase. The conditions of extraction and analysis proposed by us are described afterwards with exactitude with the aim reproduced by other researchers.

Key words Food analysis, Cholesterol, Normal phase, HPLC-DAD

1 Introduction

Cholesterol is an essential lipid molecule that is commonly found as a component in the cell membrane [1]. One of the major functions is to participate in the biosynthesis of bile acids in the liver [2, 3]. It is an important precursor for bile acids, provitamin D, and several steroidal hormones as testosterone and estrogen [2–5]. Moreover, they also take part in the production and absorption of vitamin D [3]. Due to all the aforementioned, the intake of low cholesterol levels could have benefits for human health [3]. However, high cholesterol levels are associated with a higher risk of

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_6,

hypercholesterolemia, obesity, diabetes, brain diseases, and cardiovascular diseases [3, 6, 7].

Cholesterol is mostly present in meat, meat products and derived products from livestock as eggs, cheese, milk, butter, etc. [4]. The consumption of meat and meat products provide one third to one half of daily-recommended cholesterol intake [6]. To prevent the appearance of diseases associated with cholesterol, it is necessary accurate determination of cholesterol. The development of analytical techniques for the determination and quantification of total cholesterol in meat and meat products is important mainly to inform of their quality and safety [8].

The AOAC International adopted the first validated cholesterol determination method for foods (AOAC International 976.26, [9]). This method was based on a prior lipid extraction followed by saponification, which makes it a long and tedious method. Moreover, this extraction method increases the analysis time and the consumption of dissolvents. In order to overcome these drawbacks, the method was modified, by employing direct saponification (AOAC International 994.10, [10–12]). Nowadays, new extraction methods based on direct saponification followed by extraction with organic solvents were validated with good results [4, 8, 11, 13]. The saponification step is indispensable to separate cholesterol and other unsaponifiable materials from fatty acids and to remove triglyceride interferences [12, 14]. The most suitable direct saponification conditions are the use of an alcoholic KOH solution (between 0.33 and 0.5 M) and temperatures ranging from 55 to 75 °C during 15 to 60 min [11, 12], also known as hot saponification. Despite, cold saponification (at room temperatures) procedures during several hours (overnight) could also be used [15], to limit the cholesterol degradation during heating. It is important to highlight that in hot saponification, the addition of antioxidants (i.e., Vitamin C) and the use of anaerobic conditions (air removed with nitrogen or argon) during saponification and extraction processes contribute to preventing cholesterol oxidation [6].

For cholesterol analysis several, analytical methods have been developed, including spectroscopic and gravimetric procedures, enzymatic assays, and chromatographic methods based on gas chromatography, liquid chromatography, and mass spectrometry [1, 3]. In food, the chromatographic methods were more reliable and selective because of avoidance of the interference from other sterols [1, 4, 12, 14]. One of the most used methods in meat cholesterol analysis is based on liquid chromatography coupled to photodiode array detector [4, 8, 15].

For cholesterol analysis by HPLC, both normal phase (NP) and reversed phase (RP) can be used [13, 15]. However, due to its low polarity, the use of normal phase is preferable. In this regard, some authors proposed the cholesterol analysis by normal phase

(NP) using a polar stationary phase (silica) and an apolar mobile phase (hexane) [13]. The normal phase analysis provides better chromatograms than reversed phase methods due to the low viscosity of commonly used eluents.

Thus, the aim of this book chapter is to explain in detail a procedure for determining cholesterol in meat and meat products. The extraction consists of direct saponification (hot saponification) of the meat sample with ethanolic KOH solution, a posterior extraction with hexane, and finally the chromatographic analysis by liquid chromatography.

2 Materials

Prepare all solutions using distilled water and/or analytical grade reagents. Use HPLC-grade hexane. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials (*see* **Note 1**).

2.1 Extraction Step

1. Saponification solution: 11% w/v potassium hydroxide (KOH) in a mixture of 55% v/v absolute ethanol (EtOH) and 45% v/v distilled water (H_2O). For 1 L of solution dissolve 129.4 g of KOH (85% purity) in 450 mL of distiller water and then add 550 mL of absolute ethanol. The saponification solution must be prepared/renewed every week.
2. BHT (25 ppm) solution in n-hexane: Dissolve 0.0125 g of butylated hydroxytoluene (BHT) in 500 mL of n-hexane.

2.2 Calibration and Cholesterol Identification

1. Cholesterol ($\geq$ 99%): For a concentration of 1 mg/mL, dissolve 0.05 g of cholesterol in 50 mL of n-hexane.
2. Standards for calibration curve: In order to obtain a seven-point calibration curve, different volumes (μL) of the previously described cholesterol standard were mixed with n-hexane (Table 1). The final concentrations (ppm) of calibration curve standards are those listed in Table 2.

Table 1
Volume (μL) of the cholesterol standard solution (1 mg/mL) and hexane for the elaboration of the different points of the calibration curve (final volume 1500 μL)

Compound	STD0	STD1	STD2	STD3	STD4	STD5	STD6
Cholesterol (1 mg/mL)	23.44	46.88	93.75	187.50	375	750	1500
Hexane (HPLC grade)	1476.56	1453.13	1406.25	1312.50	1125	750	0

Table 2
Final concentration (ppm) of cholesterol in each point of the calibration curve

Concentration (ppm)	STD0	STD1	STD2	STD3	STD4	STD5	STD6
Cholesterol	0.15625	0.3125	0.625	1.25	2.5	5	10

2.3 HPLC System

Separation and quantification of cholesterol are carried out using a liquid chromatograph model Alliance 2695 equipped with a 996 Photodiode Array Detector (Waters, Milford, MA, USA). The cholesterol separation was performed using a normal phase silica column (SunFireTM Prep Silica, 4.6 mm ID × 250 mm, 5 μm particle size, Waters, Milford, MA, USA). Empower 3TM advanced software (Waters, Milford, MA, USA) was used to control system operation and analyze results.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Cholesterol Extraction

The extraction was carried out according to the protocol described by Domínguez et al. [13] with minimal changes. Figure 1 shows a simplified and schematic way of the extraction phases.

1. Weigh 0.50 ± 0.02 g of homogenized meat sample in a screw Teflon-lined cap tube of 15 mL.
2. Add 0.25 g of L-ascorbic acid (to avoid the oxidative process) and 5 mL of saponification solution.
3. Apply nitrogen gas to eliminate the air from the reaction by displacement and then close the tube (*see* **Note 2**).
4. Shake until the ascorbic acid is completely dissolved with a vortex (approximately 30 s), then the samples are left to rest for 5 min and finally they are stirred again for another 30 s.
5. The saponification is carried out in a shaking water bath (THER-SPIN, Orto Alresa, Madrid, Spain) (200 rpm) at 85 °C for 45 min. After 20 min of the saponification process, the samples were vortexed. At the end of the saponification process, the samples were cooled at room temperature (about 10 min) (*see* **Note 3**).
6. Follow cooling, 1.5 mL of distilled water and 3 mL of 25 ppm of BHT solution in n-hexane are added and they are vortexed vigorously.
7. The samples are transferred to conical centrifuge tubes of 15 mL capacity and are centrifuged at 1500 × *g* for 3 min (*see* **Note 4**).
8. An aliquot of n-hexane (upper layer) is transferred into another tube. In order to ensure the complete dehydration of the

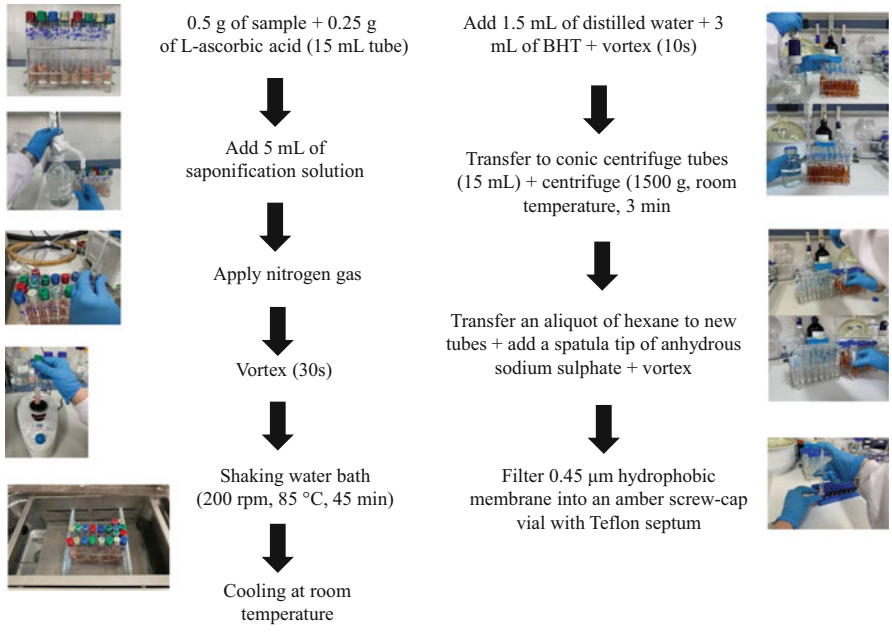

Fig. 1 Schematic and simplified representation of the steps of the cholesterol extraction phases

organic phase, a spatula tip of anhydrous sodium sulfate are added.

9. Finally, the tubes are briefly shaken (vortexed), and an aliquot of the n-hexane was filtered through a 0.45-µm hydrophobic membrane into an amber screw-cap vial with Teflon septum.

3.2 Cholesterol Identification and Quantification (HPLC-DAD)

Separation and quantification of cholesterol are carried out using a liquid chromatograph coupled with a diode array detector following the next conditions (Table 3):

1. Ten microliters of the sample (or the standard) are injected. A SunFireTM Prep Silica, 4.6 mm ID × 250 mm, 5 µm particle size, (Waters) is used for cholesterol separation. The temperature of the column oven is adjusted at 30 °C.
2. The mobile phase is adjusted at 2% 2-propanol and 98% n-hexane. The isocratic mode is used during chromatographic analysis at a flow rate of 1 mL/min.
3. The detection of cholesterol are carried out using Photodiode Array detector (DAD) at 208 nm. The total time for chromatographic analysis is 15 min.

Data acquisition, equipment control, and data analysis are carried out using the HPLC software (in our case Empower 3TM; Waters). The cholesterol in meat products is identified by comparing its retention time with the authenticated standards (Cholesterol ≥ 99%) (*see* **Notes 5** and **6**). Figure 2 shows examples of

Table 3
Summary of the chromatographic conditions used for the cholesterol analysis

Parameters	Values
Injection volume	10 µL
Flow rate	1 mL/min
Column	SunFireTM prep silica, 4.6 mm ID × 250 mm, 5 µm particle size, waters
Oven temperature	30 °C
Mobile phase	2-propanol/hexane (2:98, v/v)
Elution type	Isocratic
Detector	Photodiode Array detector
Wavelength	208 nm
Retention time	10 min
Run time	15 min

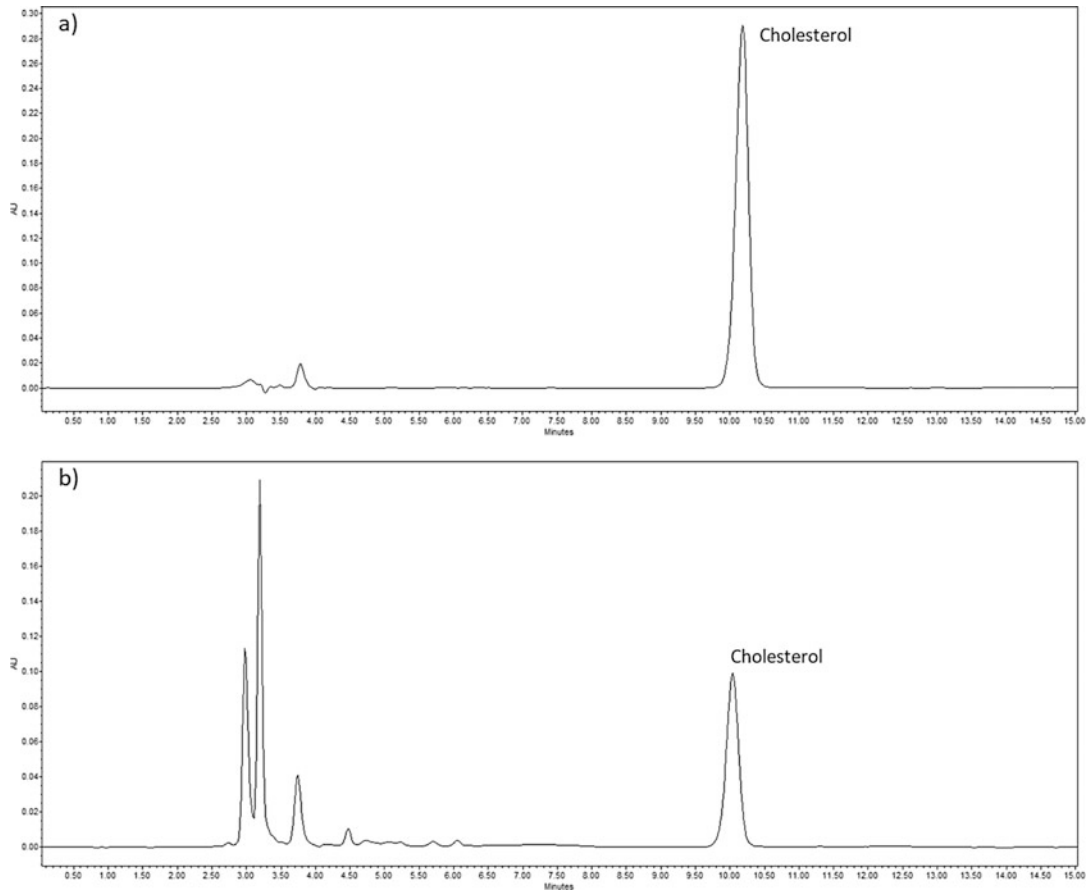

Fig. 2 Cholesterol chromatogram of a standard (**a**) and a fresh meat sample (**b**)

chromatograms of standard (a) and sample (b). The results are expressed as mg/100 g of meat.

4 Notes

1. Due to the toxic and/or corrosive nature of the solvents and reagents used in the extraction, it is necessary for the operator to take the necessary protective measures (gloves, glasses, etc.) as well as to carry out all the operations in a laboratory fume hood.
2. Apply a low pressure of nitrogen to avoid losses due to splashes.
3. The water batch should be turned on before (about 15 min) to allow the water bath to have the correct saponification temperature.
4. The emulsification of some samples may occur during the extraction process. If this happens, 0.2 mL of absolute ethanol can be added to facilitate the separation process. The ethanol is gently mixed in swirling motion, and the emulsifying mixture allowed to stand to enable the separation.
5. Standards should be injected first, for identification and to create the calibration curve. Identification/calibration standards must be prepared each time a calibration is performed. During the analysis of the samples, a blank (hexane) should be injected every 10 samples. It is recommended to inject a standard to verify retention times and the concentration each time a new sequence begins. Calibrations should be performed at least once a month, or when any maintenance is performed on the chromatograph that may affect the resolution of the equipment or the detector signal.
6. Standards prepared for calibrations can be stored frozen (−20 °C) and used to check retention times. However, these standards cannot be used for calibration, only to verify retention times. Calibration curve standards must be prepared each time a new calibration is performed.

Acknowledgments

This work was supported by Centro Tecnolóxico de la Carne de Galicia. Thanks to GAIN (Axencia Galega de Innovación) for supporting this book chapter (grant number IN607A2019/01). Authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568).

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Chapter 7

Mineral Profile

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Abstract

In recent years, there has been an increase in consumer interest in nutritional and health aspects, which has affected the consumption of animal products. Meat is a source of essential minerals for humans, among which iron stands out, as well as others, such as zinc, phosphorus, magnesium, and potassium. Moreover, meat products can contain high levels of NaCl, and this can be harmful to health. Therefore, it is very important to have an appropriate method for a correct determination of the mineral content in meat and meat products.

Although a wide variety of analytical methods are available for the analysis of minerals and trace elements in food products, Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) is one of the most used techniques, as it allows the simultaneous analysis of a large number of elements.

Taking this into account, this chapter is intended to describe in detail a procedure for mineral analysis of meat and meat products, including determination of total ash and analysis of mineral content by ICP-OES, so that it can be reproduced by other researchers.

Key words Mineral content, Meat, Calcium, Sodium, Iron, Magnesium, Phosphorous, Food analysis, Inductively coupled plasma optical emission spectroscopy (ICP-OES)

1 Introduction

In the last few decades, nutritional habits have notably changed. Specifically, meat consumption has been on the rise since the 1960s in most of countries, but especially from 1980 to the present [1–4]. Moreover, a trend has been observed in recent years: consumers concern for their health and their interest in the nutritional aspects of food has increased, which is also reflected in the consumption of meats and meat products [4, 5].

Meat provides important nutrients for the human diet, mainly proteins, vitamins, and minerals. Minerals have very varied

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_7,

functions in our body, such as electrolytes, as enzymatic constituents and as building materials (in bones and teeth). They are divided into macro minerals, trace elements (microminerals), and ultra-trace elements. The main elements (Na, K, Ca, Mg, Cl, P, S) are essential for humans in amounts >50 mg/day and trace elements (Fe, I, F, Zn, Se, Cu, Mn, Cr, Mo, Co, Ni) are essential in concentrations of <50 mg/day. Health authorities in most countries have established recommendations for daily intake levels of these essential minerals [6–8].

The mineral content in meat depends on factors like the species, breed, rearing practices, feeds composition, slaughter age, and the cut [8, 9]. According to Hermida et al. [10], the macro minerals and trace elements concentrations in tissues depend on the type of cut, the age of the animals, and other factors. Greenfield and Southgate [11] concluded that the lean/fat tissue ratio affects the levels of most nutrients, which are distributed differently in the two fractions. In addition, thermal processes can change the mineral content of meat [12].

One of the most relevant minerals in meat is iron as it is highly bioavailable. The form in which this iron is found (mainly in heme form, which is bound with myoglobin and hemoglobin) allows 20–30% of it to be absorbed. In addition, the presence of meat favors the absorption of iron forms from vegetables [13]. Another important contribution of meat is zinc as well as magnesium, potassium, copper, and other minerals and microelements [14].

On the other hand, meat products (especially dry-cured products like ham, sausages, and bacon) may contain high concentrations of sodium chloride (NaCl), since salting with NaCl is widely employed to preserve these products, contributes to achieve a characteristic flavor, gives microbial stability and improves proteins solubility [15–17]. However, an excessive consumption of salt is not advisable. Several researches have revealed that high levels of NaCl intake can raise blood pressure and promote certain diseases, which is why it is currently intended to offer consumers healthier food products with low salt content and without losing quality [15, 16]. Potassium chloride is the most common salt substitute in meat products, although calcium and magnesium chlorides also can be another alternative [17].

Considering this, it seems clear that it is important to develop a suitable method for determining the mineral content in meat and meat products.

Nowadays, there are a great variety of analytical methods for the analysis of minerals and trace elements in food. The most commonly used methods include: spectrophotometry, fluorometry, atomic absorption spectrometry (AAS), flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), hydride generation atomic absorption spectrometry (HGAAS), inductively coupled plasma optical

emission spectroscopy (ICP-OES), and inductively coupled plasma mass spectrometry (ICP-MS). The choice of analytical method generally depends on available instrumentation, laboratory experience, and analyte concentration levels [18].

Among all techniques, the use of inductively coupled plasma optical emission spectroscopy (ICP-OES) to identify and quantify minerals in meat and meat products is frequent. It is based on the measurement of the radiation of the spectral line emitted by excited atoms in an Ar plasma generated by inductive heating with a high-frequency electromagnetic field.

ICP-OES is one of the most widely used techniques around the world to determine elements in a wide variety of samples (which must be previously digested) as it allows the simultaneous analysis of a large number of elements [18, 19].

The method we have developed includes two main steps: (1) total ash determination, followed by (2) mineral content determination by ICP-OES.

With all the above, the present book chapter aims to clearly and completely describe a procedure for determining minerals elements in meat and meat products.

2 Materials

Prepare all solutions using mili-Q water and analytical grade reagents, and store stock and standard solutions at refrigerate temperature (4 °C) (*see* **Note 1**).

Follow all waste disposal regulations when disposing of waste materials.

2.1 Total Ash and Mineral Content Determination

Analytical balance (*see* **Note 2**), porcelain crucibles, desiccator for storing them, and a muffle furnace for incinerate samples are needed for total ash determination.

For mineral content determination, these are required: 110 mm quantitative paper filters (FILTER-LAB, Barcelona, Spain), plastic funnels, racks and plastic tubes for ICP-OES autosampler.

1 M HNO_3 solution is required. For this, mix 69.23 mL of nitric acid (65%) (Suprapur or Ultratrace grade) with 900 mL of miliQ water.

2.1.1 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

The quantification of mineral elements is performed using a *PerkinElmer Avio 200* optical emission spectrometer (PerkinElmer, Massachusetts, USA) with an autosampler (S23, PerkinElmer).

The main components of an ICP-OES instrument are the plasma torch, the nebulizer, and the polychromator. The torch consists of three concentric quartz tubes surrounded by a water-

cooled induction coil connected to a high-frequency generator. Plasma is created by making Ar conductive by exposing it to an electrical discharge that creates electrons and ions. Under the influence of the high-frequency electromagnetic field, the charged particles heat the argon until the plasma reaches temperatures above 9700 °C. This leads to almost complete vaporization of the analyte and high atomization efficiency. The sample is introduced through the nebulizer into the torch using a transporter flow of Ar [18].

ICP-OES is also equipped with a radio frequency source set of 27.12 MHz, a peristaltic pump, a spraying chamber, and a concentric spray nebulizer (MEINHARD glass nebulizer, type K1), using 99.996% liquid argon plasma gas (Praxair, Madrid, Spain).

A 2% nitric acid solution is needed for equipment cleaning between sample measurements.

2.1.2 Calibration

Stock solutions at 1000 mg/L for Ca, K, Mg, Na, P, Fe, Mn, Zn, and Cu (SCP-SCIENCE, Courtaboeuf, France) were used for preparing the standard solutions in 1 N HNO_3, v/v.

The concentration ranges of the standard solutions were: 5–40 mg/L for Ca and Zn, 0.5–2.5 mg/L for Cu and Mn, 2–15 mg/L for Fe, and 50–200 mg/L for K, Mg, Na and P (*see* **Note 3**). Both, final concentrations (mg/L) of each mineral and the volume (μL) of each stock solution (1000 mg/L) used for the preparation of 50 mL of each standard are those listed in Tables 1 and 2 (*see* **Note 4**).

Table 1
Concentration ranges of the standard solutions (prepared in 1 N HNO_3)

	Mineral standard (mg/L)[a]				Adjust to 50 mL with HNO_3 (1 N)[b]			
	Std1	Std2	Std3	Std4	μL (Std1)	μL (Std2)	μL (Std3)	μL (Std4)
Ca	5	10	25	40	250	500	1250	2000
Cu	0.5	1	1.5	2.5	25	50	75	125
Fe	2	5	10	15	100	250	500	750
K	50	100	150	200	2500	5000	7500	10,000
Mg	50	100	150	200	2500	5000	7500	10,000
Mn	0.5	1	1.5	2.5	25	50	75	125
Na	50	100	150	200	2500	5000	7500	10,000
P	50	100	150	200	2500	5000	7500	10,000
Zn	5	10	30	40	250	500	1500	2000

Std. standard
[a]Final concentrations (mg/L) of each element in the standards used for calibration
[b]Volume (μL) of the stock solutions (1000 mg/L) used for the preparation of 50 mL of each standard

Table 2
Sodium concentration ranges of the standard solutions (prepared in 1 N HNO_3)

	Na standard (mg/L)[a]						Adjust to 50 mL with HNO_3 (1 N)[b]					
	Std1	Std2	Std3	Std4	Std5	Std6	μL (Std1)	μL (Std2)	μL (Std3)	μL (Std4)	μL (Std5)	μL (Std6)
Na	50	100	150	200	500	1000	2500	5000	7500	10,000	25,000	50,000

Std standard
[a]Final concentrations (mg/L) of sodium in the standards used for calibration
[b]Volume (μL) of the Na stock solution (1000 mg/L) used for the preparation of 50 mL of each standard level

3 Methods

Three-gram (3.00 ± 0.05 g) samples are weighed into porcelain crucibles for mineral analysis. Afterward, the samples are incinerated in a furnace at 450 °C for 12 h (using the protocol ISO 936: 1998 [20]). Then, the crucibles are stored at room temperature in the desiccator until constant weight. The amount of ash obtained is calculated by the difference between the crucible with the sample after incinerating—empty crucible. The ash obtained is dissolved in 10 mL of 1 M HNO_3 and filtered to plastic tubes (Fig. 1).

The next step is the mineral content determination using inductively coupled plasma optical emission spectroscopy (ICP-OES) in order to determine the concentration of Ca, K, Mg, Na, P, Fe, Mn, Zn, and Cu following the protocol defined by Lorenzo et al. [21] with modifications (*see* **Note 5**). Operating conditions of the ICP-OES equipment are: vertical plasma, argon pressure: 6.5 bar, reflected power: 1400 W, sample flow rate: 1 mL/min, nebulizer gas flow: 0.7 L/min, auxiliary argon flow: 0.2 L/min, main argon flow: 10 L/min, purge gas flow: 1.5 L/min, reading time: 2 seconds, height of vertical observation: 15 mm and radial torch configuration.

The operating wavelengths are: Ca, 317.933 nm; K, 404.721 and 766.490 nm; Mg, 279.077 and 285.213 nm; Na, 589.592 nm; P, 213.617 nm; Fe, 238.204 and 239.562 nm; Mn, 257.610 nm; Zn, 206.200 and 213.857 nm; and finally Cu, 324.752 and 327.393 nm (*see* **Note 6**).

Figure 2a and b shows the calibration curves obtained for Ca, Cu, Fe, Mg Na, Zn, Mn, and P at different operating wavelengths (*see* **Note 7**).

Syngistix ICP software is used to control the system and acquire the data. The external standard procedure is used to determine the concentration of each mineral (*see* **Note 8**).

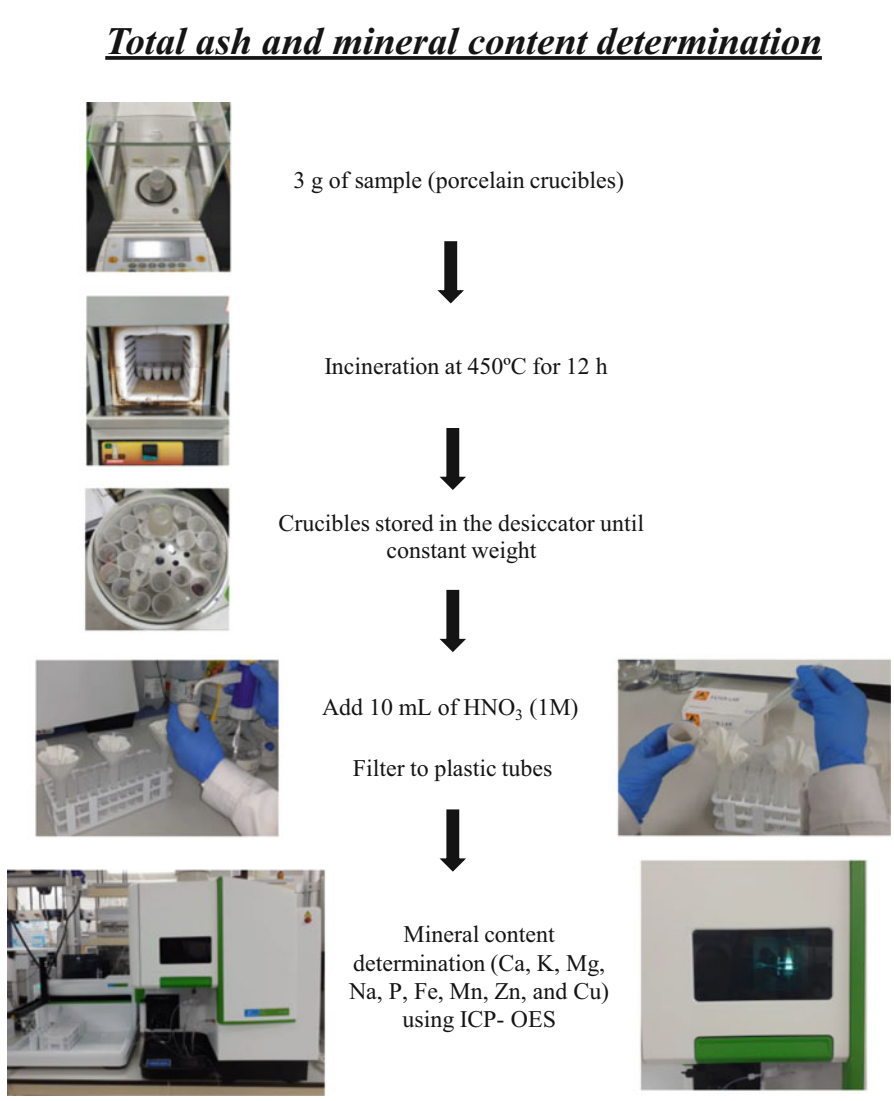

Fig. 1 Schematic and simplified representation of the steps of the total ash and mineral content determination

An example of the spectra obtained for Ca, Mg, and Na at the corresponding wavelength in a meat product sample is shown in Fig. 3. The final value for each element is obtained by calculating the average of two determinations using the Eq. 1, and the results are expressed as mg/100 g of meat.

$$\text{Sample mineral content } \left(\text{mg}/\text{100 g meat}\right) = \frac{\text{ICP mineral content } \left(\frac{\text{mg}}{\text{L}}\right)}{\text{Fresh sample weight (g)}} \quad (1)$$

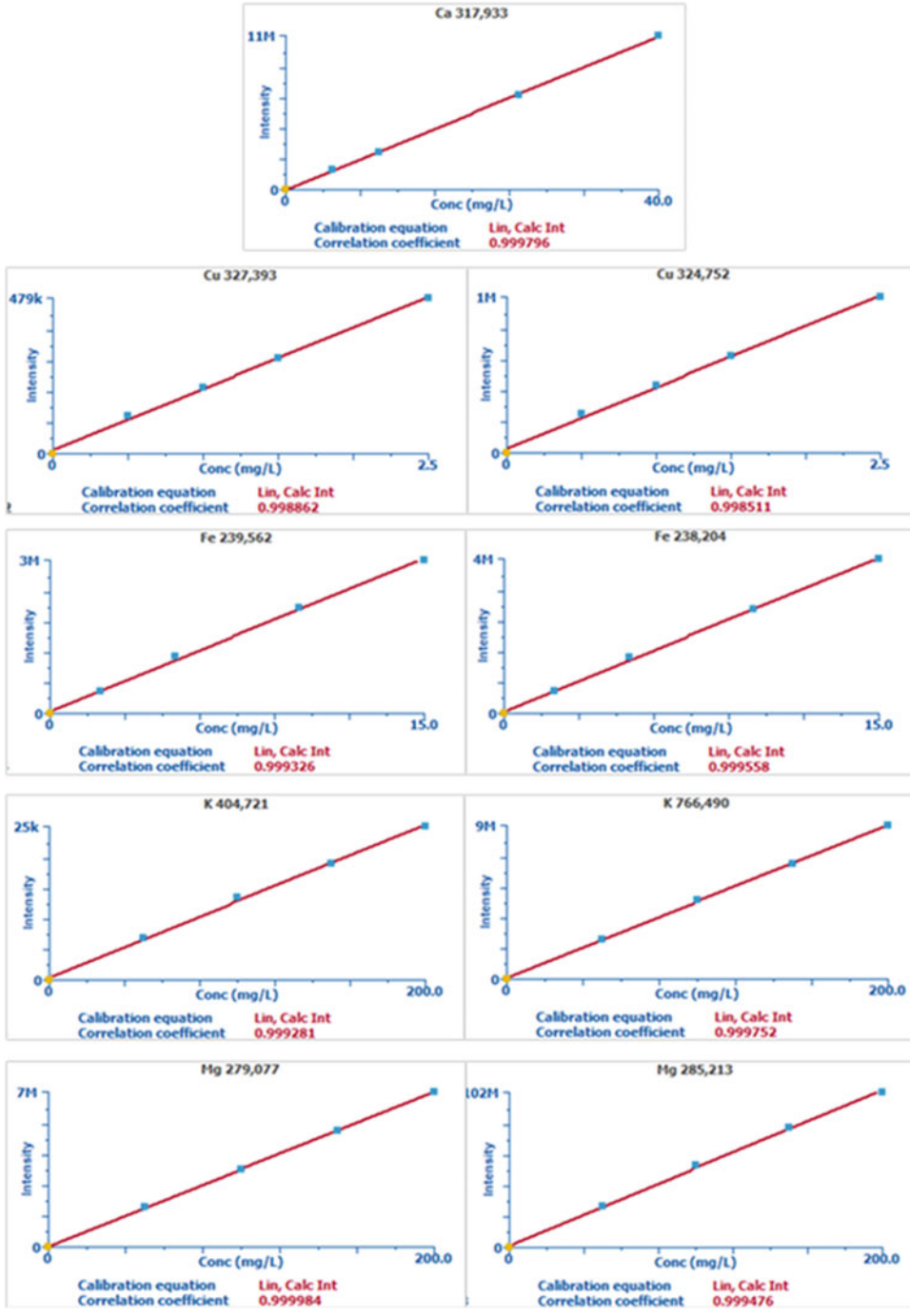

Fig. 2 (**a**) Calibration curves for Ca, Cu, Fe, and Mg at different operating wavelengths. (**b**) Calibration curves for Na, Zn, Mn, and P at different operating wavelengths

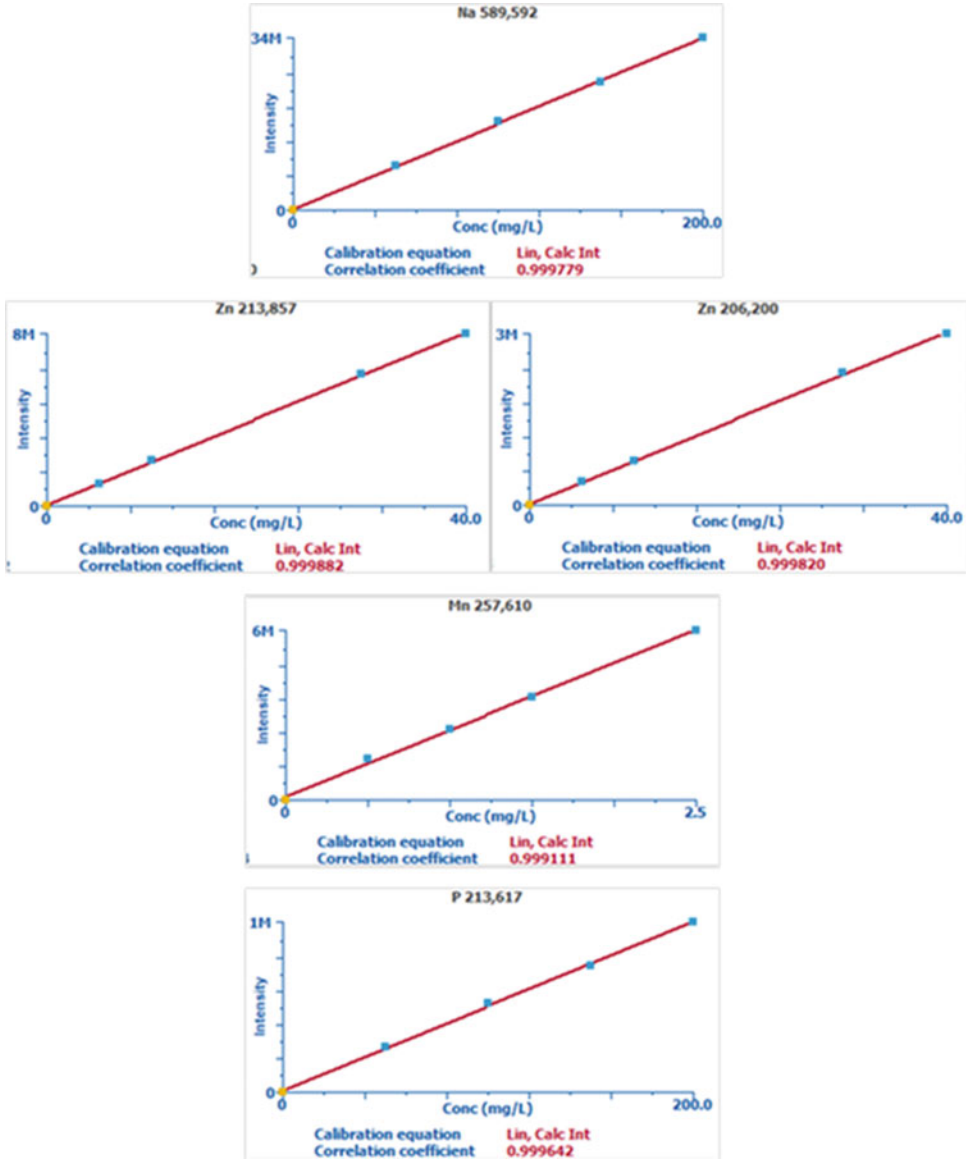

Fig. 2 (continued)

4 Notes

1. Due to the corrosive nature of the reagent used, it is necessary for the operator to take the necessary protective measures (plastic gloves) as well as to carry out all the operations in laboratory fume hoods.
2. An analytical balance with 0.1 mg resolution or higher should be used to weigh the porcelain crucibles.

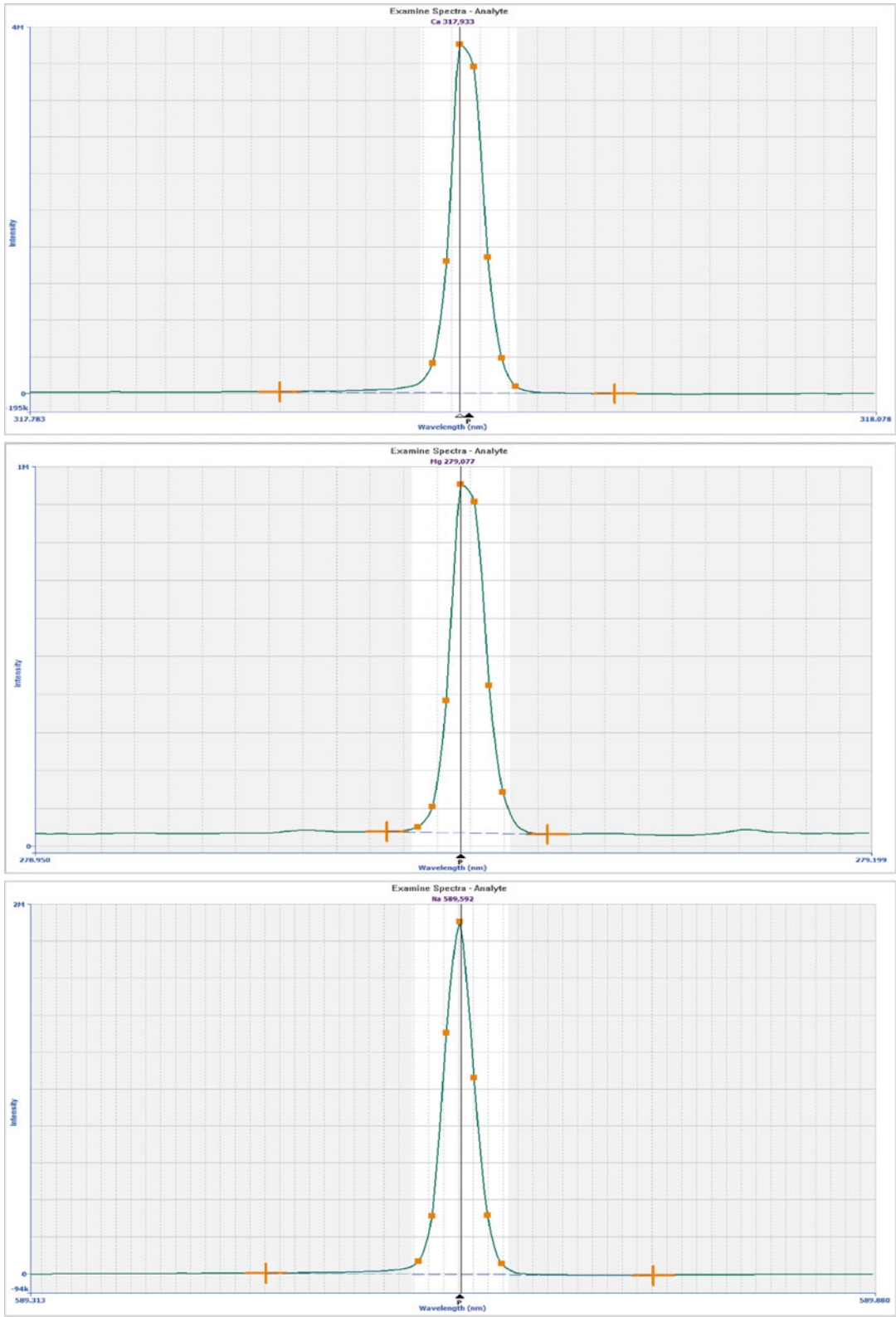

Fig. 3 Spectra for Ca, Mg, and Na at corresponding wavelength in a meat product sample

3. Due to the extremely small quantities (few μL) needed to prepare standard solutions for elements like Cu and Mn, the pipetting process must be scrupulously careful (Tables 1 and 2).
4. Stock solutions and standards prepared for calibrations can be stored refrigerated (4 °C).
5. Standard solutions and samples must be vortexed before measurement.
6. Standards should be injected first, for identification and to create calibration curves for each element at the corresponding wavelength (Fig. 2a, b).
7. It must be verified that there are no interferences that compromise the accuracy of the analytical result. Interferences arise due to differences in composition of the analyzed sample and the external standards and blanks used for calibration.
8. Calibration should be performed every day and/or when any change is made in the conditions of the equipment.

Acknowledgments

This work was supported by Centro Tecnolóxico de la Carne de Galicia. Thanks to GAIN (Axencia Galega de Innovación) for supporting this book chapter (grant number IN607A2019/01). Authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568).

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Chapter 8

Nitrate and Nitrite

Elisa Rafaela Bonadio Bellucci, Camila Vespúcio Bis Souza, José Manuel Lorenzo, Gonzalo Aleu, Alfredo Teixeira, Rubén Domínguez, and Andrea Carla da Silva-Barretto

Abstract

The determination of the residual content of sodium nitrite and sodium nitrate in meat products is related to food safety, since the excessive consumption of these additives has been associated with the increase in the risk of certain types of cancer. However, the use of these additives in the meat industry is vital, since they play an important role in the controlling of pathogenic microorganisms, reduce oxidative reactions and improve color characteristics of meat products. Moreover, due to the health implications, specific limits (residual amounts) of nitrite and nitrate were stablished for each type of meat products. Thus, in view of the importance of this determination, the use of an appropriate analytic technique in meat and meat product is very important.

Consequently, this chapter gives a comprehensive vision of the procedure for the determination of residual nitrate and nitrite in cured meat products. All stages are described in detail so that the conditions proposed by us can be reproduced by other researchers.

Key words Residual nitrite, Residual nitrate, Colorimetric analyses, Cured meat products

1 Introduction

Sodium nitrite and nitrate are ordinarily added in cured meat products because of their antimicrobial activity [1] due to inhibition of the germination of the spore of *Clostridium botulinum* and resisting the growth of *Listeria monocytogenes* [2], antioxidant activity over reduction in the rancidity of the meat product [3], and the development of the reddish-pink color and the flavor characteristics of cured meat products [4]. Despite their technological benefits, sodium nitrite and nitrate present a high risk to the health of the consumers, especially when consumed in excess. Some authors have already connected these components with the increase in the risk of certain types of cancer [5–7] since sodium nitrite may be the precursor of carcinogenic components such as nitrosamines

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_8,

[8]. Its application has been concerning the meat industry and consumers for years [9]. With all this in mind, there is a threshold limit set by the governments and health institutions for nitrite intake. Therefore, the level of nitrite present in the meat products needs to be tested to ensure food safety and protect the consumers from potential health problems [10].

The intake of nitrates and nitrites is predominantly due to the consumption of vegetables and meat products. In vegetables, the amount of nitrate present depends on the type of vegetable, whether it is the root, leaves or fruits, agricultural practices, genetic factors and, mainly, the way it is consumed (raw, cooked, or fried). In a study with the Italian population, it was concluded that vegetables contribute more to the intake of nitrate than meat products through the diet [7]. However, the use of both, nitrate and nitrite are also widely used in the meat industry. Nitrate (NO_3^-) is used in meat products that will be stored for a long period of time as a source of nitrite for curing reactions. Although nitrate has the same healing function as nitrite, it reacts more slowly and is, therefore, less used. In addition, the conversion of nitrate to nitrite requires an additional step that depends on the action of microorganisms such as starter bacteria with nitrate-reducing properties which use nitrate as a substrate for anaerobic respiration [11].

On the other hand, the formation of the characteristic color of cured meat products is obtained through specific reactions that occur in the curing process. In this regard, the nitrosylation reaction (Fig. 1) occurs when the nitric oxide formed from the nitrite reacts with the myoglobin, forming an unstable compound called nitrosomyoglobin (MbNO), bright red in color, which becomes a stable and colored compound pink (nitrous-hemochrome) after

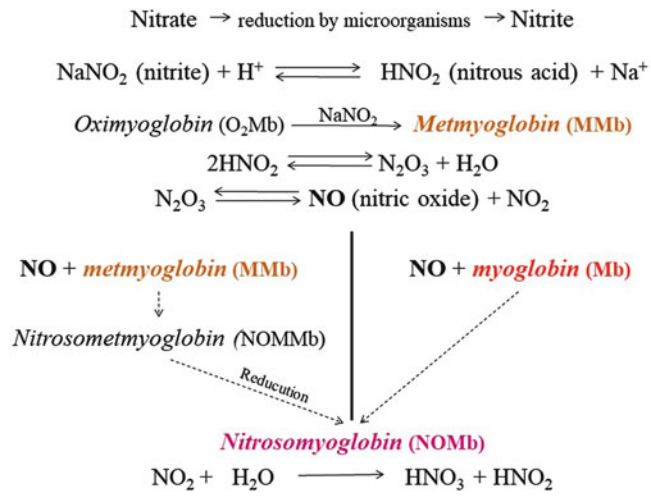

Fig. 1 Sequence of the nitrosylation reaction in cured products. Adapted from Ramos and Gomide [12]

heating [13]. However, it is difficult to control the amount of nitrite during the meat product processing due to the nature of the conditions of the manufacturing such as the heating that might lead to the conversion of nitrate into nitrite or nitrosamines. Thus, the control process is most appropriate for measuring the residual values in meat products [14].

With all the aforementioned, the present book chapter aims to fully and comprehensively describe a procedure for determining residual nitrite and nitrate in meat and meat products.

2 Materials

Prepare all solutions using distilled water and/or analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Residual Nitrite Determination

1. Sodium tetraborate solution decahydrate 5%: Dissolve 5 g of sodium tetraborate in 100 mL of distilled water.
2. Potassium ferrocyanide solution 15%: Dissolve 150 g of Potassium ferrocyanide in 1 L of distilled water.
3. Zinc acetate solution heptahydrate 30%: Dissolve 300 g of Zinc acetate heptahydrate in 1 L of distilled water.
4. Sulfanilamide (0.5%): Dissolve 1.25 g of Sulfanilamide ($C_6H_8N_2O_2S$) in 250 mL of hydrochloric acid solution (*see* **Notes 1** and **2**).
5. N-(1-naphtil)-ethylenediamine dihydrochloride (NED): Dissolve 0.5 g of alpha-naphthyl-ethylenediamine chloride in 100 mL of distilled water (*see* **Note 3**).
6. Standard solution of sodium nitrite ($NaNO_2$) (0.2 g/L): Weigh analytically 0.2 g of sodium nitrite, previously dried for 1 hour at 105 °C. Dissolve in 1 L of distilled water (*see* **Note 4**).
7. Standard work solution of sodium nitrite (8 μg/mL): Pipette 10 mL of the standard solution of sodium nitrite (0.2 g/L) in 250 mL of distilled water. Vortex the mixture.

2.2 Residual Nitrate Determination

1. Sodium tetraborate solution decahydrate 5%: Dissolve 5 g of sodium tetraborate in 100 mL of distilled water.
2. Potassium ferrocyanide solution 15%: Dissolve 150 g of Potassium ferrocyanide in 1 L of distilled water.
3. Zinc acetate solution heptahydrate 30%: Dissolve 300 g of Zinc acetate heptahydrate in 1 L of distilled water.
4. Sulfanilamide (0.5%): Dissolve 1.25 g of Sulfanilamide ($C_6H_8N_2O_2S$) in 250 mL of hydrochloric acid solution (*see* **Notes 1** and **2**).

5. N-(1-naphtil)-ethylenediamine dihydrochloride (NED): Dissolve 0.5 g of alpha-naphthyl-ethylenediamine chloride in 100 mL of distilled water (*see* **Note 3**).
6. Disodium ethylenediaminetetraacetate dehydrate (EDTA) 5% w/v: Dissolve 5 g of EDTA in water and complete the volume to 100 mL (*see* **Note 5**).
7. Buffer solution (pH: 9.6–9.7)—Dilute 20 mL of hydrochloric acid in 700 mL of water. Add 50 mL of ammonium hydroxide. Adjust the pH to 9.6–9.7 if necessary using HCl or ammonium hydroxide and complete the volume to 1 L.
8. Standard solution of sodium nitrate (1 g/L): Weigh analytically 0.1 g of sodium nitrate, previously dried for 1 h at 105 °C. Dissolve in distilled water and add 50 mL of buffer solution (pH: 9.6–9.7). Complete the volumetric flash to 100 mL with distilled water.
9. Standard work solution of sodium nitrate (10 μg/mL): Dilute 1 mL of standard solution of sodium nitrate in 100 mL volumetric flash. This work solution must be prepared in the analysis time.

3 Methods

3.1 Residual Nitrite Determination: Samples Reading

The nitrite determination through the colorimetric methodology involves the diazotization of nitrite with sulfanilic acid and coupling with N-(1-naphtil)-ethylenediamine dihydrochloride forming the pink colored alpha-naphthylamino-p-azobenzene-p-sulfonic [15]. Figure 2 shows in a simplified and schematic steps of the nitrite extraction.

1. Weigh 10 g of a mixed and homogenized sample in a 200 mL beaker and add 5 mL of sodium tetraborate solution decahydrate 5%. Mix with a glass stick.
2. Add 50 mL of hot distilled water (80 °C).
3. Place the samples in a hot water bath (60–70 °C) for 15 min. The samples must be mixed constantly (*see* **Note 6**).
4. Transfer the contents to a 200 mL volumetric flask with the aid of a funnel and a glass stick.
5. Wash the beaker with 50 mL of hot distilled water (80 °C) and add this content in the volumetric flask.
6. Let the samples cool to room temperature.
7. Add 5 mL of potassium ferrocyanide solution 15% and 5 mL of zinc acetate solution heptahydrate 30% (*see* **Note 7**).
8. Complete the volume of the volumetric flask with distilled water.

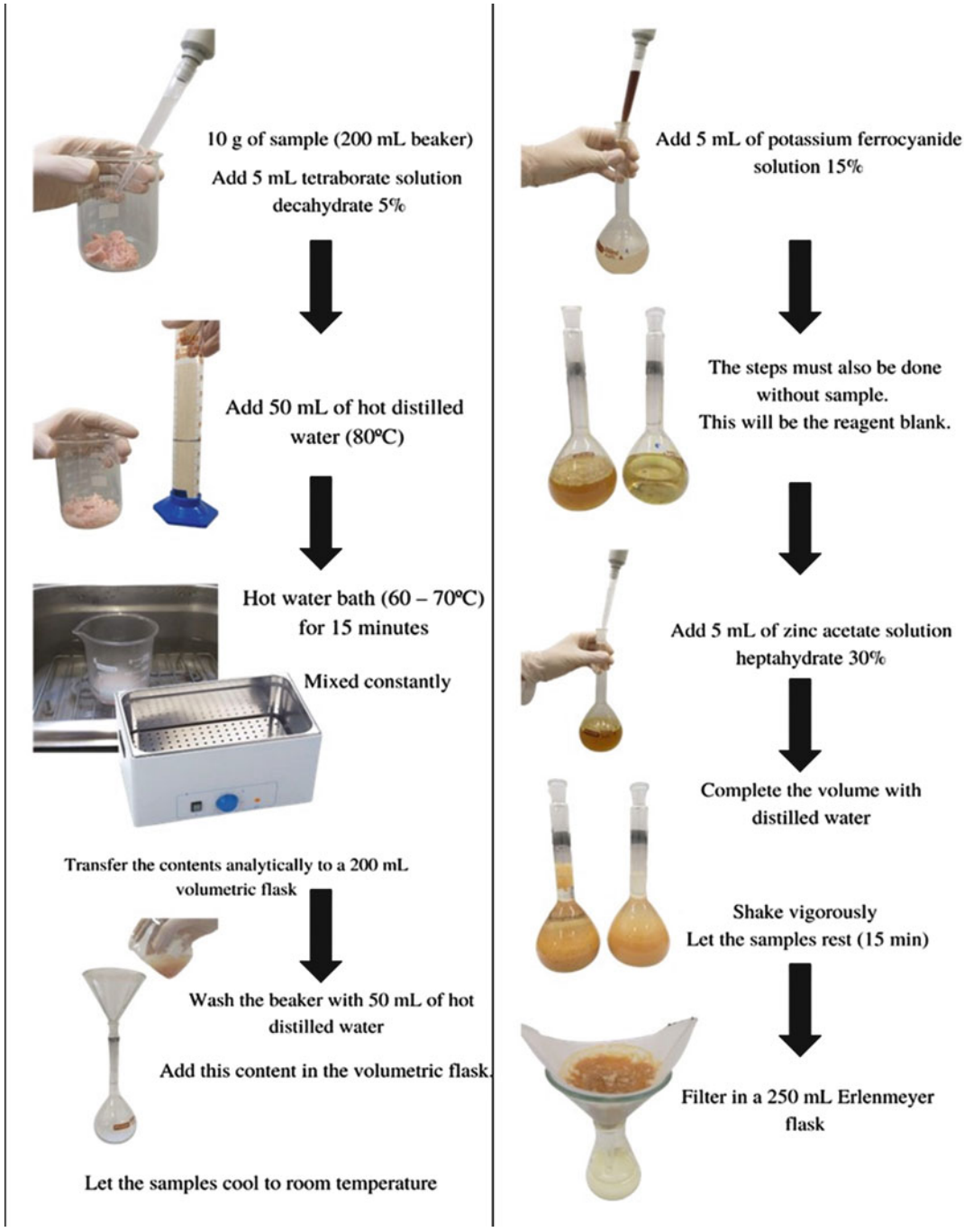

Fig. 2 Schematic steps of the nitrite extraction

9. Shake vigorously and let the samples rest for 15 min.
10. Filter on qualitative filter paper in a 250 mL Erlenmeyer flask (*see* **Note 8**).

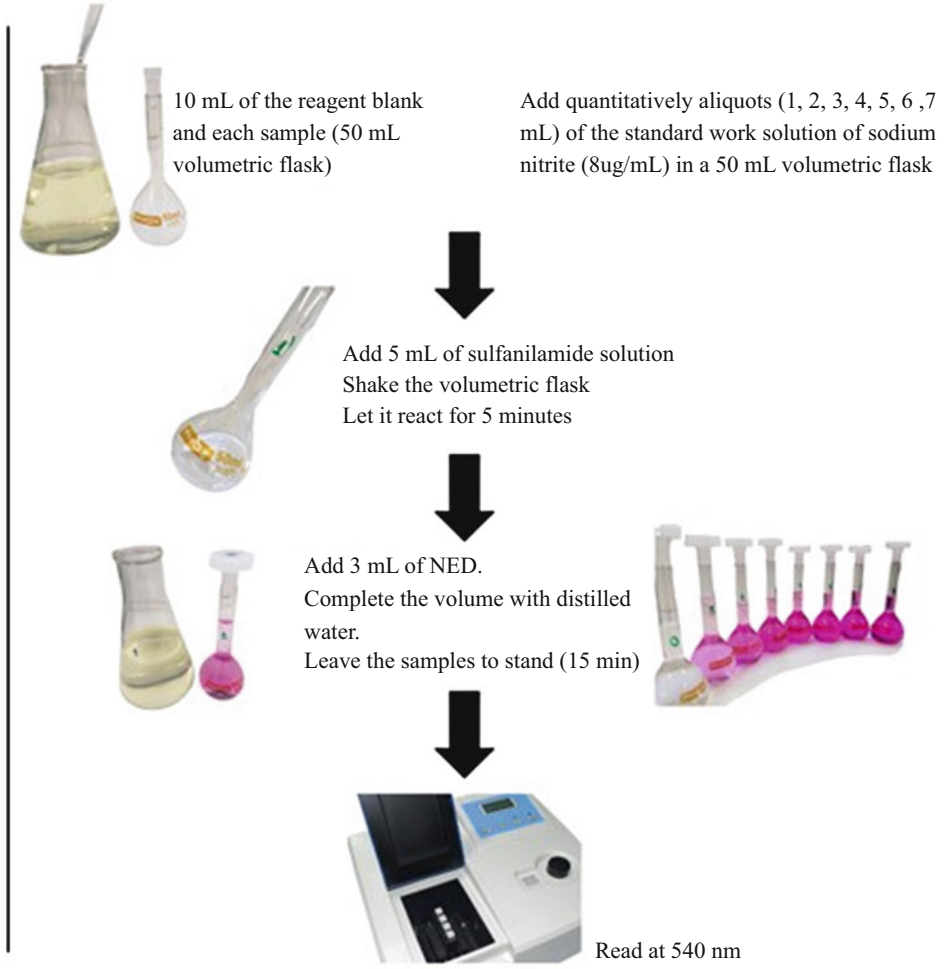

Fig. 3 Schematic steps for a reaction of sample and aliquots for standard curve with reagents to form a colored azo dye for residual nitrite determination

11. Add 10 mL of the reagent blank and each sample in a 50 mL volumetric flask. Figure 3 shows in a schematic way the steps for a reaction of sample with reagents to form a colored azo dye.
12. Add 5 mL of sulfanilamide solution. Shake the volumetric flask and let it react for 5 min.
13. Add 3 mL of NED. Shake each volumetric flask. Complete the volume (50 mL) with distilled water.
14. Leave the samples to stand for 15 min.
15. Read on a spectrophotometer at 540 nm of absorbance (*see* **Note 9**).

3.1.1 Residual Nitrite Determination: Analytical Curve

1. Add different aliquots (1, 2, 3, 4, 5, 6, 7 mL) of the standard work solution of sodium nitrite (8 μg/mL) in a 50 mL volumetric flask. Figure 4 shows an example of analytical curve for nitrite determination.
2. Add 5 mL of sulfanilamide solution. Shake the volumetric flask and let it react for 5 min.
3. Add 3 mL of NED. Shake each volumetric flask. Complete the volume (50 mL) with distilled water.
4. Leave the samples to stand for 15 min.
5. Read on a spectrophotometer at 540 nm of absorbance (*see* **Note 9**).
6. Construct the curve with the absorbance values on the *y*-axis and sodium nitrite concentration on the *x*-axis (*see* **Note 10**) (Fig. 4).
7. Calculate the linear and angular coefficient of the line (Fig. 4) (*see* **Note 11**).
8. Use Eq. 1 to quantify the sample's residual nitrite value.

$$\text{Residual sodium nitrite}\left(\frac{\text{mg}}{\text{kg}}\right) = \frac{(\text{ABS} - b) \times 1000}{p \times a} \qquad (1)$$

Being that:ABS: Sample absorbance, *b*: linear coefficient of the line obtained in the standard curve, 1000: dilution factor, *p*: sample mass in grams, *a*: absorptivity (Angular coefficient of the line obtained in the standard curve).

3.2 Nitrate Determination

For nitrate analysis, nitrate is reduced to nitrite through a cadmium column in an alkaline medium and nitrite amounts is determinate as described in Subheadings 3.1 and 3.2. Another sample (aliquot) is used to determine residual nitrite without the reducing step. The difference between the amount of residual nitrite of sample reduced of nitrate (through the passage in cadmium column) and the residual nitrite is the commonly method to quantify nitrate in cured meat products samples [14].

1. Weigh 10 g of a mixed and homogenized sample in a 200 mL beaker and add 5 mL of sodium tetraborate solution decahydrate 5%. Mix with a glass stick.
2. Add 50 mL of hot distilled water (80 °C).
3. Place the samples in a hot water bath (60–70 °C) for 15 min. The samples must be mixed constantly (*see* **Note 6**).
4. Transfer the contents to a 200 mL volumetric flask with the aid of a funnel and a glass stick.
5. Wash the beaker with 50 mL of hot distilled water (80 °C) and add this content in the volumetric flask.

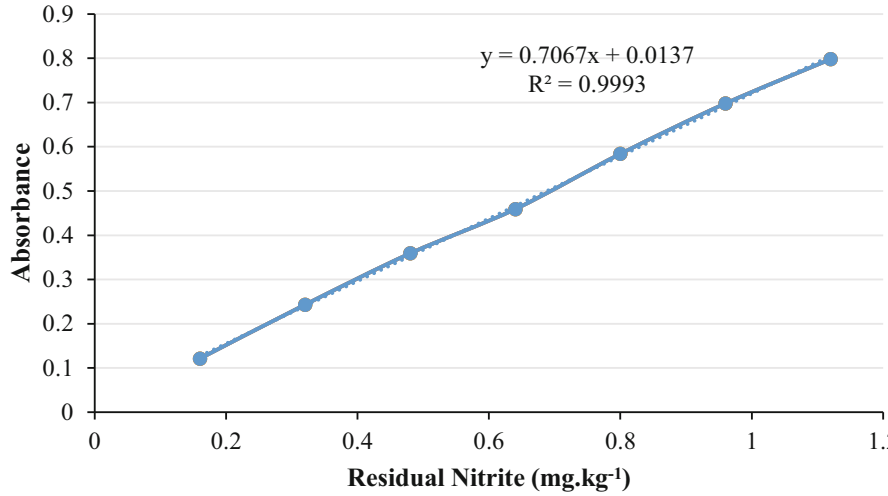

Fig. 4 Example of a calibration curve (absorbance "*y*-axis" vs. sodium nitrite concentration "*x*-axis")

6. Let the samples cool to room temperature.
7. Add 5 mL of potassium ferrocyanide solution 15% and 5 mL of zinc acetate solution heptahydrate 30% (*see* **Note 7**).
8. Complete the volume of the volumetric flask with distilled water.
9. Shake vigorously and let the samples rest for 15 min.
10. Filter on qualitative filter paper in a 250 mL Erlenmeyer flask (*see* **Note 8**).
11. Transfer 20 mL of the filtrate to a 100 mL beaker (first blank, then sample aliquots).
12. Add 5 mL of the buffer solution (*see* **Note 12**).
13. Pass through the cadmium column and collect in a 100 mL volumetric flask (*see* **Notes 13** and **14**).
14. Pass water through the column until completing 100 mL in the volumetric flask.
15. Add 10 mL of the reagent blank and each sample in a 50 mL volumetric flask. Figure 4 shows in a schematic way the steps for a reaction of sample with reagents to form a colored azo dye.
16. Add 5 mL of sulfanilamide solution. Shake the volumetric flask and let it react for 5 min.
17. Add 3 mL of NED. Shake each volumetric flask. Complete the volume (50 mL) with distilled water.

18. Leave the samples to stand for 15 min.
19. Read on a spectrophotometer at 540 nm of absorbance (*see* **Note 9**).

3.2.1 Nitrate Determination: Analytical Curve

At first, the efficiency of cadmium column must be tested by passing the standard solution of sodium nitrate in the cadmium column to determinate the amount of nitrite formed.

Cadmium Column Efficiency

1. In a 100 mL beaker, add 20 mL of standard work solution of sodium nitrate (10 μg/mL) and 5 mL of buffer solution (pH: 9.6–9.7).
2. Pass through the cadmium column (*see* **Notes 13** and **14**) and collect in a volumetric flash of 100 mL.
3. Pass water through the column until completing 100 mL in the volumetric flask.
4. Add 10 mL of the reagent blank and each sample in a 50 mL volumetric flask.
5. Add 5 mL of sulfanilamide solution. Shake the volumetric flask and let it react for 5 min.
6. Add 3 mL of NED. Shake each volumetric flask. Complete the volume (50 mL) with distilled water.
7. Leave the samples to stand for 15 min.
8. Read on a spectrophotometer at 540 nm of absorbance. Reset the spectrophotometer with the blank of reagents (passed through the column) (*see* **Note 15**).
9. Use Eq. 2 to calculate the efficiency (%):

$$\text{Efficiency } (\%) = \frac{(\text{ABS} - b) \times 30.8}{p \times a} \tag{2}$$

Being that: ABS: Standard sodium nitrate absorbance, b: linear coefficient of the sodium nitrite standard curve, p: sample mass in grams, a: absorptivity (Angular coefficient of the sodium nitrite standard curve), 30.8: factor (dilution and conversion of sodium nitrate/sodium nitrite).

3.2.2 Calculation of Amount of Sodium Nitrate

Construct the analytical curve using sodium nitrite as standard construct the graphic with the absorbance values on the y-axis and sodium nitrite concentration on the x-axis and calculate the linear and angular coefficient of the line for residual nitrite as described in Subheading 3.1.1.

Use Eqs. 1, 3, and 4 to quantify the amount of nitrate in the aliquots (*see* **Note 10**).

$$\text{Total sodium nitrite}\left(\frac{\text{mg}}{\text{kg}}\right) = \frac{(\text{ABS} - b) \times 5000}{p \times a} \tag{3}$$

$$\text{Sodium nitrate}\left(\frac{\text{mg}}{\text{kg}}\right) = (\text{Eq.3} - \text{Eq.1}) \times 1.231 \qquad (4)$$

Being that: ABS: Sample absorbance, *b*: linear coefficient of the line obtained in the standard curve, *p*: sample mass in grams, *a*: absorptivity (Angular coefficient of the line obtained in the standard curve), 1000: dilution factor, 5000: dilution factor of sample to calculation of total sodium nitrite, 1.231: correction factor from nitrite to nitrate.

4 Notes

1. Dilute 125 mL of hydrochloric acid PA in 125 mL of distilled water.
2. This solution can be made weekly and stored under refrigeration in amber glass.
3. Store in amber glass under refrigeration.
4. This standard stock solution is stable for 2 weeks in amber glass at 4 °C.
5. EDTA solution must be stored in polyethylene bottles.
6. Make a sample blank, using the same reagents and conditions but without adding the meat sample or meat product. This will be the reagent blank.
7. Shake by rotation after adding each reagent.
8. The mixture to be filtered can clog the filters and become slow, so the filter can be changed periodically until the end of the filtration or use a vacuum pump.
9. Use the reagent blank to reset the equipment.
10. Sodium nitrite concentration: 0.16; 0.32; 0.48; 0.64; 0.80; 0.92; and 1.12 μg/mL of sodium nitrite.
11. The quantification limit of residual sodium nitrite for this analysis is 0.032 μg/mL in the analysis pliers, with deviations of 3.7%.
12. If after adding the buffer solution (pH: 9.6–9.7) the solution becomes cloudy, 2 mL of the 5% EDTA solution should be added.
13. The flow in the cadmium column should not exceed 6 mL/min.
14. Wash the walls of the cadmium column at least 5 times with approximately 15 mL of distilled water.
15. If the recovery is less than 90%, regenerate the column.

Acknowledgments

This work was supported by Department of Food Technology and Engineering. Authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568).

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Chapter 9

Biogenic Amines

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Abstract

The control and determination of biogenic amines in different kinds of foods in particular in meat products is important to assure their safety and high level of quality. Having precise analysis techniques, with few limitations and easy to perform and reproduce is a challenge for analysts, researchers, control laboratories, and companies.

This chapter includes a detailed protocol for a chromatographic simultaneous determination of the most common biogenic amines (BA) (tyramine, β-phenylethylamine, histamine, putrescine, cadaverine, tryptamine, agmatine, spermidine, and spermine) that can be found in meat and meat products. This protocol description includes both the acid samples extraction step of the BA and their determination by liquid chromatography which employs a cation-exchange column for BA separation and with a post-column system for the derivatization process using o-phthalaldehyde as a derivatizing reagent.

This method showed an appropriate, precise, fast, and versatile procedure to determine nine BA simultaneously in different matrices of meat products.

Key words Biogenic amines, HPLC post-column, Ion chromatography, OPA, Meat and meat products

1 Introduction

Biogenic amines (BA) are biologically active low-molecular-weight basic nitrogenous compounds which are present in great majority of foods (fish, meat, cheese, vegetables, soy bean, etc.) [1–3]. Their control in foods is important for safety and quality reasons. The consumption of food containing high concentrations of biogenic amines has been associated with toxic effects and constitutes a potential risk for the human health ("scombroid poisoning" or "histamine poisoning," "tyramine poisoning" or "cheese poisoning," etc.) [3–6]. Besides, BA have been employed as quality indices in food, mainly in meat and fish. They allowed to assess the success of the applied technological process and shelf life of the products during conservation [3, 7–10].

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_9,

Biogenic amines are produced by decarboxylation of free amino acids from the action of microbial amino acid decarboxylase enzymes [3]. Its greater or lesser presence in food depends on various factors associated with food composition, processing, and storage conditions that can affect the formation of these BA (free amino acids, microorganisms, medium, processing and preservation conditions, etc.) [3, 8, 11].

Therefore, controlling, detecting, and quantifying these biogenic amines in the different kinds of foods is important to assure a high level of quality and food safety [3, 7, 8]. In this regard, it is worth mentioning that there are specific legislations designed in order to control these compounds [12–14].

Numerous procedures have been developed for the determination of BA in various types of foods. Methods range from the determination of individual amines to the joint simultaneous determination of various amines present in food [1, 3, 7, 15, 16]. Many methods can be found in the literature, from traditional ones, such as colorimetric and fluorometric methods focused mainly on determining histamine individually, to modern ones, such as gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), HPLC-tandem mass spectrometry, flow injection analysis (FIA), capillary electrophoresis, etc., that allow the simultaneous determination of several biogenic amines in the same food [3, 16]. Of all the techniques/methods used for the determination of BA, nowadays HPLC is the most common [3]. The chromatographic determination by HPLC generally uses pre- and post-columns with reverse phase or ion exchange columns and with different derivatizing reagents (ninhydrin, dansyl chloride, ortho-phthalaldehyde (OPA), etc.) to increase sensitivity of the HPLC method, since BA are characterized by their low volatility and lack of chromophores. Depending on the derivatizing reagent, different detection systems are used: UV/Vis, diode array, this latter being the most used fluorescence detector [1, 3, 17, 18].

The determination of biogenic amines also requires a more or less complex extraction process which depends on the type of matrix, the technique, and the separation and identification of the BA. Indeed, many different solvents, such as hydrochloric acid, trichloroacetic acid, perchloric acid, methanol, and other organic solvents, have been used to extract BA from meat products.

Therefore, the methodology proposed in the present chapter for the determination of BA includes the extraction process, the chromatographic separation, the identification, and the quantification of nine BA simultaneously following the methodology performed by Triki et al. [16].

2 Materials

All solutions must be prepared with ultra-pure water and analytical grade reagents. Preparation of all reagents is performed at room temperature and storage is carried out at 4 °C.

2.1 Biogenic Amines Extraction

The determination of biogenic amines in solid foods frequently requires extraction or concentration procedures prior to separation and analysis. This is a critical stage for adequate recovery of all BA due to the complexity of the sample's matrices and the fact that they do not contain only one BA [3, 15].

2.2 Biogenic Amines Standard Solutions for Identification and Quantification

1. The trichloroacetic acid (TCA) was supplied by Panreac (Barcelona, Spain) (*see* **Note 1**).

   TCA 7.5%: Dissolve 75 g of TCA in 1 l of ultra-pure water (*see* **Note 2**).

   The biogenic amines included in these standard solutions are: tyramine hydrochloride (Tyr), histamine dihydrochloride (His), 2-phenylethylamine hydrochloride (Pea), putrescine dihydrochloride (Put), cadaverine dihydrochloride (Cad), tryptamine-crystalline (Try), agmatine sulfate salt (Agm), spermidine trihydrochloride (Spd), and spermine tetrahydrochloride (Spm). They were purchased from Sigma-Aldrich (Spain).
2. A BA stock solution of 1000 mg/l was prepared, as a free base with TCA 7.5% in a volumetric flask:
   (a) A stock solution of 1000 mg/l of each separate BA was prepared in a volumetric flask (*see* **Note 3**).
   (b) Afterwards, a 1000 mg/l stock solution of all biogenic amines was prepared from the previous separate stock solutions in a volumetric flask and transferred to 5 ml glass tubes. Then, from the glass tubes, the solution was transferred again to vials (*see* **Note 3**).
3. BA intermediate solution of 100 mg/l was prepared with TCA 7.5% from the stock solution (1000 mg/l):
   (a) 2.5 ml of the BA stock solution were poured in a 25 ml volumetric flask and the rest was filled with 7.5% TCA until reaching the mark of the flask (*see* **Note 3**).
4. BA working solutions (Standard solutions):
   (a) From the BA intermediate solution, appropriate working solutions from 0.05–12 mg/l of mixed BAs with TCA 7.5% were prepared to be used for the standards calibration. For example, the 4 mg/l working solution was prepared in a 10 ml volumetric flask where 0.4 ml of the

intermediate solution were poured and the rest was filled with TCA 7.5% (*see* **Note 3**).

(b) The working solutions were also filtered through a 0.22 μm Nylon Syringe filter (Teknokroma, Barcelona, Spain) into 2 ml amber vials with screw caps (PTFE/silicone) (Perkin Elmer Life and Analytical Sciences, USA) and kept in refrigeration until their use. They will be placed in the auto-sampler which will take a certain volume that will be injected into a high-performance liquid chromatograph (HPLC).

2.3 Mobile Phases

1. The trichloroacetic acid (TCA) was supplied by Panreac (Barcelona, Spain) and the ultra-pure water was obtained from Milli-Q system (Millipore, France).
2. Methanol and 2-propanol for high-performance liquid chromatography, potassium phosphate dibasic, potassium hydroxide, potassium chloride, acetic acid, and Brij 35 solution (30% w/v) were obtained from Sigma-Aldrich (Spain).
3. O-phthalaldehyde (OPA, ref. O120), Thiofluor Chromatographic Grade (N,N-Dimethyl-2mercaptoethylamine-hydrochloride), OPA diluent (ref OD104: 3% potassium hydroxide, 3% boric acid, 94% water, pH = 10.40), and for the mobile phases, potassium phosphate phase A buffer (ref K600: 11% 2-propanol, 0.9% potassium phosphate dibasic, 0.3% acetic acid, 87.8% water, pH = 6.00), phase B (ref K563: 5% potassium chloride, 4% 2-propanol, 0.9% potassium phosphate dibasic, 0.3% acetic acid, 89.8% water, pH = 5.63), and the potassium regenerating column, phase C (ref: K130: 0.7% potassium chloride, 4% 2-propanol, 0.5% potassium hydroxide, 94.8% water, pH = 13.00) were all purchased from Pickering laboratories (CA, USA) (*see* **Note 4**).

2.4 Derivatizing Reagent OPA Preparation

1. The post-column derivatizing reagent OPA was prepared with 975 ml of OPA solution (ref OD 104) and 0.100 mg of OPA (OPA, ref. O120) dissolved in 10 ml methanol, 2 g Thiofluor, and 3 ml of Brij 35 solution, which were purchased from Sigma-Aldrich (Spain) (*see* **Note 5**).
   (a) A part from OPA solution (ref OD 104) (approx. 800 ml) was poured in an opaque bottle (OPA bottle) which was degassed with Helium for 5 min (*see* **Note 6**).
   (b) 0.100 mg of OPA (OPA, ref. O120) were weighted in a beaker covered with aluminum paper (to avoid contact with light).
   (c) 10 ml of methanol were added to the beaker and shaken until the OPA (OPA, ref. O120) dissolved completely.

(d) The obtained solution was then added to the degassed OPA bottle.

(e) The beaker was cleaned with the rest of the OPA solution (ref OD 104) (that was not poured in the OPA bottle).

(f) 2 g of Thiofluor were weighted and dissolved in the rest of the OPA solution (ref OD 104) (which was used to clean the beaker).

(g) The obtained solution was added to the degassed OPA bottle (*see* **Note 7**).

(h) 3 ml of Brij 35 solution were added to the degassed OPA bottle (*see* **Note 7**).

(i) The whole content in the degassed OPA bottle was thoroughly mixed (*see* **Note 8**).

(j) The whole solution was then degassed again (*see* **Note 6**).

2.5 High-Performance Liquid Chromatography Equipment (HPLC)

The chromatographic determination of BAs was performed using liquid chromatography consisting of: a quaternary pump (series 200, Perkin Elmer, SL Spain), an auto-sampler (series 200, Perkin Elmer Life and Analytical Sciences, USA), a Pickering PCX 3100 post-column system (Pickering Laboratories, CA, USA) containing a cation-exchange column (K^+, 4 mm × 150 mm) with a 10 μm particle diameter, and a pre-column (K^+, 3 mm × 20 mm) also with a 10 μm diameter particle (Pickering Laboratories, CA, USA). The whole system was degassed and pressurized with helium (*see* **Note 9**).

3 Methods

3.1 Biogenic Amines Extraction

The extraction of BA from meat and meat products samples was performed following the steps mentioned hereafter:

1. 15 g of the sample were weighted in an omnimixer glass (Ovni International, Waterbury, CT, USA) and 30 ml of TCA (7.5%) were then added.
2. The mixture was blended in the omnimixer at 20,000 rpm during 3 min.
3. Afterwards, the obtained blend was centrifuged at 5000 × *g* for 15 min at 4 °C in a desktop centrifuge (Sorvall RTB6000B, DuPont, USA).
4. The obtained supernatant was then filtered through a Whatman n° 1 filter.
5. The filtrate was poured in a 50 ml volumetric flask which was filled with TCA (7.5%).

6. The filtrate was also passed through a 0.22 μ Nylon filter (Millipore, Ireland) and then placed in opaque vials (2 ml) in the auto-sampler which were stored at 2 °C, until their use (within the next 24 h).

3.2 Biogenic Amines Identification and Quantification (HPLC)

BA Separation and quantification are carried out using a HPLC, according to the following conditions:

1. BA were separated using an elution gradient programmed with potassium reagents K600, K563, and K130 following the method used by Ruiz-Capillas and Moral [7] and Triki et al. [16]. With the exception of **step 2**, where an isocratic gradient curve was applied (curve N° 1), the rest of the program was based on a linear gradient (without curve) (Table 1) (*see* **Note 10**).
2. The column and pre-column temperatures were programmed at 40 °C. In the reaction chamber, the post-column reagent (OPA) flow rate was 0.3 ml/min.
3. The applied temperature of the reaction chamber was 45 °C.
4. Detection was performed using an LC 240 fluorescence detector (Perkin Elmer Life and Analytical Sciences, USA) at 330 nm excitation and 465 nm emission lengths.
5. All the chromatographic systems were controlled using a PE Nelson data integrator (Perkin Elmer Life and Analytical Sciences, USA). Data acquisition was carried out using Total-Chrom software (Perkin Elmer Life and Analytical Sciences, USA).
6. BA standards working solution were placed in the injector together with the samples for the BA identification and to create calibration curves (Figs. 1 and 2). BA identification and quantification was done by comparing and extrapolating retention times with a calibration curve performed with different standard solutions (Fig. 3). The results were expressed as mg/kg of sample (*see* **Note 11**).

4 Notes

1. Since the trichloroacetic acid (TCA) is very hygroscopic, once it is opened, it must be very well covered and stored in a dry place.
2. TCA must be handled carefully; it is extremely corrosive and harmful to the environment. During the handling, the operator must wear goggles and protective equipment. The preparation of the TCA 7.5% must be performed in the laboratory hood.

Table 1
Elution gradient program for the chromatographic separation of biogenic amines in meat and meat products

Step	Time (min)	Flow (ml/min)	Mobile phases			Gradient curve
			K 600 (%)	K 563 (%)	K 130 (%)	
0	0	0.8	100	0	0	0
1	6	0.8	100	0	0	0
2	9	0.8	0	100	0	1
3	6	0.8	0	100	0	0
4	3	0.8	0	0	100	0
5	7	0.8	100	0	0	0

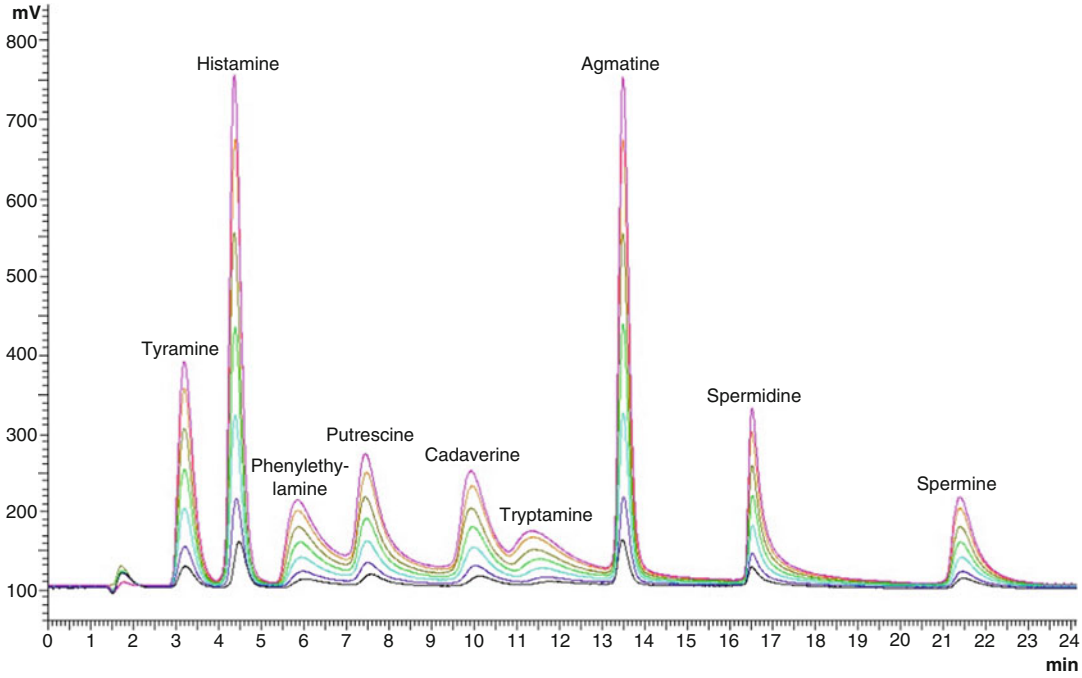
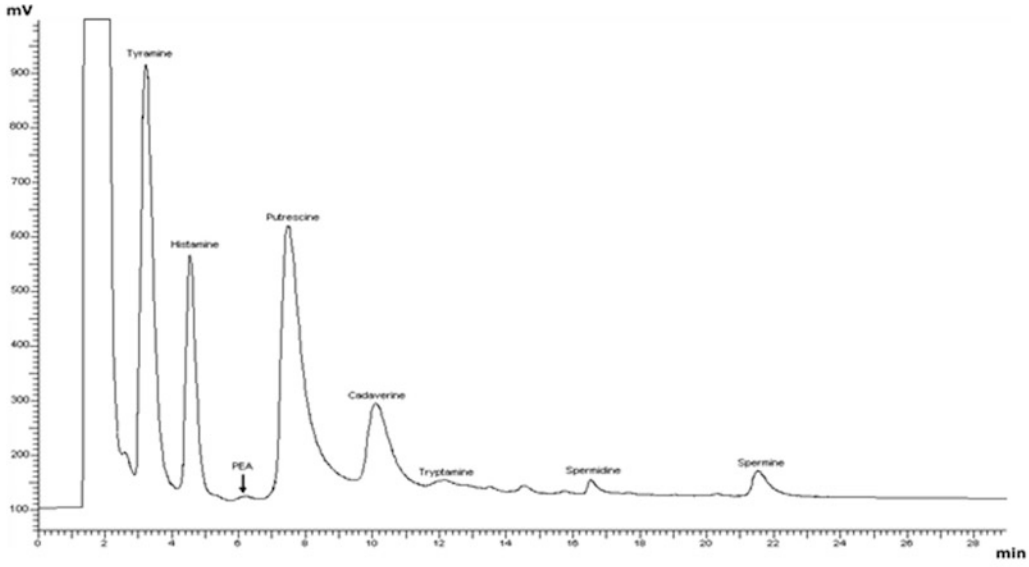


Fig. 1 Chromatograms of biogenic amines (tyramine, β-phenylethylamine, histamine, putrescine, cadaverine, tryptamine, agmatine, spermidine, and spermine) of different standard solutions (0.05–12 mg/l) for the preparation of the calibration line

3. Solutions must be well covered and stored in refrigeration until their later use.
4. Since in chromatographic separation with ion exchange columns, the pH of the mobile phases is a key factor in resolving the various peaks, the effects produced by pH variations related to the mobile phases were taken into account.

a) "Chorizo"



b) Frankfurter

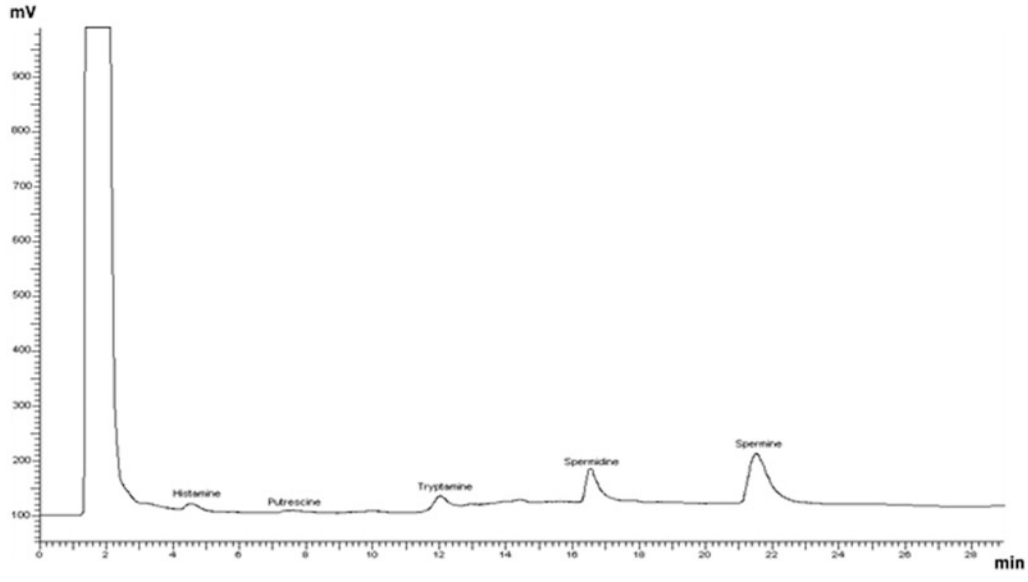


Fig. 2 Chromatograms of biogenic amines (tyramine, β-phenylethylamine, histamine, putrescine, cadaverine, tryptamine, agmatine, spermidine, and spermine) in "chorizo" (**a**) and Frankfurter (**b**)

5. This solution is prepared again weekly at the start of the working week if, in the meantime, it was not already finished.
6. The bottle must be opaque or covered with aluminum foil, avoid contact with light.

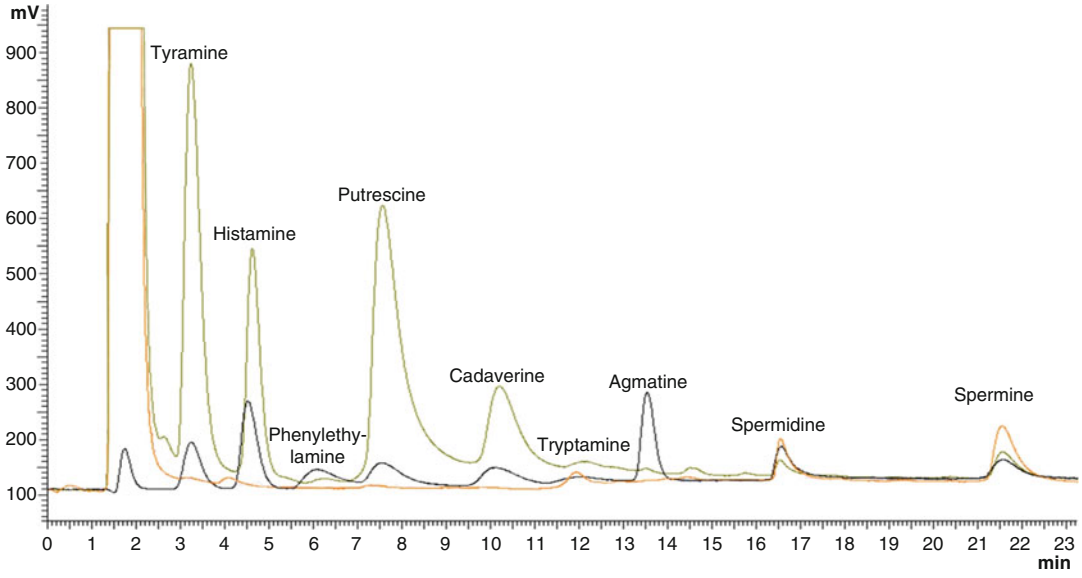


Fig. 3 Chromatograms of biogenic amines (tyramine, β-phenylethylamine, histamine, putrescine, cadaverine, tryptamine, agmatine, spermidine, and spermine) in meat products "Chorizo" (green), "Frankfurter" (orange), and standard solution 4 mg/l (black)

7. Prior to the addition of solutions, the gas of the system should be off.
8. Attention! This solution makes a lot of foam. It must be left for a while to stabilize.
9. Before starting the determination and once the phases have been degassed, the pump and the post-column system must be purged efficiently. The equipment must be left to stabilize for at least 10 min before starting with the injections.
10. It is very important to regenerate the column (K130) in order to obtain a stable balance for subsequent determinations.
11. The standard working solution should be injected every time before a series of samples injections to be more accurate when quantifying the BA in the samples since the column is very sensitive to slight changes in the conditions of the experiment. For example, the standard working solution can be injected again following a 5-h pause of injections or after performing injections non-stop for 10 h.

Acknowledgments

This research was funded by the Spanish Ministry of Science and Innovation (PID2019-107542RB-C21), by the CSIC Intramural projects (grant number 201470E073 and 202070E242), CYTED (grant number reference 119RT0568; Healthy Meat network).

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Chapter 10

Spectrophotometric Analysis of Protein Carbonyls

Francesca Soglia, Giulia Baldi, Alberto González-Mohino, Silvia Díaz-Velasco, Massimiliano Petracci, and Mario Estévez

Abstract

Oxidation of proteins is a major threat to their functionality and to the sensory properties and nutritional value of meat and meat products. Moreover, the intake of protein oxidation products may also involve a potential health risk for consumers. The accurate analysis of food protein oxidation seems to be an issue of scientific and technological relevance. Protein carbonylation is one of the most remarkable modifications in oxidized proteins and protein carbonyls are commonly used as markers of the oxidative damage to meat proteins. Regardless of its documented drawbacks, the dinitrophenylhydrazine (DNPH) method is the most widely used among food scientists for the quantification of protein carbonyls. Therefore, this chapter aims to provide a detailed description of the abovementioned determination which involves a simultaneous quantification of total protein carbonyls and total protein content in a given sample.

Key words Protein oxidation, Carbonyls, Hydrazones, 2,4-Dinitrophenylhydrazine, DNPH, Spectrophotometric method

1 Introduction

Proteins play a crucial role in foods from various perspectives including functionality (i.e., water absorption and retention, gelation, viscosity, emulsification, foam formation, etc.), sensory properties (i.e., texture and rheological properties, flavor-binding properties, enzymatic browning), and nutritional value (e.g., contribution with essential amino acids) [1]. Yet, a number of post-translational modifications in proteins affect their composition and native structure, leading to impairments in their functional properties and nutritional value.

Oxidation is a major threat to protein stability and functionality as the attack of reactive oxygen species (ROS) or reactive Maillard-dicarbonyls such as glyoxal and methylglyoxal, among others, leads to severe chemical changes in proteins, namely, peptide fragmentation, amino acid side chains oxidation, and formation of protein cross-links [2]. These chemical modifications occur all the way from

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_10,

food collection/harvesting until culinary treatment prior to consumption [3]. Yet, protein oxidation is particularly promoted during severe and recurring processing such as the combination of some of the following treatments: mincing, slicing, application of high temperatures, radiation, high-pressure, microwaves, packaging in high-oxygen atmospheres, etc. [1]. The consequences of severe protein oxidation are commonly negative as it is linked to increased toughness in meats, loss of essential amino acids and reduced protein digestibility, among others [4]. Additionally, recent reports emphasized the potential toxicity of oxidized proteins and amino acids and therefore, the intake of protein oxidation products may also involve a potential health risk [5].

In view of the aforementioned statements, the analysis of food protein oxidation seems to be an issue of scientific and technological relevance. The quantification of protein oxidation may be essential to identify the extent to which food proteins have suffered oxidative damage; the effectiveness of a given antioxidant strategy may be assessed by confirming its ability to reduce the amount of protein oxidation products [6]. Since protein oxidation is a complex phenomenon and is manifested as manifold chemical changes, an assortment of analytical procedures has been proposed to evaluate its occurrence in food systems [2]. Among all of them, the quantification of protein carbonyls is, without any doubt, the most common method to assess protein oxidation in biological samples. The occurrence of carbonyls in proteins is an unquestionable indication of an oxidative damage as the oxidative deamination of certain alkaline amino acids, such as lysine, arginine, and proline, leads to the formation of protein-bound carbonyls [7]. While several analytical procedures have been proposed to detect and quantify protein carbonyls, the spectrophotometric method that involves prior derivatization of the protein sample with dinitrophenylhydrazine (DNPH), is, again, the most widely used among food scientists (Fig. 1). The key to this procedure is to facilitate the exposure of protein-bound carbonyls to the DNPH and remove all exceeding reagent so that the numbers from quantification are as accurate as possible. To fulfill this objective, some cleaning steps are required to remove lipids and any other substrate and/or fluorophore that may interfere with the spectrophotometric measurement at 370 nm. The method has been criticized for being time-consuming, poorly accurate and intricate in the way samples have to be handled during the entire method, which facilitates a failed sampling from homogenates, loss of sample during washing steps or a failed derivatization. Even if the method is applied correctly, the nature and origin of the protein carbonyls remains unknown as these can derive from the oxidation of amino acid side chains (primary carbonyls) or from the oxidation of unsaturated lipids (i.e., malondialdehyde, MDA) which appear as protein-bound carbonyls upon reaction and covalent linkage with protein

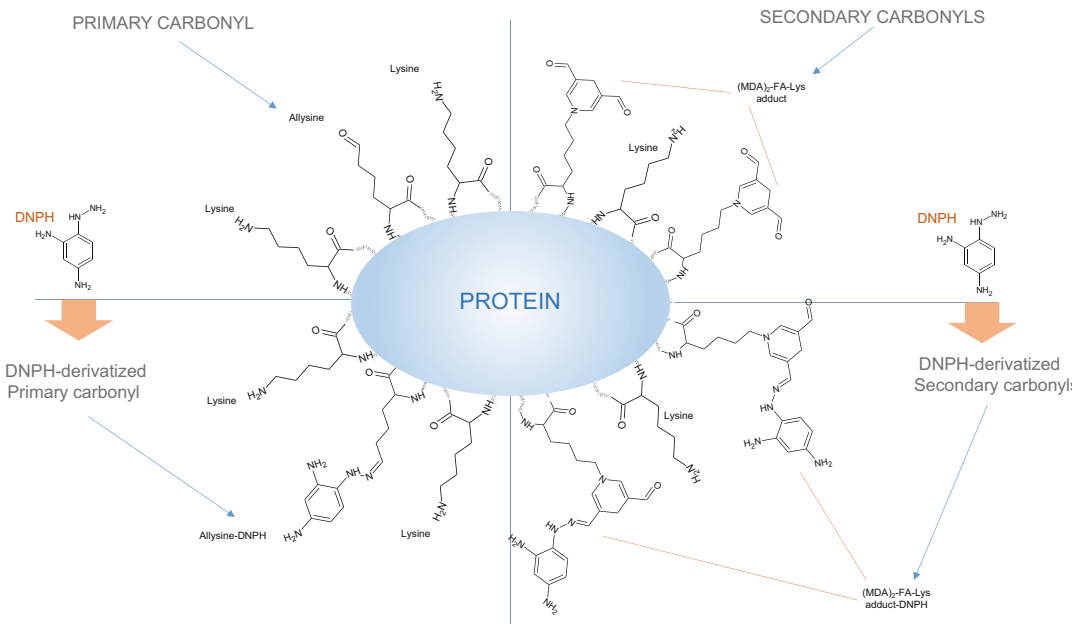

Fig. 1 Schematic representation of the DNPH-derivatization of primary (allysine) and secondary (protein-bound MDA) protein carbonyls (Adapted from Estévez et al. [8])

amines [8]. Figure 1 shows derivatization of DNPH with one primary carbonyl (allysine) and one secondary carbonyl (protein-bound MDA). The method, that involves a simultaneous quantification of total protein carbonyls and total protein content in a given sample, is described in the following sections.

2 Materials

Prepare all solutions using distilled water and/or analytical grade reagents. Prepare and store all the solutions and reagents at 4 ± 1 °C (unless indicated otherwise) with the only exception of Sodium Dodecyl Sulfate (SDS) 5% (w/v) which should be stored at room temperature to avoid crystallization. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Protein Extraction

1. KCl 0.15 M: Dissolve 11.18 g of potassium chloride (≥98%) in 1 L of distilled water (*see* **Note 1**).
2. TCA 10% (w/v): Dissolve 100.0 g of trichloroacetic acid (≥98%) in 1 L of distilled water (*see* **Note 2**).

2.2 Carbonyl Groups Exposure

1. SDS 5% (w/v): Dissolve 50 g of sodium dodecyl sulfate (≥98%) in 1 L of distilled water (*see* **Note 3**).

2.3 Derivatization

1. HCl 3 M: Mix 248 mL of hydrochloric acid (≥37%) with 752 mL of distilled water (*see* **Notes 2** and **4**).
2. DNPH 0.3% (w/v) in 3 M HCl: Dissolve 6.0 g of 2,4-Dinitrophenylhydrazine (~50%) in 1 L of HCl 3 M (*see* **Notes 2** and **5**).

2.4 Washing

1. TCA 40% (w/v): Dissolve 400.0 g of trichloroacetic acid (≥98%) in 1 L of distilled water (*see* **Note 2**).
2. Ethanol-ethyl acetate (1:1): Mix 500 mL ethanol with 500 mL ethyl acetate (*see* **Note 6**).
3. Guanidine hydrochloride 6 M in NaH_2PO_4 20 mM: Add 573.18 g of guanidine hydrochloride (≥99%) to 1 L of sodium phosphate buffer (*see* the following **item 4**) and adjust the final pH of the solution to 6.5 (*see* **Note 7**).
4. NaH_2PO_4 20 mM: Add 2.40 g of Sodium phosphate monobasic (≥98%) to 1 L of distilled water.

3 Methods

Carry out all procedures at room temperature, unless otherwise specified. Carbonyl content should be measured following the traditional spectrophotometric DNPH-based method described by Levine et al. [9] with the modifications proposed by Soglia et al. [10]. As aforementioned, the spectrophotometric quantification of protein carbonyls at 370 nm is made simultaneously to the spectrophotometric quantification of total protein content at 280 nm since results are commonly expressed as per protein unit. All the steps of protein extraction, carbonyl groups exposure and subsequent quantification are outlined in Fig. 2.

3.1 Protein Extraction

1. Weigh 1.0 g of sample in a 50 mL centrifuge tube and add 10 mL of ice-cold 0.15 M KCl solution (*see* **Notes 1** and **8**).
2. Homogenize the samples by Ultra-Turrax (IKA-WERKE, Labortechnik, Staufen, Germany) or similar apparatus at 9500 rpm for 30 s (*see* **Note 8**).
3. Per each sample, prepare five microcentrifuge tubes of 2 mL in which aliquots of homogenate (100 μL/each) are mixed with 1 mL 10% TCA (*see* **Notes 1** and **9**).
4. Centrifuge the samples at 5000 × *g* for 5 min at room temperature (*see* **Note 10**).

3.2 Carbonyl Groups Exposure

1. After removing and discarding the supernatant, add 400 μL of 5% SDS to the pellet.

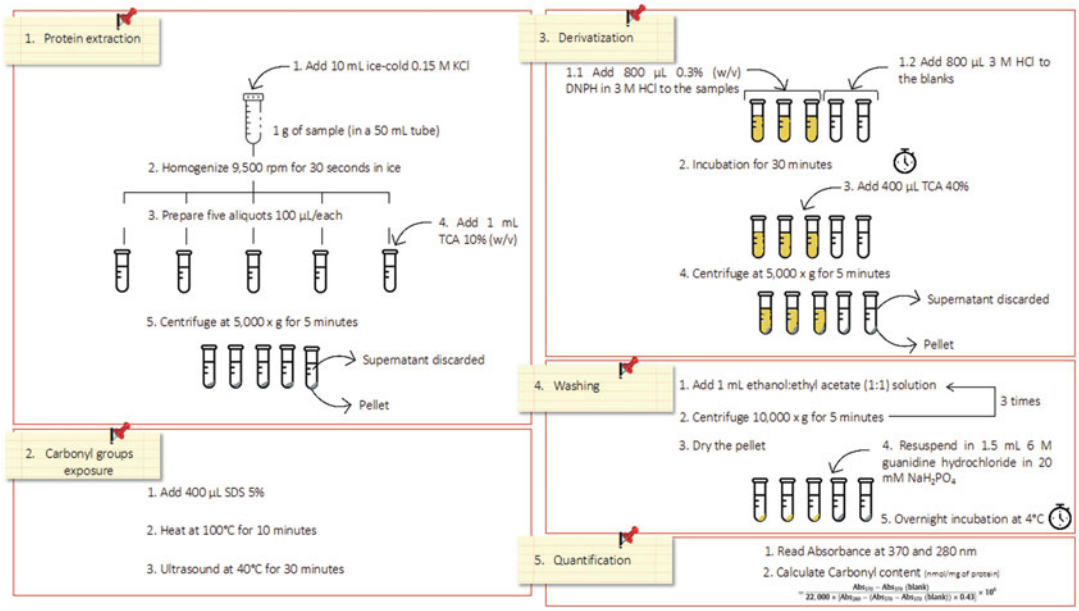

Fig. 2 Schematic and simplified representation of the steps of carbonyls quantification involving protein extraction, carbonyl groups exposure, derivatization, and quantification in the presence of DNPH

2. Heat the samples in a dry bath at 100 °C for 10 min (*see* **Note 11**).
3. Sonicate the samples in an ultrasonic cleaner at 40 °C for 30 min.
4. The implementation of the abovementioned steps has been recently studied and introduced by Soglia et al. [10]. In detail, these steps, inducing protein unfolding, result in the exposure of even the carbonyl groups that being buried in the inner core of the proteins' structure would have not react with DNPH, thus leading to an underestimation of the carbonyls development following protein oxidation.

3.3 Derivatization

1. Add 800 µL of 0.3% DNPH in 3 M HCl to each sample, while the same volume (800 µL) of 3 M HCl is added to the blanks (*see* **Note 2**).
2. Incubate the samples for 30 min at room temperature.
3. Following incubation, add 400 µL 40% TCA to each tube (*see* **Note 2**).

3.4 Washing

1. Centrifuge the samples at 5000 × *g* for 5 min (at room temperature) to precipitate the proteins and separate (and discard) the supernatant (*see* **Note 10**).
2. Add 1 mL of ethanol:ethyl acetate solution (1:1) to each tube (*see* **Note 6**).

3. Centrifuge the samples at 10,000 × *g* for 5 min (at room temperature) and discard the supernatant (*see* **Notes 6** and **10**).
4. Repeat **steps 2** and **3** two more times in order to remove unbound DNPH reagent.
5. After removing the supernatant resulting from the third washing step, evaporate any residual solvent under N_2 flux or allow the pellet to dry under the fume hood (*see* **Note 6**).
6. Resuspend the pellet in 1.5 mL 6 M guanidine hydrochloride in NaH_2PO_4 20 mM (pH 6.5) (*see* **Note 2**).
7. Incubate the samples at 4 ± 1 °C, overnight.

3.5 Quantification

1. Transfer the samples into semi-micro, UV-grade cuvettes (1.5 mL).
2. Read the absorbance of the samples and their respective blanks at 370 nm.
3. Read the absorbance of the samples (only) at 280 nm.
4. Calculate carbonyl content (expressed as nmol/mg of protein) according to the formula:

$$\text{Carbonyl content} = \frac{\text{Abs } 370 - \text{Abs } 370_{\text{blank}}}{22,000 \times [\text{Abs } 280 - (\text{Abs } 370 - \text{Abs } 370_{\text{blank}}) \times 0.43]} \times 10^6$$

4 Notes

1. Depending on the nature of the sample (liquid, solid, semi-solid) and the solubility of the sample's proteins in water, the use of specific buffers and sodium chloride (>2 M) to increase the ionic strength of the solution may be required. In very dry samples (i.e., dry-cured products) and very low protein solubility (i.e., collagen/elastin), the homogenization parameters may be properly modified (e.g., longer time and/or higher speed of homogenization), and the application of urea (>2 M) may be required. The protein extraction should be optimized to guarantee that each sampling aliquot from the homogenate (1) accurately represents the original sample and (2) contains the same protein content.
2. Due to the toxic and/or corrosive nature of the reagents used for protein precipitation, it is necessary for the operator to take the required protective measures (gloves, glasses, etc.) as well as to carry out all the operations in laboratory fume hoods.
3. SDS is an anionic surfactant capable of denaturing proteins' structure. Due to its detergent nature, air bubbles easily develop when the solution is transferred from the beaker, used to dissolve it, to a volumetric flask. To avoid/limit the

formation of air bubbles, gently heat up the solution to a temperature lower than 68 °C while stirring with a magnetic stirrer to assist dissolution.

4. Exothermic reaction. Insert 500 mL of distilled water into a 1 L beaker and slowly add 248 mL of hydrochloric acid (≥37%) while stirring with a magnetic stirrer. Wait for the mixture to cool down to room temperature, then add the remaining 252 mL of distilled water.
5. The eventual water added to moisten and stabilize the reagent should be considered in the calculation. As an example, if about 50% of water is used to moisten DNPH, then 6.0 g (instead of 3.0 g) of reagent should be dissolved in 1 L of HCl 3 M to achieve the final concentration of 0.3%.
6. Due to the irritant nature of the reagents used for washing the pellet, it is necessary for the operator to take all the required protective measures (gloves, glasses, etc.) as well as to carry out all the operations in laboratory fume hoods.
7. Endothermic reaction. The solution should be prepared with constant magnetic stirring and gentle heating (<40 °C) and allowed to equilibrate to room temperature before transferring it to a volumetric flask and fill it up to the mark.
8. Depending on the concentration of protein in the sample as well as on the extent of the oxidative damage, the amount of sample, the sample:dispersing solution ratio, and the homogenization intensity and time may vary.
9. Before pipetting the aliquots in the microcentrifuge tubes, the homogenate should be vortexed (few seconds) to have a homogeneous sample.
10. Depending on the protein nature and concentration, the centrifugation force ($\times g$) as well as the TCA concentration added may be adjusted. The pellet formed upon protein precipitation in these steps should be (1) strong enough to allow the easy removal of supernatant but (2) mild enough to guarantee a complete dissolution in the next step for carbonyl exposure, derivatization, or washing.
11. Once that the microcentrifuge tubes are placed in the dry bath, gently individually raise and shake them to ensure that the pellet is located in the bottom of the tube and submerged with the SDS solution.

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Chapter 11

Lipid Oxidation (Primary and Secondary Products)

Yasmim S. V. Leães, José Manuel Lorenzo, Alexandre J. Cichoski, Roger Wagner, Eva María Santos, Jorge F. Reyes, and Paulo C. B. Campagnol

Abstract

Lipid oxidation is one of the major causes of deterioration in the quality of meat and meat products. In addition to impairing the sensory quality, it is also responsible for reducing the nutritional quality and producing toxic compounds. The quantification of both the primary and secondary oxidation products is essential to establish strategies to prevent quality losses.

Several methodologies have been used to assess the lipid oxidation in foods, and the methods most commonly used to determine the primary and secondary products of lipid oxidation of meat and meat products will be discussed in this chapter. The chapter will describe in detail the procedures for determining the peroxide values, conjugated compounds, TBARS, and hexanal levels of meat and meat products.

Key words Lipid oxidation, Meat, Meat products, Peroxide values, Conjugated compounds, TBARS, Hexanal

1 Introduction

Lipid oxidation is one of the major causes of deterioration of meat and meat products. In addition to reducing the nutritional quality due to the degradation of vitamins and essential fatty acids, the lipid oxidation reactions lead to the appearance of undesirable compounds that impair the sensory quality of the products. The main sensory modifications include rancid flavor and aroma, unpleasant color, and texture changes in some cases [1]. Furthermore, the oxidation reactions produce toxic compounds that are associated with the emergence of various human pathologies, including various types of cancer [2].

The most important mechanism of lipid oxidation in meat is auto-oxidation, which is triggered by the reaction of oxygen with the double bonds of fatty acids, with the free radical's formation as the main initiator [3]. This mechanism is divided into three steps:

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_11,

(a) initiation, producing peroxides and hydroperoxides as the main compounds, which will later originate oxidation products responsible for the rancid flavor and aroma; (b) propagation, which consists of the displacement of double bonds during the initiation step, with the formation of conjugated dienes and trienes. During this step, hydrogens are subtracted from fatty acids and transformed into free radicals, triggering an autocatalytic process that accelerates the peroxidation reaction, with structural changes in the fatty acid molecules; (c) and termination, which consists of the reaction of free radicals with each other and with primary compounds, originating stable, non-reactive, and potentially toxic secondary products, such as aldehydes, especially malonaldehyde, and ketones. At this step, the rancid flavor and aroma are accentuated and perceptible to human senses [4].

Considering the importance of lipid oxidation reactions for the quality of meat and meat products, it is necessary to determine both the primary and secondary products, aimed to establish strategies to prevent quality losses [2]. Several methodologies have been used to assess the lipid oxidation of foods, and this chapter will discuss the protocols most commonly used to determine the primary and secondary products of this reaction in meat and meat products.

Hydroperoxides are the main primary products of lipid oxidation. The determination of hydroperoxides, also called peroxide value (PV), is one of the indicators used to evaluate the formation of primary oxidation compounds in meat and meat products. An increase in PV is observed in the early stages of lipid oxidation, which can decrease in the more advanced stages due to the decomposition of hydroperoxides [2]. Thus, a low PV may not be indicative of good oxidative stability. PV can be determined by volumetric methods, UV-visible spectral methods, chromatographic techniques, iodide values, and ferrous oxidation method. Among these methods, ferrous oxidation, proposed by Shanta and Decker [5], is considered a relatively simple method with greater stability, as it has lower sensitivity of ferrous ions to spontaneous oxidation by oxygen present in the air. The determination of conjugated dienes and trienes has also been used to monitor the formation of primary compounds of lipid oxidation, once it is a simple and fast technique that uses a small amount of organic solvents [2]. In this technique, first, the conjugated compounds are extracted from the sample using organic solvents, and then the concentration of conjugated dienes and trienes in the organic phase is measured at 234 and 266 nm, respectively, using a UV-Vis spectrophotometer [6]. Recent studies have proven that the combined determination of PV and conjugated compounds can be a very effective strategy to monitor the changes in the early stages of lipid oxidation of meat and meat products [7–10].

Malonaldehyde (MDA) is one of the major by-products of lipid oxidation and is considered a marker of lipid oxidation, as it confers

a rancid aroma even at low concentrations (>2 mg MDA/kg of sample) [2]. The TBARS (Thiobarbituric Acid Reactive Substances) assay is used to quantify MDA. First, MDA is extracted from the sample, and then MDA reacts with the TBA reagent (2-thiobarbituric acid), forming a red complex that is measured at 532 nm in a UV-Vis spectrophotometer. Whereas the TBA reagent can also react with other compounds, this methodology is not specific to determine MDA. However, the TBARS assay is one of the most used methodologies to assess the lipid oxidation of meat and meat products, once it is a simple methodology and the results are well correlated with deterioration in sensory quality.

Hexanal has been reported for its positive correlation with MDA concentrations [11], and quantitatively figures as the main volatile compound of lipid oxidation in meat and meat products, making it an important oxidative marker. The increase in hexanal concentration, as well as MDA concentrations, has an inverse relationship with the sensory acceptance of meat and meat products. The main precursors of hexanal are the polyunsaturated fatty acids (PUFA), including linoleic, linolenic, and arachidonic acids [12]. Other straight-chain aldehydes (C5–C9), saturated or unsaturated, also contribute to the same purpose but are often found in lower concentrations. Both hexanal and other volatile oxidation products can be selectively determined by gas chromatography-mass spectrometry (GC-MS). Although different procedures are used for sample preparation, headspace solid-phase microextraction (HS-SPME) has proven to be a viable alternative for volatiles extraction, as it is an easy-to-apply, solvent-free sampling technique, using mild temperature conditions, which minimize the degradation reactions of the meat matrix during extraction. In this context, the combination of HS-SPME and GC/MS for separation and quantification of hexanal, as well as other volatile compounds, has been used as an important tool in monitoring the lipid oxidation reactions in meat and meat products.

Given the above, this chapter will discuss the procedures for (1) determination of PV and conjugated compounds; (2) determination of TBARS; and (3) determination of hexanal in meat and meat products, with emphasis on the preparation of solutions, materials, care, and protocols.

2 Peroxide Values and Conjugated Dienes and Trienes

2.1 Materials

All solutions should be prepared using distilled water and volumetric glassware established in the protocols and/or according to the amount of solution necessary for the analysis. For different volumes, the rule of three should be used to calculate the amount of reagent to be used. Although the solutions have no expiration date, a short-term estimate of the volume required should be

performed. The solutions should be stored at room temperature except those requiring refrigeration, which should be informed in the protocols.

2.1.1 Lipid Extraction

1. BHT-stabilized chloroform solution: Dissolve 0.2 g of butylated hydroxytoluene (BHT) in 1000 mL of chloroform. The procedure must be carried out in a fume cupboard. Store the solution at room temperature.
2. 1.5% sodium sulfate solution: Dissolve 15 g of anhydrous sodium sulfate in 1000 mL of distilled water. Store the solution at room temperature.
3. Sodium sulfate anhydrous.
4. Methyl alcohol.

2.1.2 Determination of Peroxide Value

Solutions and Reagents

1. Barium chloride solution: Dissolve 0.4 g of barium chloride dihydrate in 50 mL of distilled water.
2. Ferrous sulfate solution: Dissolve 0.5 g of iron (II) sulfate heptahydrate in 50 mL of distilled water.
3. 10 N Hydrochloric Acid: In a 50 mL volumetric flask, add 8.2 mL of distilled water and top up with 37% hydrochloric acid. Store the solution in an amber bottle, under refrigeration (±5 °C).
4. Ferrous chloride solution: In a beaker, mix 50 mL of barium chloride solution and 50 mL of ferrous sulfate solution under constant agitation. Add 2 mL of 37% hydrochloric acid and wait for the precipitation of barium sulfate. Filter the solution and store in an amber bottle, under refrigeration (±5 °C).
5. 30% ammonium thiocyanate solution: Dissolve 30 g of ammonium thiocyanate and in 100 mL of distilled water. Store the solution in an amber bottle, under refrigeration (±5 °C).
6. Chloroform: methanol (7:3): For 1000 mL solution, mix 700 mL of chloroform and 300 mL of methanol. Store the solvent mixture in an amber bottle under refrigeration (±5 °C).

Solutions and Reagents for the Curve

Solution 1: Dissolve 0.2421 g of ferric chloride hexahydrate in 50 mL of chloroform: methanol (7:3). The iron concentration in the mixture is 1.0 mg/L. Store the solvent mixture under refrigeration (5 °C).

Solution 2: Dilute 1 mL of Solution 1 to 100 mL using the mixture of chloroform and methanol (7:3). The iron concentration in the mixture is 1.0 μg /L. Store the solvent mixture under refrigeration (5 °C).

Solutions 5 and 6 described above are also used to prepare the standard curve.

2.1.3 Determination of Conjugated Dienes and Trienes

This analysis requires only the cyclohexane ACS reagent.

2.2 Methods

All procedures must be performed at controlled room temperature (±20 °C) and low lighting. Due to the use of organic solvents, it is necessary to use an appropriate chemical resistant mask with a filter for organic gases, nitrile gloves, and goggles. All chemical procedures must be carried out in a fume cupboard.

2.2.1 Lipid Extraction

The determinations of PV and conjugated dienes/trienes require at least 100 μg of fat per sample. To extract the necessary amounts of fat from the samples, cold extraction is performed according to the method of Bligh and Dyer [13]. Figure 1 shows the simplified scheme with a brief description of each step.

The technique to determine PV only requires fat extraction. However, the determination of fat content is necessary to determine conjugated dienes and trienes, as the fat content is used to calculate the results. Thus, the drying and weighing steps will be described, as well as other necessary protocols before analysis.

Preparation of Materials

1. Identify and place beakers in an oven set at 105 °C for 2 h.
2. Remove the beakers from the oven using tongs and place them into a desiccator. Wait 30 min or until completely cooled.
3. On an analytical balance, weigh the beakers and put them back into the desiccator until analysis.

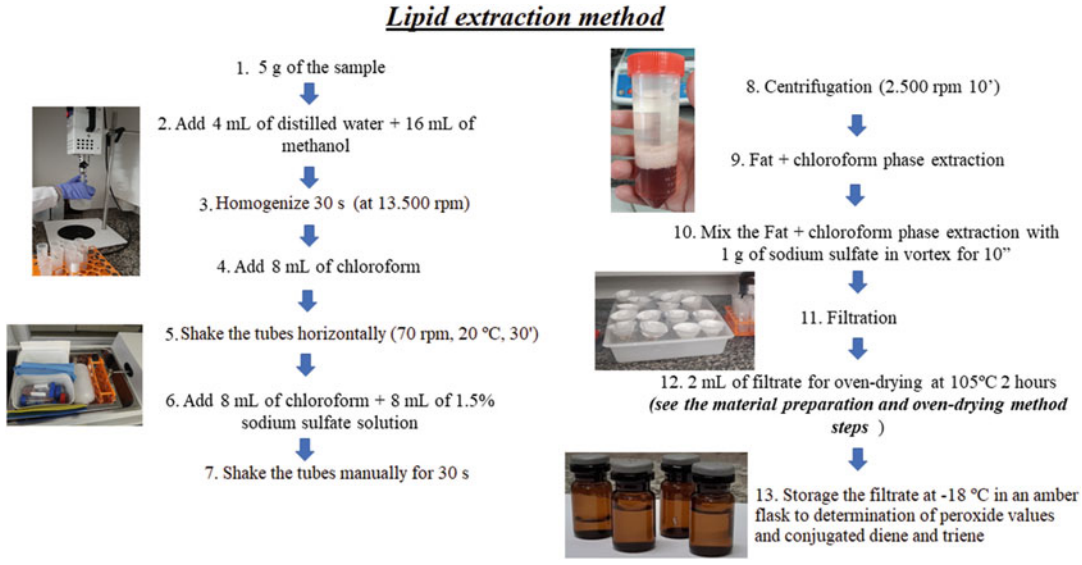

Fig. 1 Schematic and simplified representation of the steps of the fat extraction

4. In 15 mL screw cap test tubes, add approximately 1 g of sodium sulfate. Close the tubes and reserve for the following steps.

Determination Procedures

1. On an analytical balance, weigh 5 g of sample into 50 mL polypropylene centrifuge tubes and note the exact weight of the samples. It is desirable to respect a maximum deviation of ±0.03 g.
2. Add 4 mL of distilled water.
3. Add 16 mL of methanol, and close tubes to prevent volatilization. Remove tube caps only in **step 4**.
4. Homogenize the samples in an ultraturrax at 13,500 rpm for 30 s, with the tube immersed in ice water to prevent heating and increasing the oxidation rate. Clean the dispersion tool shaft between samples (*see* **Note 1** of Subheading 2.3).
5. Add 8 mL of BHT-stabilized chloroform to each tube and close the cap to prevent volatilization.
6. Shake the tubes horizontally on a shaking table at 70 rpm for 30 min at 20 °C. Make sure the tubes are properly closed to prevent sample leakage.
7. Add 8 mL of chloroform and 8 mL of 1.5% sodium sulfate solution, and close the lid. Shake the tubes manually for 30 s.
8. Centrifuge the tubes at 2500 rpm for 10 min. It is not necessary to use a refrigerated centrifuge. It is important to balance the tubes before centrifugation, by forming pairs with similar weights (*see* **Note 2** of Subheading 2.3). Centrifugation allows the separation of the contents into two phases, consisting of a phase with methanol plus the food matrix and another phase at the bottom of the tube corresponding to the chloroform containing the extracted fat.
9. Pipette the chloroform + fat phase into the test tube containing the sodium sulfate, obtained in **step 4** of Subheading "Preparation of Materials", and homogenize by vortexing for 10 s. Insert the pipette carefully, pressing it into the wall of the Falcon tube until it reaches the bottom. At this step, approximately 10 mL of the chloroform + fat phase can be pipetted.
10. Filter the samples. Place 2 mL in the beakers separated in **step 3** of Subheading "Preparation of Materials". Store the remaining filtrate in amber vials sealed with a stopper and lid. Apply a layer of Parafilm® around the entire bottle cap to avoid the volatilization of chloroform. Store the filtrate under freezing until analysis of the peroxide values (± −18 °C).
11. Preheat the oven at 105 °C so that it reaches the desired temperature for the drying step.

12. Before placing the beakers containing 2 mL of sample in the oven, it is important to place them in the fume cupboard for chloroform evaporation. Then, place the samples in the oven at 105 °C for 2 h.
13. Remove the beakers from the oven using tongs and place them into a desiccator. Wait 30 min or until completely cooled. Weigh the beakers containing the dried samples.

The fat content is calculated considering the difference between the weight of the empty beaker and the beaker containing the dried sample. The results are expressed in g of fat/100 g of sample.

2.2.2 Determination of Peroxide Values

The determination of peroxide values described below is based on the methodology of Shanta and Decker [5], with some modifications. It is important to emphasize that there is no need to perform a standard curve for each repetition when the same standard Fe^{3+} solution (Solution 2, Subheading 2.1.2) is used. When a new standard solution is required, a new standard curve must be performed. The procedures to prepare the standard curve and to determine the peroxide values are described below, and a simplified scheme with a brief description of each step is shown in Fig. 2a.

Preparation of the Standard Curve

1. For the standard curve, a blank without the Fe^{3+} standard solution, containing the mixture of chloroform and methanol

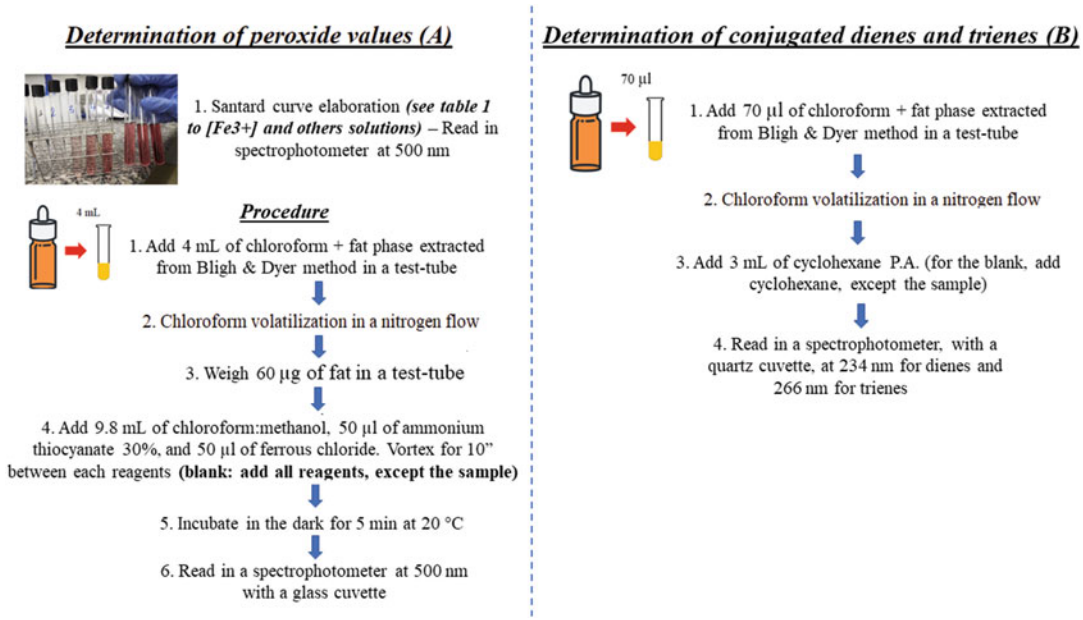

Fig. 2 Simplified scheme with a brief description of each step of the determination of peroxide values (**a**) and conjugated dienes and trienes (**b**)

Table 1
Concentrations of each reagent used to construct the standard curve of the determination of peroxide values

	Fe^{3+} concentration (μg)	Volume of Fe^{3+} standard solution	Volume of chloroform: methanol (mL)	Volume of ammonium thiocyanate (μL)
Blank	0	0	9.8	50
1	0.5	50 μL	9.7	50
2	1	100 μL	9.7	50
3	5	0.5 mL	9.3	50
4	10	1 mL	8.8	50
5	15	1.5 mL	8.3	50
6	20	2 mL	7.8	50
7	30	3 mL	6.8	50
8	40	4 mL	5.8	50

and ammonium thiocyanate should be made, as well as eight points with different standard solution concentrations (0.5, 1, 5, 10, 15, 20, 30, and 40 μL). The volume of chloroform: methanol decreases with increasing the volumes of standard solution, and the volume of ammonium thiocyanate solution is fixed at 50 μL. In the end, a colorimetric curve is obtained, in which the greater the pink color, the greater the representation of the oxidized sample. Table 1 shows the concentrations of each reagent to construct the standard curve. Absorbance readings should be performed at 500 nm.

2. The absorbance values obtained for each Fe^{3+} concentration are used to calculate the standard curve, and an ideal R^2 value should be above 0.99.

Determination Procedures

1. Prepare the water bath at 30 °C. This temperature is indicated to accelerate the chloroform volatilization process, and cannot be exceeded, aiming to prevent the fat oxidation of the sample.
2. In a test tube, add 4 mL of the phase containing chloroform + fat, extracted by the Bligh and Dyer method [13] previously described.
3. Use a nitrogen flow to dry the sample, until only a light-colored viscous liquid remains at the bottom of the test tube (fat) (*see* **Note 3** of Subheading 2.3).
4. In a test tube, weigh 60 μg of sample using an analytical balance.

5. Add 9.8 mL of chloroform: methanol (7:3) mixture. Close the tube cap and homogenize by vortexing for 10 s.
6. Add 50 μL of 30% ammonium thiocyanate solution. Close the tube cap and homogenize by vortexing for 10 s.
7. Add 50 μL of ferrous chloride solution. Close the tube cap and homogenize by vortexing for 10 s.
8. Place the samples in a dark environment for 5 min at controlled room temperature (±20 °C).
9. Prepare the blank with all reagents except the sample. Absorbance readings should be performed in a spectrophotometer at 500 nm. A glass cuvette can be used due to the absorbance range used.
10. The results are calculated according to Eq. 1 and expressed in milliequivalent of peroxides/kg of lipids (meq peroxides/kg of lipids).

$$\text{Peroxide value} = (\text{Abs } 500\text{ nm} \times m0)/(55.84 \times m1 \times 2) \quad (1)$$

where: Abs 500 nm = absorbance of the sample in the spectrophotometer; $m0$ = 1/slope of the standard curve/55.84 = molar weight of iron; $m1$ = weight of fat (g); 2 = peroxide meq factor.

2.2.3 Determination of Conjugated Dienes and Trienes

The determination of conjugated dienes and trienes is based on the methodologies of Semb [14] and Recknagel and Glende [6]. Figure 2b shows a simplified scheme with a brief description of each step.

Determination Procedures

1. Repeat **step 1** of the peroxide determination.
2. In a test tube, place 70 μL of the chloroform + fat phase, extracted by the Bligh and Dyer method as previously described.
3. Dry the samples in nitrogen flow (*see* **Note 3** of Subheading 2.3).
4. In a fume cupboard, add approximately 3 mL of cyclohexane to the tubes containing the sample and homogenize by vortexing. Prepare the blank using only 3 mL of cyclohexane.
5. Absorbance readings should be performed simultaneously in a spectrophotometer, using a quartz cuvette, at 234 (dienes) and 266 nm (trienes).
6. The results are calculated according to Eqs. 2 and 3, and expressed in mg of lipids/mL of cyclohexane). It is worth noting that the results of the Bligh and Dyer analysis are used for the calculation.

$$\text{Conjugated dienes} = \frac{\text{Abs } 234 \text{ nm} \times \text{mg fat of the sample}}{3 \text{ mL cyclohexane}} \quad (2)$$

$$\text{Conjugated trienes} = \frac{\text{Abs } 266 \text{ nm} \times \text{mg fat of the sample}}{3 \text{ mL cyclohexane}} \quad (3)$$

2.3 Notes

1. Before cleaning the dispersion tool shaft of the ultraturrax, it is necessary to ensure that no sample residue is retained. If there is any residue on the shaft, remove the sample with the aid of a needle and place it in the Falcon tube, since sample loss affects the result and the fat yield for the determination of peroxides and conjugated dienes/trienes. Then, wash the shaft with distilled water and dry it with a paper towel. It is not necessary to wash each replicate. At each different treatment, washing must be done to avoid interference of one sample with another. At the end of use, sanitize the stem according to the manufacturer's instructions.
2. When using the centrifuge, there may be a variation in the weight of the tubes due to the lack of standardization (size, weight, tube model), as well as a difference in the weight of the samples. Therefore, before inserting the tubes into the centrifuge rotor, weigh each one and note the weight, grouping the tubes with approximate weight, and adjusting the weights with distilled water when necessary, for proper use of the centrifuge.
3. It is important to check whether the amount of fat in the food matrix using 4 mL of the chloroform: fat phase will be sufficient to carry out the analysis. Therefore, it is important to standardize the amount of sample subjected to drying so that all samples have the minimum μg needed for analysis.

3 Thiobarbituric Acid Reactive Substances (TBARS)

The simplified steps of the TBARS assay are shown in Fig. 3. The analytical protocol was based on the methodology of Bruna et al. [15] and Ripollés et al. [16].

3.1 Materials

Use distilled water and analytical grade reagents to prepare all solutions (*see* **Note 1** of Subheading 3.3).

1. 5% TCA solution: Dissolve 5.0 g of trichloroacetic acid (TCA) in 100 mL of distilled water.
2. 0.08 M TBA solution: Dissolve 1.3 g of thiobarbituric acid (TBA) and 5.0 g of sodium hydroxide in 100 mL of a 50% acetic acid solution (*see* **Note 2** of Subheading 3.3).

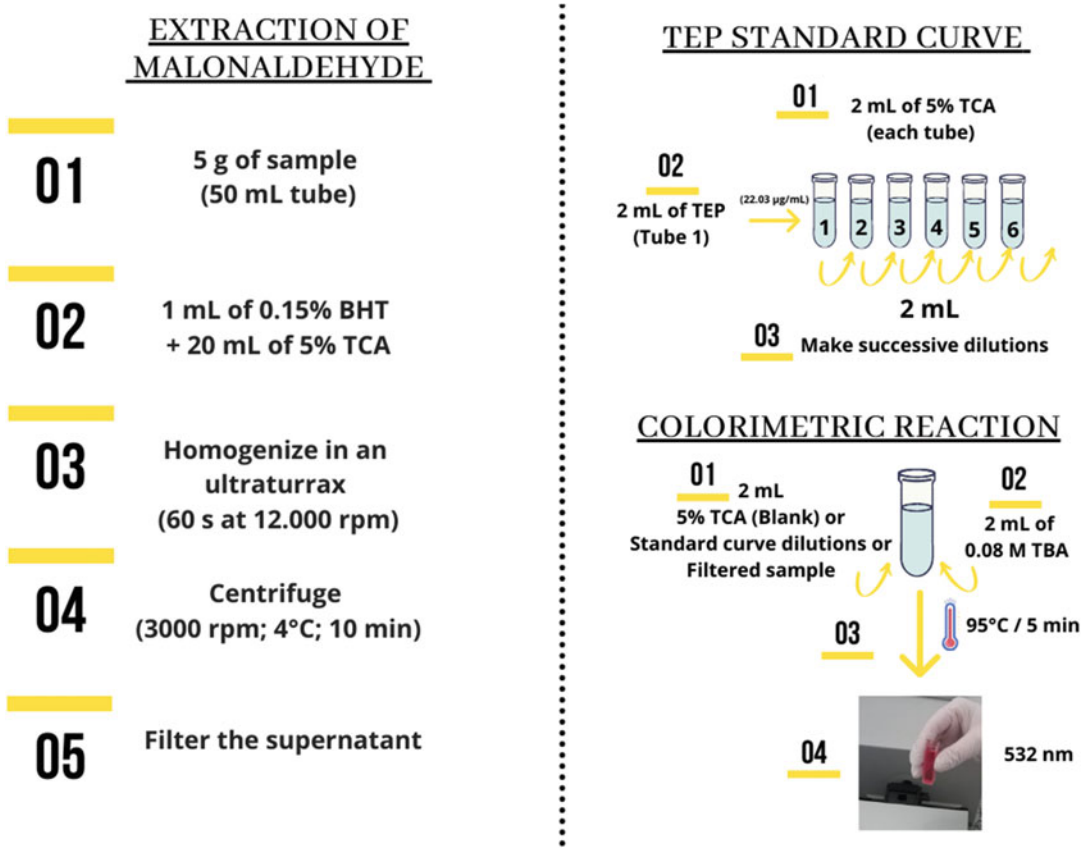

Fig. 3 Schematic and simplified representation of the steps of the TBARS assay

3. 0.15% BHT solution: Dissolve 0.75 g of butylated hydroxytoluene (BHT) in 500 mL of ethanol.
4. 1 mM TEP standard solution (220.3 µg/mL): Exactly weigh 227.1 mg of 1,1,3,3-tetraethoxypropane (TEP) (97% purity) and dissolve in distilled water using a 1 L volumetric flask.
5. TEP working solution (22.03 µg/mL): Transfer 10 mL of the standard TEP solution (220.3 µg/mL) to a 100 mL volumetric flask and complete the volume with distilled water.

3.2 Methods

3.2.1 Extraction of Malonaldehyde from the Samples

1. Weigh 5 g of the previously homogenized sample (note the weight) into 50 mL centrifuge tubes.
2. Add 1 mL of 0.15% BHT solution and 20 mL of 5% TCA solution.
3. Homogenize the tubes for 60 s in an ultraturrax at 12,000 rpm (*see* **Note 3** of Subheading 3.3).
4. Centrifuge at 4 °C for 10 min at 3000 rpm and filter the supernatant.

3.2.2 Preparation of the TEP Standard Curve

1. Add 2 mL of 5% TCA solution into six 15 mL tubes.
2. Add 2 mL of TEP working solution (22.03 μg/mL) and homogenize by vortexing. This tube contains 11.015 μg/mL of TEP.
3. Make successive dilutions to the desired concentration. For example, remove 2 mL from the tube with the concentration of 11.015 μg/mL and place in another tube containing 2 mL of 5% TCA solution (Concentration of 5.50 μg/mL). Discard 2 mL from the last tube.

3.2.3 Colorimetric Reaction

1. In 15 mL tubes add:
   (a) 2 mL of 5% TCA solution for the blank
   (b) 2 mL of standard curve dilutions
   (c) 2 mL of centrifuged and filtered sample.
2. Add 2 mL of 0.08 M TBA solution to all tubes.
3. Allow reacting for 5 min at 95 °C (*see* **Note 4** of Subheading 3.3).
4. Cool to room temperature.
5. Measure the absorbance at 532 nm in a spectrophotometer using the blank to zero the equipment.

3.2.4 Calculation of Malonaldehyde Concentration

1. The acid hydrolysis of 1 mol of TEP generates 1 mol of malonaldehyde, thus 220.3 g of TEP releases 72.03 g of malonaldehyde. This relationship is used to calculate the malonaldehyde concentration for each point of the standard curve by dividing the TEP concentration by 3.057.
2. Using specific software, construct a graph of the standard curve, by placing the malonaldehyde concentration on the X-axis and the absorbance on the Y-axis and generate the equation of the line.
3. Substitute the absorbance of the samples in the equation of the line.

For example : $y = 1.1419x + 0.024$ Sample absorbance : 0.30

$0.30 - 0.024 = 1.1419x$

$x = 0.24$ μg of malonaldehyde (*see* **Note 5** of Subheading 3.3)

4. Calculate the malonaldehyde concentration in the extract

$Z1 = (x \times (\text{Volume TCA used in the extraction})/$
$(\text{Volume of TCA used in thecolorimetric reaction})$

$Z1 = (0.24 \times 20)/2$

$Z1 = 2.4$ μg of malonaldehyde (*see* **Note 6** of Subheading 3.3)

5. Calculate the malonaldehyde concentration in the sample

Malonaldehyde concentration = $Z1$/sample weight in grams

Malonaldehyde concentration = 2.4/5

Malonaldehyde concentration = 0.48 μg of malonaldehyde/g of sample (*see* **Note 7** of Subheading 3.3).

3.3 Notes

1. Use personal protective equipment and prepare the solutions in a fume cupboard due to the toxic and corrosive nature of the reagents.
2. Use magnetic stirrer to dissolve TBA. The dissolution of TBA must not be carried out with heating and the presence of light to prevent degradation. The TBA solution should be used immediately after preparation.
3. During homogenization, the tubes must be kept in an ice bath to prevent heating of the sample. It is recommended to stop the agitation every 20 s to remove the sample residues from the dispersion tool shaft of the ultraturrax.
4. The colorimetric reaction must be carried out at 40 °C for 80 min for samples containing sugar in its composition.
5. Amount of malonaldehyde present in 2 mL of filtrate added to the colorimetric reaction tube (Subheading 3.2.3).
6. Amount of malonaldehyde present in 20 mL of TCA used to extract malonaldehyde from the sample (5 g) in the extraction step (Subheading 3.2.1).
7. Final result of the TBARS values of the sample. The result can be expressed as μg malonaldehyde/g sample or per mg malonaldehyde/kg sample.

4 Hexanal Determination

The steps of hexanal determination in meat and meat products through headspace solid-phase microextraction (HS-SPME) and gas chromatograph-mass spectrometry (GC/MS) are shown in Fig. 4. The analytical protocol is based on the methodologies of Wagner and Franco [17] and Domínguez et al. [12]. This protocol allows obtaining quantitative information of hexanal, and the result is expressed as area units of extraction ion chromatogram (AU-EIC) per gram of sample (AU-EIC $\times$ 10^4 g of sample). Thus, this technique allows evaluating the oxidation products in different meat matrices for various purposes, including manufacturing processes, or evaluation of raw materials or new

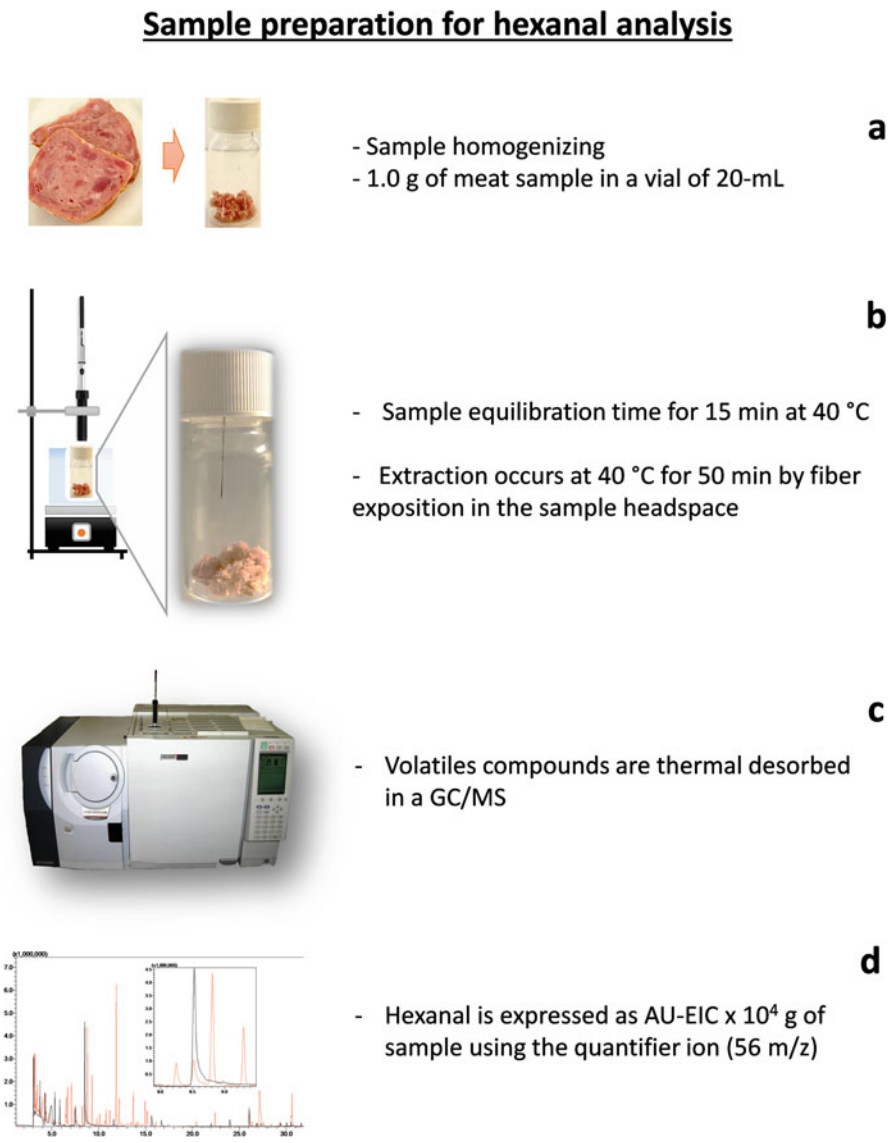

Fig. 4 Schematic representation of the steps of the hexanal extraction and GC/MS analysis

ingredients, as well as the shelf life of meat products. Additionally, the protocol comprises established chromatographic conditions, which allow the analyst to investigate other metabolites of the lipid or protein oxidation, through the identification of the mass spectra of other analytes, if applicable.

4.1 Materials

Use analytical grade reagents to prepare the methanolic hexanal solutions (*see* **Note 1** of Subheading 4.3).

1. Hexanal solutions: Prepare a methanolic solution of hexanal (S1) by mixing 10 μL of hexanal and 1 mL of methanol. Then,

prepare the qualitative solution using 100 μL of methanolic solution S1 and 10 mL of methanol (S2).

2. SPME fiber installed in a GC-autosampler or a manual holder: Use SPME fused-silica fiber (10 mm length) coated with a 50/30-μm thickness of DVB/Car/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) (Supelco, Bellefonte, PA, USA) (*see* **Note 2** of Subheading 4.3).
3. The volatile compounds of meat samples should be analyzed in a gas chromatograph coupled to a mass spectrometer Shimadzu GC/MS QP2010 (Shimadzu Corporation, Japan). The GC injection port should be equipped with an SPME liner (internal diameter of 0.75 mm). The separation of analytes should be carried out in a ZB-Wax fused-silica capillary column (60 m × 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, USA).

4.2 Methods

Meat samples should be ground in a food multiprocessor to enhance the sample homogeneity and particle size reduction.

4.2.1 Sample Preparation

1. Weigh 1 g of homogenized sample in a 20-mL extraction vial and immediately screw-capped with a laminated Teflon-rubber septum (Fig. 4a). For the analysis of the hexanal standard, 1 μL of the methanolic hexanal solution (S2) should also be placed in a 20-mL vial.
2. The extractions of volatile compounds should be carried out at 40 °C for 50 min of fiber exposition in the sample headspace. To obtain this temperature, it is recommended to use a water bath with a controlled temperature and accuracy of 0.2 °C. Before extraction, the sample remains at this same temperature for 15 min, to reach a homogeneous temperature inside the vial. After the time of extraction, the fiber should be withdrawn into the needle, and then posteriorly inserted into the GC injection port for volatile compounds analysis (Fig. 4b).

4.2.2 Hexanal Determination

The hexanal as well as other volatile compounds extracted by SPME fiber are separated, identified, and quantified using a gas chromatograph as following:

1. The volatile compounds are thermally desorbed from SPME fiber by inserting it into the injection port of the GC/MS at 260 °C for 10 min (Fig. 4c). The thermal desorption of the volatile compounds occurs in a split/splitless injector operating in a splitless mode for 1 min (after the splitter valve is open at 20:1). Helium is used as the carrier gas under a constant flow of 1.2 mL/min. After each injection, the SPME fiber should be cleaned in the SPME conditioning station at 260 °C for 2 min to ensure that the fiber is completely clean before the next extraction.

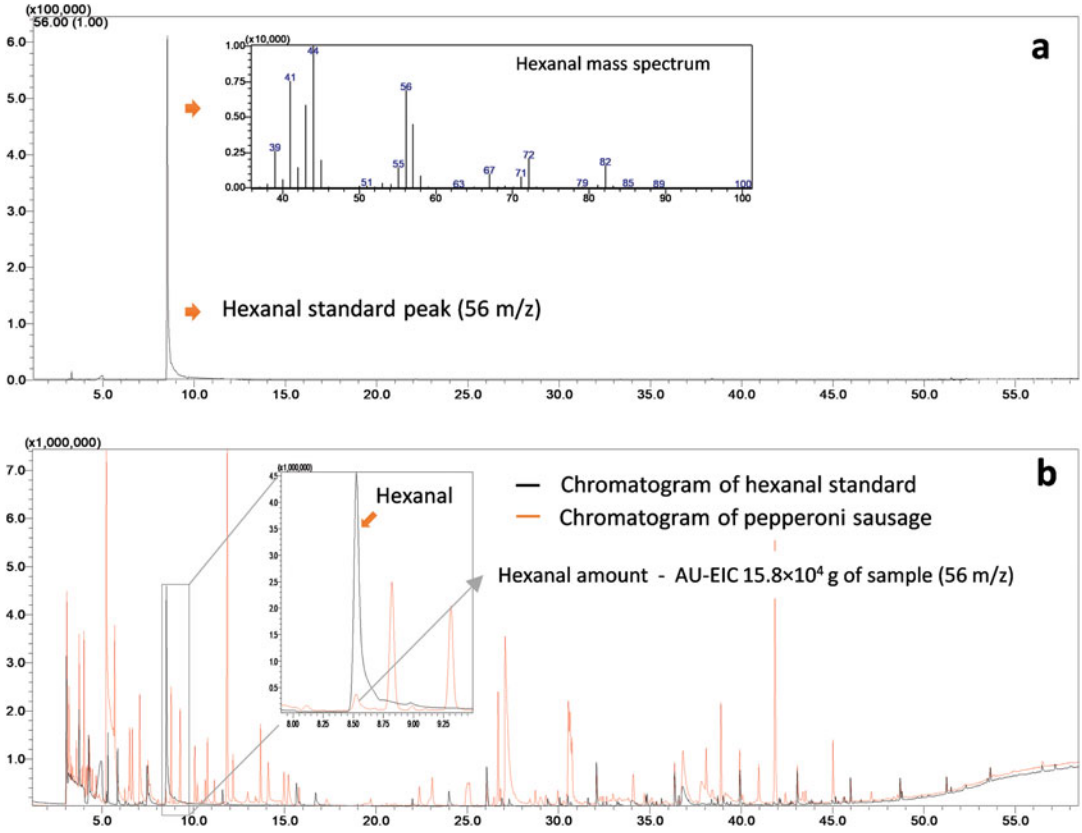

Fig. 5 Chromatograms of hexanal standard (**a**) and a pepperoni sausage sample red line, and hexanal standard—black line (**b**)

2. The hexanal and other volatile compounds are separated in a capillary column of fused-silica ZB-Wax. The oven temperature is programmed as initial oven temperature of 35 °C, remaining for 5 min, increasing at 2 °C/min until 80 °C, followed by an increase of 5 °C/min until 230 °C, held for 3 min.
3. The GC/MS interface temperature and ion source of MS detector are maintained both at 230 °C. The instrument is operated in the electron ionization mode (+70 eV) with a single quadrupole mass analyzer operating in full scan mode, collecting 2 scans/s over the range of 35–350 *m/z*.
4. The hexanal identification in the sample is done by comparing the retention times and the spectra of the chromatographic peak of the sample and hexanal standard (*see* **Note 3** of Subheading 4.3). The hexanal mass spectrum is shown in Fig. 5a. Due to greater selectivity, it is suggested to use the ion *m/z* 56 (82% of intensity) as quantitative ion and ions 57 (39%), 72 (18%), and 82 (17%) as qualitative ions.

5. The final results are expressed as area units of the extraction ion chromatogram (AU-EIC) per gram of sample (AU-EIC $\times 10^4$ g of sample) using the quantifier ion of m/z 56 (Fig. 5b).

4.3 Notes

1. Use personal protective equipment and prepare the solutions in the fume cupboard due to the toxic and corrosive nature of the reagents.
2. Before the analysis, the SPME fiber should be conditioned by heating in an SPME fiber conditioning station or a GC injection port (in split mode) at 260 °C for 30 min, according to the manufacturer's instructions.
3. Chromatographic selectivity must be verified when determining the hexanal. This can be assessed by spectral comparisons of ions of the hexanal standard and the corresponding peak of the samples. The similarity index between the spectra must be greater than 95%.

Acknowledgments

This work was supported by National Council for Scientific and Technological Development (CNPq) and Coordination for the Improvement of Higher Education Personnel (CAPES finance code 001). Authors are members of the HealthyMeat network, funded by CYTED (ref. 119RT0568).

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Chapter 12

Volatile Organic Compound Profile

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Abstract

The intake of meat and meat products provide certain benefits to the consumer as these foods are an important source of vital nutrients such as minerals, fatty acids, vitamins, essential amino acids and proteins. However, it must be taken into account that due to their characteristics, this type of foods is susceptible to deterioration, either by enzymatic degradation, lipid oxidation or by the action of the microorganisms. The spoilage reactions can produce textural and color changes and also the formation of volatile organic compounds that could origin nasty odors in meat which can lead to rejection by consumers. To avoid this, it is necessary to develop new faster and easier analytical techniques to determine volatile organic compounds in meat and meat products.

A procedure for the analysis of volatile organic compounds in meat and meat products is detailed below, which includes an extraction stage by solid-phase microextraction with fused-silica fiber coated with divinylbenzene/carboxen/polydimethylsiloxane and its subsequent analysis by gas chromatography with mass spectrometry. All steps and conditions proposed by us are described clearly with the aim reproduced by other researchers.

Key words Volatile compounds, Meat, Meat products, SPME extraction, Gas chromatography-mass spectrometry, Food analysis

1 Introduction

Meat and meat products are foods very consumed. This is consumed largely because they constitute an important source of protein in the diet [1, 2]. However, their consumption is in great part affected by their nutritional quality and sensory perception [3, 4]. These characteristics specifics of each kind of meat and meat products are greatly influenced by diverse factors such as the animal characteristics (sex, feed, breed, muscles, rearing systems, genotypes, etc.) [5, 6], the meat processing (freezing, cooking, smoking process, mincing degree, etc.), the ingredients (salt amount, adding of species, meat/fat ratio, etc.), and the ripening

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_12,

conditions (time, temperature, and relative humidity) [5, 7, 8]. All these factors have an influence over the meat and meat products aroma.

The aroma is formed by a hundred molecules called volatile organic compounds (VOCs) [7]. The principal VOCs that can be found in meat and meat products can be separated into different chemical families between including alcohols, aldehydes, esters, furans, hydrocarbons, ketones, lactones, organic acids, and terpenes [8]. The importance of each compound in the aroma depends on the concentration of each VOC and the olfactory threshold [8, 9].

For their features, meat and meat products are foods highly susceptible to spoilage and the formation of unwanted odors and flavors that are produced as a result of microorganisms degradation or the lipid oxidation and enzymatic processes [4]. Some volatile organic compounds (VOC) are created through these types of reactions that are considered a critical factor of food flavor quality [10, 11]. In addition, it should be noted that not only do these processes give rise to unwanted tastes/odors, but in the case of cured meat, for example, these processes release desirable volatile compounds, which are part of the characteristic flavor of meat products. Therefore, it is vital to correctly monitor the evolution and release of volatile compounds, both from the point of view of seeing a possible degradation and to monitor the correct development of the desired aroma/flavor [8].

Nowadays, is necessary the development of new methods of analysis of volatile organic compounds in meat and meat products to control their quality due to increasing consumer demands for safe and high-quality food [4, 12]. However, the quantification of these compounds in this kind of food is not easy because the sampling techniques and analytic instrumentation used are complex [11]. Several sampling techniques are available for the analysis of VOCs released during meat and meat products manufacture, such as the solvent extraction, simultaneous steam distillation–extraction, static and dynamic headspace and solid-phase microextraction (SPME) [4]. However, the most common technique used in meat and meat products is solid-phase microextraction (SPME) [13]. This fact is caused for SPME a rapid and inexpensive analytical technique enabling VOCs sampling in the headspace of a sample [12]. Nevertheless, to insure a high efficiency of SPME is necessary to control several parameters as the type of fiber, the sample volume, time, temperature, and stirring [14]. Regarding the analysis technique, the gas chromatography (GC) coupled to mass spectrometry (MS) has been the most used technique for VOCs analysis in different meat samples and meat products. Recent studies such as those cited below in beef [15, 16], pork [17, 18], lamb [10, 19], bovine [11], chicken [12], and meat products [8, 20–22] evidence this.

With this in mind, the objective of this book chapter is to clearly and fully explain a procedure for determining and quantifying volatile organic compounds in meat and meat products so that they can be reproduced in the future by other researchers. In this case, two stages are specified, the solid-phase microextraction extraction (SPME) of the volatile organic compounds and the subsequent chromatographic analysis by gas chromatography-mass spectrometry (GC-MS).

2 Materials

2.1 Volatile Organic Compounds Extraction

- Fused-silica fiber (10 mm length) coated with a 50/30-mm thickness of DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) (Supelco, Bellefonte, PA, USA).
- 20 mL vials with screw-capped with a laminated Teflon-rubber disc (Agilent Technologies, Santa Clara, CA, USA).

2.2 Gas Chromatograph

The extraction of volatile organic compounds (VOC) was performed using solid-phase microextraction (SPME) with an autosampler Pal RTC-120 (Agilent Technologies) and the separation, identification, and quantification of them were carried out using a gas chromatograph Agilent mod. 7890B (Agilent Technologies) equipped with a mass selective detector Agilent mod. 5977B (Agilent Technologies). For the separation of the volatile organic compounds, a DB-624 capillary column (30 m, 250 μm i.d., 1.4 μm film thickness; Agilent Technologies) was used.

3 Methods

3.1 SPME Extraction

Prior to the analysis, the fiber (fused-silica fiber DVB/CAR/PDMS) has conditioned by heating in an SPME Fiber Conditioning Station at 270 °C for 30 min.

For headspace SPME (HS-SPME) extraction, weigh 1 ± 0.02 g of each sample in a 20-mL vial and then screw-capped with a laminated Teflon-rubber disc (*see* **Notes 1–3**).

The SPME extraction was carried out at 37 °C for 30 min, after sample equilibration time at the same temperature for 15 min, to ensure a homogeneous temperature for the sample and headspace. A resume of SPME extraction conditions is shown in Table 1.

Figure 1 shows a summary of the extraction process of volatile compounds in meat samples and meat products by SPME extraction.

Table 1
Summary of SPME extraction for the volatile compound analysis

SPME extraction	
Fiber type	Fused-silica fiber (10 mm length) coated with a 50/30-mm thickness of DVB/CAR/PDMS
Fiber conditioning	270 °C during 30 min
Sample incubation	37 °C during 15 min
Sample extraction	37 °C during 30 min

3.2 Volatile Organic Compounds Identification and Quantification (GC-MS)

Separation, identification, and quantification of volatile organic compounds were carried out using a gas chromatograph–mass spectrometer, following the next conditions (Table 2) (*see* **Notes 4** and **5**):

1. After the extraction process, the fiber was transferred to the injection port of the gas chromatograph–mass spectrometer (GC–MS) system. Then the volatile organic compounds were desorbed from the SPME fiber in the injection port at 260 °C for 8 min (splitless mode; helium pressure 9.59 psi). After each injection, the SPME fiber was clean for 2 min at 270 °C in the SPME Conditioning Station to guarantee that fiber is completely clean before the next sample extraction. Helium was used as carrier gas at a constant flow of 1.2 mL/min (9.59 psi). A DB-624 capillary column (30 m, 0.25 mm i.d., 1.4 μm film thickness; Agilent Technologies) was used for volatile compound separation. This column has excellent resolution and a wide operating temperature range.
2. The oven temperature program was isothermal for 10 min at 40 °C, raised to 200 °C at 5 °C/min, then raised to 250 °C at 20 °C/min and held for 5 min.
3. The mass detector transfer line was maintained at 260 °C. The ion source used was an Extraction Source Xtr EI 350 (Agilent Technologies, Santa Clara, CA, USA). The mass spectra were obtained using a 5977B mass selective detector working in electronic energy at 70 eV, with an electron multiplier voltage of about 900 V (gain factor = 1) and collecting data at 2.9 scans/s over the range m/z 40–550 in scan acquisition mode. The mass source was maintained at 230 °C while the mass quad was set at 150 °C. The total time for chromatographic analysis is 49.5 min. Figure 2 shows an example of volatile organic compounds chromatogram of *Cecina*.

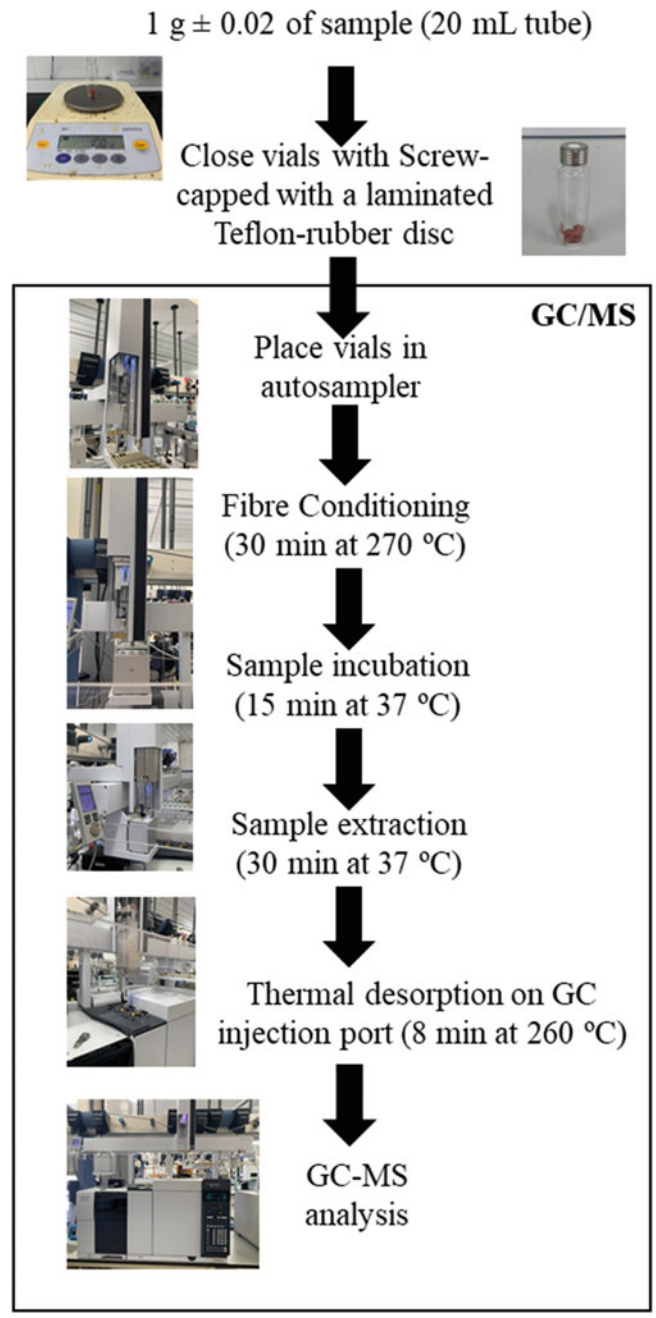

Fig. 1 Schematic and simplified representation of the steps of the volatile organic compounds extraction

3.3 Data Processing

Data acquisition and equipment control was carried out using GC software MassHunter Acquisition B07.05, while data were analyzed with MassHunter Quantitative analysis B.07.01 (Agilent Technologies).

Table 2
Summary of the chromatographic and mass spectrometer conditions used for the volatile compound analysis

Chromatographic and mass spectrometer conditions				
Inlet	*Temperature (°C)*	*Pressure (psi)*	*Total flow (mL/min)*	*Desorption time*
Splitless mode	260	9.59	19.2	8 min
Oven	*Rate (°C/min)*	*Temperature (°C)*	*Hold time (min)*	*Total time (min)*
Initial	–	40	10	10
Ramp 1	5	200	–	42
Ramp 2	20	250	5	49.5
Column	*Characteristics*	*Temperature (°C)*	*Pressure (psi)*	*Flow (mL/min)*
DB-624	30 m × 250 µm × 1.4 µm	40 (Initial)	9.59	1.2
Transfer line temperature		260 °C		
Detector	*Temperature (°C)*	*m/z range*	*Mass source temperature*	*Mass quadrupole temperature*
MS	260	40–550	230	150

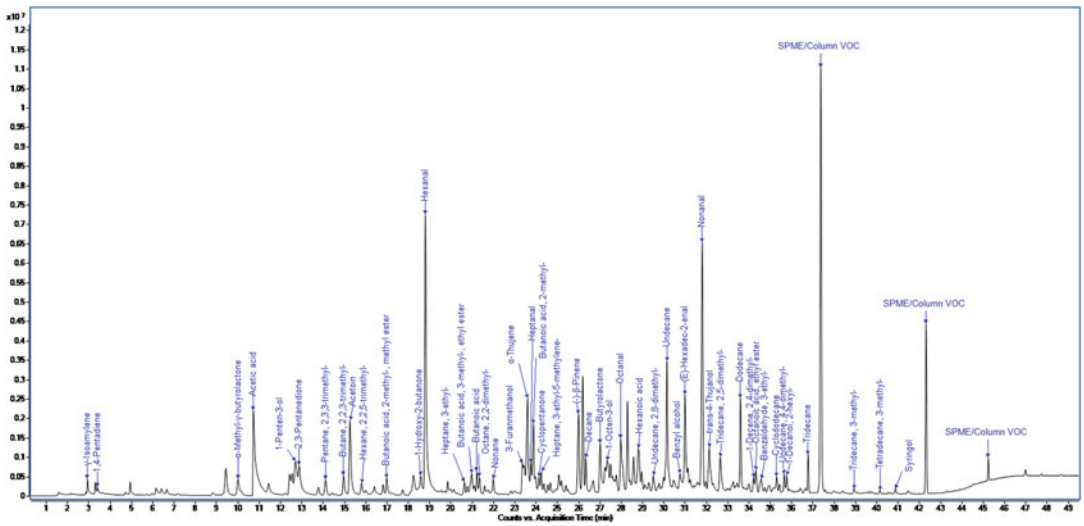

Fig. 2 Volatile organic compounds chromatogram of *Cecina*

After samples analysis, a new method from acquired scan data with library search was created. For the integration Agile2 algorithm was used, while the peak detection was carried out by deconvolution. For method creation, a representative number of samples must be used. The compounds identification was carried out by

comparison of their mass spectra with those contained in the NIST14 library (National Institute of Standards and Technology, Gaithersburg). The identification was considered correct only for the peaks with a match factor $> 85\%$. After integration, peak detection and identification of each compound, the extracted ion chromatogram (EIC) from the quantifier ion was obtained from each peak. The final results were expressed as area units of the EIC $\times 10^4$ per gram of sample (AU-EIC $\times 10^4$/g of sample).

4 Notes

1. Homogenize the sample well to ensure a correct extraction of all the volatile compounds. In samples without chopping, it is necessary to grind (using a mincer) to achieve greater homogenization. However, it is necessary to avoid overheating of the sample since increasing the temperature of the sample could generate new and unwanted volatile compounds (*see* **Note 6**).
2. Make sure that the sample is well homogenized and that it remains at the bottom of the vial to prevent the fiber from touching the sample.
3. It the samples are not analyzed immediately, it is necessary to preserve them under vacuum and keep them frozen until use. However, it is advisable to analyze them fresh, since these processes can lead to unwanted changes.
4. A blank should be injected to compare the peaks/spectra with the injected samples, and to be able to detect potential equipment or SPME fiber problems.
5. To ensure a correct operation of the mass spectrometer, it is necessary to carry out a Tune Evaluation approximately once a week (to check the mass properties) and an Etune once a month to calibrate the masses. These tests apply different voltages to adjust various parameters of the mass spectrometer through the infusion of a tuned compound, commonly perfluorotributylamine (PFTBA). With this, it is possible to increase the sensitivity of the equipment and the signal/noise ratio.
6. Take into account that the fiber supports approximately between 90 and 100 injections, from that number it is necessary to replace it with another.

Acknowledgments

This work was supported by Centro Tecnolóxico de la Carne de Galicia. Thanks to GAIN (Axencia Galega de Innovación) for supporting this book chapter (grant number IN607A2019/01). Authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568).

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Chapter 13

Proteomics

María López-Pedrouso, José Manuel Lorenzo, and Daniel Franco

Abstract

This text provides a brief overview of specific issues in the meat industry which may be resolved using the current proteomics-based methodology. Proteins as key to understanding biological processes as well as to change the characteristics of meat products offer enormous potential in the context of meat science. The quantification, analysis and curation of proteomics data, and particularly, the industrial applications of these new findings will become a more essential tool in the coming years.

The methodology described in this chapter presents the basis for quantification, analysis, and curation of proteomics data, representing the pillars of main proteomic methods. The main steps include 1. Extraction and quantification of proteins; 2. Separation of proteins by isoelectric point; 3. Separation of proteins by molecular weight; 4. Staining of proteins; 5. Tryptic digestion with enzymes; and 6. identification of proteins by mass spectrometry.

More recent proteomics approaches are beyond the scope of this chapter. Therefore, all steps are described in detail so that the conditions proposed by us can be reproduced by other researcher groups. Although protocols and methodology are specifically obtained from our lab, these can be easily adapted for every laboratory.

Key words 2-DE, Protein isoelectric point, MALDI-TOF, Meat products

Abbreviations

2-DE	Two-dimensional electrophoresis
CHAPS detergent	(3-((3-cholamidopropyl) dimethylammonium)-1-propanesulfonate)
DTT	Dithiothreitol
IPG	Immobilized pH gradient
MALDI-TOF	Matrix-assisted laser/desorption ionization-time of flight
Mr.	Molecular weight
p*I*	Isoelectric point
PMF	Peptide Mass Fingerprint
PSA	Persulfate ammonium
SDS	Sodium dodecyl sulfate

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_13,

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic
TEMED	Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane

1 Introduction

The protein content of meat products has a critical influence on nutritional quality, sensory and textural properties. Important quality traits of fresh meat, like tenderness, color, and water holding capacity are closely linked to their proteins and posttranslational modifications. Indeed, during meat aging, the rate of pH fall is influencing the protein denaturation, myofibrillar structures, and cellular shrinkage causing variations in meat quality [1, 2]. Moreover, protein changes such as protein oxidation has an important role in sensory attributes and other quality traits of the meat products [3], as well as proteolysis caused by enzymes during the ripening of dry-cured meat products [4]. Other processes like protein carbonylation, breakdown, and aggregation also imply solubility and functionality changes that lead to a reduction of digestibility, gelation, emulsification, and water holding capacity [5]. On the other hand, the quality of processed meats is particularly linked to the nutritional value of proteins, composed of essential amino acids and bioactive peptides. Indeed, it has been reported that peptides released by digestion gastric or enzymatic hydrolysis from animal proteins may achieve a high biological activity and bioavailability [6]. Within the meat industry, quality control should be performed using protein biomarkers determining phenotypes and predicting meat quality features. The aim is to identify proteins whose expression or abundance is linked to a phenotypic trait [7], influencing the final meat product quality. Briefly, it has been described an overview of specific issues in the meat industry which may be resolved using the current proteomics-based methodology. For the above reasons, the knowledge of meat proteins and their modifications need to be investigated further for the improvement of meat quality in all these issues.

The growing use of omics technologies including proteomics brings significant benefits to food sciences. In this regard, proteomics as the large-scale analysis of proteins has mainly been developed for gel-based techniques and mass spectrometry in these years. The identification of protein patterns using two-dimensional electrophoresis (2-DE) for protein separation implies a first separation based on their isoelectric point (pI) and a second step separated based on molecular weight (Mr). These two parameters are independent resulting in an effective separation method. The analysis of 2-DE gel is performed by software comparing the images to

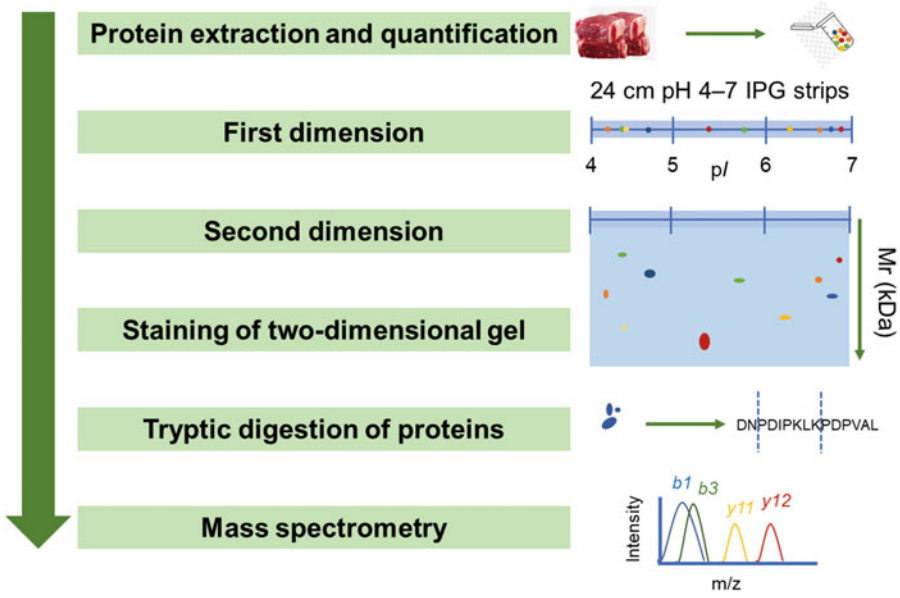

Fig. 1 Main steps to carry out separation and identification of proteins

identify different spots in quantitatively and qualitatively terms. Afterwards, the preferred method to identify the proteins in gels is Peptide Mass Fingerprint (PMF) using matrix-assisted laser/desorption ionization-time of flight (MALDI-TOF) mass spectrometry. For this purpose, it is necessary to carry out enzymatic digestion that breaks down protein with great specificity. This specific fingerprint is used to achieve the identification of the protein searching in databases.

The in-depth knowledge of protocols for each food matrix is a key step towards reliable data of proteins. 2-DE is the most common technique used in food proteomics as it can separate different isoforms or even detecting post-translation modifications of proteins by using commercial dyes. However, variability associated with the technique should be minimized through a rigorous procedure in the laboratory. The main steps (Fig. 1) are: (1) Extraction of proteins and initial quantification by commercial kit; (2) First dimension or separation of proteins by p*I*; (3) Second dimension or separation of proteins by Mr.; (4) Staining of gels and comparison of gel images using a specific software package; (5) Tryptic digestion with commercial enzymes; and (6) Identification of proteins by mass spectrometry (e.g., MALDI-TOF).

Particularly in muscle sample tissues, before the proteomic analysis, a protein extraction protocol for 2-DE analysis should be carried out by TCA-acetone precipitation avoiding contaminants (mainly salts and detergents) and increasing concentration of the proteins in solution [8]. The proteins should be solubilized in a denaturing buffer with neutral chaotropic, zwitterionic or neutral

detergent and reducing agents. Currently, the first dimension (known as isoelectric focusing) is often performed on an immobilized pH gradient (IPG) submitted to a high voltage where the proteins can move towards the pH produces a zero-net charge on protein. The second-dimension gel is based on the movement of the unfolded protein which contains SDS through a gel pore resulting in a separation by protein size. Inside the gel, the proteins are stained using Coomassie blue, SYPRO, or silver stain producing gel imaging. Thus, the protein spots could be detected by gel image analysis and the quantitative changes measured reflecting the expression of the proteins. After all, mass spectrometry as MALDI-TOF MS is being used to identify the proteins after tryptic digestion.

To sum up, it has been presented an overview of proteomic methodology to identify and quantify proteins in meat products. In the next sections, detailed information about the main steps will be provided. The methodology described in this chapter presents the basis for quantification, analysis, and curation of proteomics data, representing the pillars of main proteomic methods. Thus, protocols and methodology were specifically obtained from our lab, these can be easily adapted for every laboratory.

2 Material and Methods

In the next sections, we will provide a detailed description of the main steps indicated in Fig. 1, developed in our lab.

2.1 Extraction and Measurement of Protein Content in the Solution

After the collection of meat samples, they must be excised in small pieces, lyophilized and maintained at −80 °C to ensure the conservation before protein extraction. Afterwards, meat proteins will be precipitated using the TCA-acetone protocol. The protein content in the solution will be carried out to know exactly the amount of protein necessary to carry out the proteomic experiment. In our lab, we use the commercial CB-X protein assay kit (GBiosciences, USA) according to the instructions of the manufacturer using a microplate reader, but other protein quantification kits would also be valid.

Solutions and buffers: Prepare all solutions using ultrapure water and analytical grade reagents.

- *Acetone* (90%).
- *Extraction buffer* (10% TCA and 0.07% dithiothreitol (DTT) in acetone): Dissolve 6 g of TCA in 60 mL of acetone and add 4.2 mg of DTT.
- *Lysis buffer* (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT and 2% Pharmalytes): Dissolve 10.5 g of urea, 3.8 g of thiourea,

1 g of CHAPS, 38 mg of DTT, and 0.5 mL of Pharmalyte™ pH 3–10 (GE Healthcare, Uppsala, Sweden) in 25 mL and make up to 25 mL with water. Stir for hours to dissolve completely at room temperature.

- *Wash buffer* (0.07% DTT in acetone): Dissolve 14 mg of DTT in acetone.

2.1.1 Protocol

1. An amount of 50 mg of lyophilized meat tissue is suspended in 10 mL of extraction buffer (10% TCA and 0.07% DTT in acetone).
2. A sonication step should be considered for cell disruption and homogenization of the solution in refrigerated conditions. The sonication should be in short pulses at 0 °C.
3. The solution is maintained at −20 °C for at least 1 h.
4. The solution is centrifuged at 4500 × *g* at 4 °C for 30 min and the pellet is recovered.
5. The pellet is rinsed two times with 1 mL of wash buffer (0.07% DTT in acetone) followed by centrifugation as described in **step 4**.
6. The last washing is using acetone (90%) and centrifugation (**step 4**).
7. The final pellet is eluted in 1 mL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT, and 2% Pharmalytes) which should be stirred to dissolve completely in an Eppendorf Thermomixer.
8. The concentration of protein in the final solution should be determined using the commercial CB-X protein assay kit (GBiosciences, USA) according to the instructions of the manufacturer in a microplate reader. A microplate reader is necessary to look up the absorbance at 595 nm. This method is compatible with detergents and other substances like reducing sugars, thiols, and chelating agents (protein standard is specifically provided to prepare a calibration plot).

2.2 First Dimension or Separation of Proteins by pI

Isoelectric focusing with IEF by using Protean IEF Cell (Bio-Rad Laboratories, Inc., USA) and immobilized pH gradients is employed for the first-dimension separation (Fig. 2). The protein can be loaded on 11-cm, 18-cm and 24-cm Ready Strip IPGs (Bio-Rad Laboratories). In this sense, IPG strips with different linear pH linear or non-linear gradients (3–10 and 4–7) can be chosen according to the experiment.

Solutions and buffers: Prepare all solutions using ultrapure water and analytical grade reagents.

Fig. 2 Protean IEF Cell (Bio-Rad Laboratories)

- *Tris solution* (50 mM Tris pH 8.8): Dissolve 181.5 g of Tris in 750 mL of ultrapure water, adjust the pH to 8.8, and make up to 1000 mL with water.
- *Equilibration solution* (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, and 2% SDS): 6.7 mL of Tris solution, 72.1 g of urea, and 60 mL glycerol are vigorously stirred for 30 min. Finally, 4 g of SDS is added and made up to 200 mL with ultrapure water. In the case of equilibration solution with 1% of DTT, dissolve 60 mg of DDT in 6 mL of equilibration solution for each strip, and in case of 2.5% of iodoacetamide, dissolve 150 mg of iodoacetamide in each 6 mL of equilibration solution for each strip.

2.2.1 Protocol

1. According to the previous protein quantification, a volume containing 250 μg of total protein is dissolved in lysis buffer up to the total volume recommended by the manufacturer for each strip length (Table 1).
2. The rehydration solution is completed by the addition of 0.6% DTT and 1% IPG buffer (Bio-Rad Laboratories). A complete dissolution takes place after a vigorous agitation.
3. This sample should be distributed along a lane of the focusing tray from one electrode to another. Carefully place the IPG strips contacting the gel side with the sample. Finally, cover with mineral oil along the lane to avoid evaporation during the first electrophoresis according to Table 1. Place the plastic lid onto the focusing tray and introduce it into the Protean IEF Cell (Bio-Rad Laboratories, Inc., USA).
4. The focusing conditions and ramping protocols have been optimized for this quantity of meat samples. A previous step of active rehydration with a voltage of 50 V during 12 h should

Table 1
Total volume of buffer for rehydration for each strip

Strip length (cm)	Total volume recommended (μL)	Volume of mineral oil (mL)
7	125	1
11	200	1.5
17	300	2
18	315	2
24	450	2.5

Table 2
Focusing conditions for programming the unit, using a linear ramp in each step

	Voltage (V)	Time (min)
Step 1	250	30
Step 2	500	60
Step 3	1000	60
Step 4	4000	120
Step 5	8000	120
Step 6	10,000[a]	

[a]A voltage of 10,000 V is applied until to reach final voltage in the last step

be performed and the settings for IEF are shown in Table 2. The final voltage depends on the length and pH of each IPG strip, therefore, necessary to consult the manual.

5. The equilibration of the strips is necessary to run the second dimension ensuring that the cysteines are reduced and alkylated. The strips should be placed in the equilibration tray for better treatment. The focused strips should be incubated firstly in equilibration solution (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, and 2% SDS) with 1% DTT for 15 min and secondly in equilibration solution (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, and 2% SDS) with 2.5% iodoacetamide for 15 min.

2.3 Second Dimension or Separation of Proteins by Mr.

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the protein unfolded and charged by anionic detergent SDS is carried out in an Ettan Dalt six gel system (GE Healthcare, Uppsala, Sweden) (Fig. 3). The gel pore size could be differently adjusted by percentages of polyacrylamide and cross-linker. In this regard, the more common percentage ranged from 10% to 15% w/v polyacrylamide gels.

Solutions and buffers: Prepare all solutions using ultrapure water and analytical grade reagents.

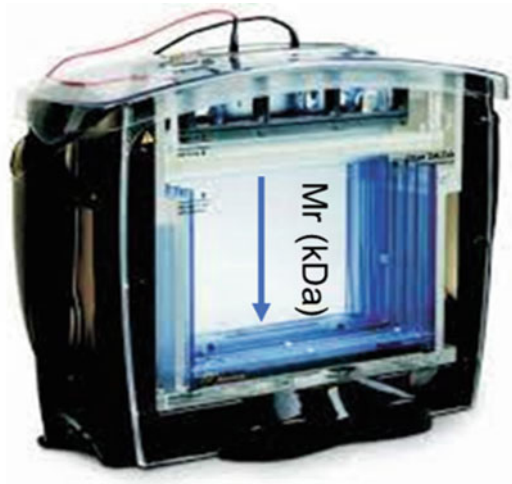

Fig. 3 Ettan Dalt six gel system (GE Healthcare, Uppsala, Sweden)

Table 3
Composition (volume) of polymerization solution according to acrylamide percentage

Percent of acrylamide	10%	12%	15%
Acrylamide solution	136 mL	171.3 mL	207 mL
Tris solution (50 mM Tris pH 8.8)	104 mL	103 mL	104 mL
Ultrapure water	162 mL	127 mL	95 mL
SDS (10%)	4.1 mL	4.1 mL	4.1 mL
PSA (persulfate ammonium) (10%)	4.1 mL	4.1 mL	4.1 mL
TEMED (N,N,N′,N′-tetramethyl-ethyl-1,2-diamine)	82 µL	64 µL	48 µL

Values are calculated for the preparation of six gels in the Gel Caster (Ettan Dalt six gel system, GE Healthcare, Uppsala, Sweden)

- *Acrylamide solution:* Dissolve 7.8 g of bis-acrylamide and 292.2 g of acrylamide in ultrapure water to reach a total volume of 1 L in dark conditions.
- *Polymerization solution*: The polyacrylamide gels consist of the polymerization from acrylamide and bis-acrylamide initiated by TEMED and PSA to free radicals. Thus, the mixture for different percentages of acrylamide should contain the amounts shown in Table 3. This solution should be prepared with constant agitation and without interruptions. Finally, the solution is poured into the Gel Caster and covered with water-saturated isobutanol. The polymerization occurs for approximately 6 h at room temperature.
- *10× Electrophoresis buffer (50 mM Tris base, 384 mM glycine and 0.2% SDS)*: Dissolve 30.2 g of Tris, 144.2 g of glycine, and 10 g of SDS in distilled water reaching a total volume of 1 L. Thus,

1× electrophoresis buffer is obtained by adding 1 part of this solution to 9 parts of the distilled water and 2× electrophoresis buffer by adding 2 parts of this to 8 parts of the distilled water.

- *Agarose solution:* Heat to boiling 0.5 g of agarose in 100 mL of 1× electrophoresis buffer for its dissolution and add traces of bromophenol blue.

2.3.1 Protocol

1. The IPG strips should be transferred to gels polymerized according to the previous section. It should be embedded the strip on the top of the second dimension and sealing the place, with molten agarose solution and bromophenol blue to ensure good contact between the gel and the IPG strip and to track the electrophoresis with the dye.
2. The electrophoresis unit (Ettan Dalt six gel system, GE Healthcare, Uppsala, Sweden) is assembled according to the manual.
3. The upper chamber is filled with 1 L of 2× electrophoresis buffer meanwhile the lower chamber needs 4.5 L of 1× electrophoresis buffer.
4. Electrophoresis runs applying 5 mA/gel, 2 h; 10 mA/gel for 3 h and finally 16 mA/gel until the color blue reaches the gel bottom indicating the end of the electrophoresis.
5. After the electrophoresis, the gels are placed in plastic boxes to proceed to the staining.

2.4 Staining of Gels and Comparison of Gel Images Using a Specific Software Package

Gel staining protocols should be chosen depending on the specific needs. Protein staining should be mainly carried out using Coomassie blue, silver staining or Sypro Ruby® stain (Bio-Rad Laboratories). In the case of silver staining, it is a very excellent sensitive method as well as very simple and cheap. The method is based on the selective reduction of silver in the proximity of protein molecules [9]. However, it is less used due to its incompatibility with the mass spectrometry necessary for protein identification. In this sense, coomassie blue has been widely employed to stain the proteins in-gel for many years. The main benefit of this stain is economical, and the equipment used is a simple densitometer, but the protein detection is poor about 30 ng. Hence, this methodology is being implemented to improve these aspects [10]. On the other hand, fluorescent staining is often preferred by its high sensitivity and dynamic range. Sypro Ruby® stain (Bio-Rad Laboratories) is the most widely used achieving a protein detection of 2 ng, but it is necessary the use of GelDoc XR System (Bio-Rad Laboratories) (Fig. 4) or similar at least.

2.4.1 SYPRO® Ruby Protein Stain According to the Instruction Manual

Gels are stained with SYPRO Ruby® stain (Bio-Rad Laboratories), following the manufacturer indications, and digitalized using the GelDoc XR System (Bio-Rad Laboratories). The volume of

Fig. 4 GelDoc XR System (Bio-Rad Laboratories)

solutions will be determined by box-sized considering that the gel must be immersed in the solution. Furthermore, a rocker is necessary for gentle mixing of the gel in the solutions.

Protocol

1. Gel is rinsed in a fixed solution (ethanol 40% and acid acetic 10%) for 3 h.
2. Gel is washed with distilled water for 30 min three times.
3. In dark conditions, the gel is immersed in fluorescent stain overnight.
4. Finally, the gel is rinsed twice with distilled water in darkness for 30 min.
5. The gel is digitalized in GelDoc XR System (Bio-Rad Laboratories).

Afterwards, the 2-DE images can be analyzed using PDQuest 2-D Analysis Software (Bio-Rad Laboratories).

In Fig. 5 two examples of polyacrylamide gels (12%), employing strips of 24 cm in the pH range 4–7 stained with SYPRO Ruby and obtained from our lab are displayed. The p*I* and Mr. of spots

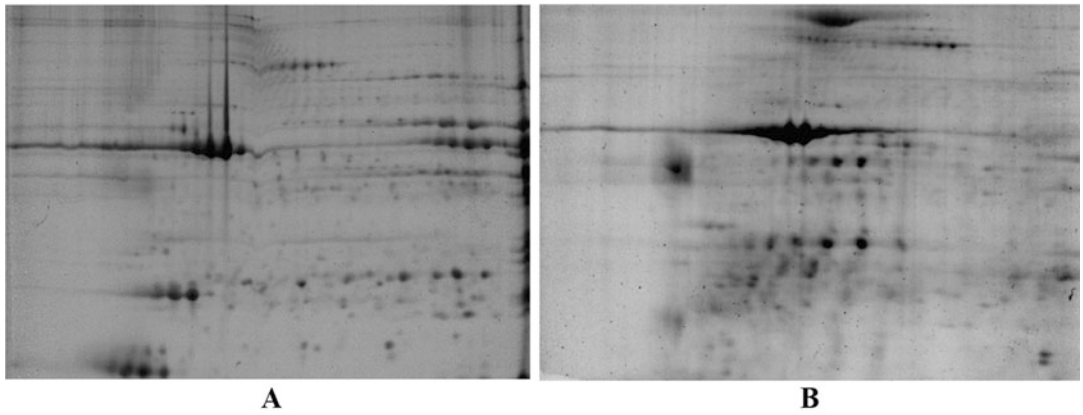

Fig. 5 Representative 2-DE gel protein profiles of fresh meat from *Longissimus thoracis* of *Bos taurus* (**a**) and dry-cured ham of *Sus scrofa* (**b**)

are determined from their position on the IEF-strips and standard molecular mass markers ranging from 15 to 200 kDa (Fermentas, Ontario) could be added, respectively.

2.5 Tryptic Digestion of Gel Spots

In a comparative proteomic analysis, the protein spots are chosen to be identified by mass spectrometry, thus, the protein spots are manually excised from the gel using a pipette tip or similar.

Solution:

- *Ambic buffer*: 50 mM ammonium bicarbonate in 50% methanol.

2.5.1 Protocol

1. The excised spots are cut into pieces and washed with Milli-Q water.
2. The pieces are twice rinsed with Ambic buffer for 20 min after centrifugation (600 rpm), the supernatant is discarded.
3. The gel pieces are dehydrated with 100 μL acetonitrile for 10 min and subsequently dried in a vacuum centrifuge.
4. Following a reduction step with 10 mM DTT in Ambic buffer for 30 min at 56 °C after centrifugation (600 rpm), the supernatant is discarded.
5. Repeat **step 2**.
6. The alkylation step is produced by 55 mM iodoacetamide in Ambic buffer freshly prepared for 20 min in darkness.
7. Repeat **step 2**.
8. Modified porcine trypsin (Promega) is added to the dry gel pieces at a final concentration of 20 ng/μL in Ambic buffer incubating it at 37 °C for 16 h. Afterwards, centrifugation (600 rpm) is used to discard the supernatant.
9. Peptides are extracted thrice by 20 min incubation in 40 μL of 60% acetonitrile in 0.5% HCOOH under constant agitation.

The supernatant is dried and stored at −20 °C for further analysis.

In the next and final main step, a MALDI-TOF MS is used to identify the proteins.

2.6 Identification of Proteins by Mass Spectrometry

Matrix solution is prepared using 0.5 μL of peptides and 3 mg CHCA dissolved in 1 mL of 50% acetonitrile in 0.1% TFA. The final solution is placed on the Opti-TOF MALDI plate (Applied Biosystems). The mass spectrometry was carried out using a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems) by reflectron positive-ion mode with a Nd:YAG, 355 nm wavelength laser, averaging 1000 laser shots and at least three trypsin autolysis peaks used as internal calibration. For tandem mass spectrometry (MS/MS), the data are performed by resolution of 300 (FWHM) and metastable suppression. GPS Explorer Software v3.6 using Mascot software v2.1. (Matrix Science) identify the proteins by peptide mass fingerprinting (PMF) and peptide fragmentation spectra data in a protein database. The parameters most used are 30 ppm precursor tolerance, 0.35 Da MSMS fragment tolerance, CAM (carbamidomethyl cysteine) as fixed modification, oxidized methionine as variable modification and allowing 1 missed cleavage. Finally, only the protein identifications are considered in the case of the score are greater than 56 and when protein score CI (Confidence Interval) was above 98%. For MS/MS, the total ion score CI should achieve above 95%.

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Chapter 14

Antioxidant Capacity

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Abstract

The requirements of the meat industry have made the determination of antioxidant capacity acquire special interest today, because these tests help to know the antioxidant status of meat and meat products. For this reason, the analysis of the antioxidant capacity represents a useful tool when studying the influence of diverse procedures in meat production (from animal husbandry to meat products elaboration), since together with other techniques it allows the election of procedures that favor the extension of the shelf life of these foods. However, despite this importance, the antioxidant capacity tests lack adequate standardization, because even using the same assays and fundamentals, different conditions and standards can be employed by researchers. This fact makes it very difficult to compare scientific results. On the other hand, the determination of the antioxidant capacity has been relatively little studied in products of animal origin, making it even more difficult to standardize the methods for a matrix as complex as meat. Taken together, these facts can hinder the advances pursued by the meat industry, generating economic losses in the sector.

Therefore, this book chapter aims to share in detail both the procedure of a general extraction method of antioxidant compounds and the methodology of three of the most used antioxidant capacity assays (FRAP, DPPH, and ABTS), in order to facilitate the comparison of outcomes.

Key words Antioxidants compounds, Antioxidant assays, FRAP, DPPH, ABTS, Absorbance, Spectrophotometric methods

1 Introduction

At present, the determination of the antioxidant capacity plays a considerable role in meat and meat products due to the importance that oxidative deterioration processes have in these foods [1, 2]. In this sense, the complementary use of the traditional oxidation analyses and the antioxidant capacity techniques is of special interest because these last tests allow to know the antioxidant status of meat and meat products [3]. At the same time, the antioxidant capacity assays permit to implement enhancements in the meat industry based on the improvement of the oxidative stability of

José Manuel Lorenzo et al. (eds.), *Methods to Assess the Quality of Meat Products*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2002-1_14,

these foods through the study of different strategies such as the enrichment in antioxidant substances using certain animal diets [4] and animal breeds [5] or employing particular antioxidant compounds in the preparation of meat-based products [6].

Given that oxidative stability improvement strategies are one of the main approaches addressed in the meat industry with the aim of avoiding the oxidation processes and extending the shelf life of meat and meat products, it seems clear that the determination of antioxidant capacity is an analysis that can be of crucial importance. On this matter, there are different methodologies that allow to know the antioxidant status of foods [7]. However, these analytical techniques are currently far from being standardized. In addition to this disadvantage, the determination of the antioxidant capacity in foods of animal origin has been studied to a lesser extent compared to other foods [8, 9], which makes their suitable application and utility in meat and meat products more difficult. For this reason, the methodology proposed in this book chapter tries to provide analysts with a unifying tool, explaining the possible considerations and differentiations to take into account when performing antioxidant capacity assays in these foods.

Prior to determining the antioxidant capacity of meat and meat products, an extraction of the antioxidant substances present in these products must be carried out. Currently, there is a great variety of methodologies for this purpose in terms of the steps and solvents employed. However, all processes are usually based on a solid-liquid extraction, the main difference being the solvent utilized that affects the extracted compounds [10–13]. Furthermore, the extraction of antioxidant compounds of meat and meat-based products can include some additional step that helps to promote the release of these substances. The present chapter describes a feasible and easy extraction technique with the use of a reagent commonly employed in the laboratory (80% methanol) together with the application of ultrasounds [4]. This simple technology favors the liberation of the antioxidant compounds due to the elongation of the pores and rupture of the cells.

Once the extraction process of the antioxidant compounds has been carried out, the determinations of the antioxidant capacity can be performed in the meat extract obtained. Although there are different methods, the most used in meat and meat products are the ferric ion reducing antioxidant power (FRAP) test, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging assay, and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS) scavenging probe due to their easy implementation [3]. These three methods are spectrophotometric tests based on the reactions between a colored complex or free radical with the antioxidant molecules capable of reducing an oxidizing substance and/or donating a hydrogen atom. Concretely, the FRAP technique is a typical electron transfer-based method because this test

measures the ability of antioxidants to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) employing the ferric 2,4,6-tripyridyl-*s*-triazine complex $[Fe^{3+} - (TPTZ)_2]^{3+}$ present in the FRAP reagent [7]. Thus, the colorless ferric complex $[Fe^{3+} - (TPTZ)_2]^{3+}$ can be reduced in the presence of antioxidant compounds to the navy blue colored ferrous complex $[Fe^{2+} - (TPTZ)_2]^{2+}$, which has a maximum of absorbance at 593 nm. In this way, the measurement of the increase in absorbance at this wavelength can be carried out by an UV/Vis spectrophotometer, which allows to monitor the formation of the $[Fe^{2+} - (TPTZ)_2]^{2+}$ complex and relates this value to the total reducing capacity of electron-donating antioxidants present in the meat sample extract [14]. On the other hand, the DPPH and ABTS methods are based on the combination of electron transfer and hydrogen donation reactions, being known as mixed-based methods, although the dominant reactions in the case of the DPPH assay are the electron transfer reactions [15]. Specifically, the DPPH test is based on the neutralization of the 2,2-diphenyl-1-picrylhydrazyl free radical, which has a violet coloration at 515–528 nm that is lost when contacting with an antioxidant substance. This loss of coloration can be related to the presence of antioxidants in the meat extract by monitoring the absorbance spectrophotometrically at 515 nm, since this parameter depends linearly on the concentration of antioxidants compounds [16, 17]. Similarly, the ABTS assay is based on the neutralization of the ABTS free radical, which has a blue-green color with a maximum absorbance at 730 nm. This test allows to measure the ability of antioxidants to reduce the ABTS radical (previously generated), which can be monitored by loss of blue-green coloration through the measurement of absorbance at 730 nm in an UV/Vis spectrophotometer [18].

With all that previously reported, the intention of this book chapter is to serve as a guide for the determination of the antioxidant capacity in meat and meat products (including the initial extraction step), thus allowing the standardization of the existing tests to favor their use in the laboratory and the correct comparisons in the analysis of these foods for the benefit of the meat industry.

2 Materials

Prepare all solutions using distilled water and/or analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Volumetric flasks were always used to adjust the volume of the reagents and standard solutions (unless indicated otherwise). When samples or reagents must be protected from light during their preparation and storage, they are protected by covering them with aluminum or using opaque material.

Diligently follow all waste disposal regulations when disposing of waste materials (*see* **Note 1**).

2.1 Sample Preparation for Analysis

1. Professional mill KN 295 Knifetec (Foss).
2. Homogeniser HM 294 (Foss).
3. Cutting board.
4. Knife.

2.2 Extraction of Antioxidant Compounds

1. Analytical balance, with a resolution of 0.0001 g, mod. ME 614S (Sartorius).
2. Polypropylene tubes of 20 and 40 mL.
3. Tube rack.
4. Borosilicate glass graduated pipette of 25 ± 0.1 mL.
5. Vortex stirrer (*see* **Note 2**).
6. Rocking shaker, mod. SW-3D-E (OVAN).
7. Ultrasound water bath apparatus, mod. 8510E-DTH (Bransonic®).
8. Centrifuge, mod. Allegra™ X-22R (Beckman Coulter).
9. Qualitative filter paper (*see* **Note 3**).
10. Aluminum foil (*see* **Note 4**).
11. 80% Methanol (v/v): Mix 800 mL of pure methanol with 200 mL of distiller water.

2.3 Ferric Ion Reducing Antioxidant Power (FRAP) Assay

1. Analytical balance, with a resolution of 0.0001 g, mod. ME 614S (Sartorius).
2. Precision balance with a resolution of 0.01 g, mod. TE 612 (Sartorius).
3. Volumetric flasks and beakers of borosilicate glass.
4. Aluminum foil (*see* **Note 4**).
5. Microtubes of 1.5 mL.
6. Opaque rack for microtubes (*see* **Note 5**).
7. Magnetic stirrer bars and magnetic stirrer plate.
8. Calibration Check pH Bench Meter, mod. HI 221 (Hanna).
9. Temperature controlled water bath, mod. 6001197 (JP Selecta™).
10. Eppendorf Research Micropipettes of 10–100, 20–200, 100–1000, and 500–5000 μL.
11. Quartzglass cuvettes of 1000 μL (Hellma Analytics).
12. UV/Vis spectrophotometer, mod. UV-1800 (Shimadzu).
13. Buffer solution pH 7.00 ± 0.02.

14. Buffer solution pH 4.00 ± 0.02.
15. 40 mM hydrochloric acid: Mix 331 μL of 37% hydrochloric acid with distilled water up to 100 mL.
16. FRAP reagent: Mix 100 mL of 0.3 M acetate buffer solution (pH 3.6) with 10 mL of 10 mM 2, 4, 6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution, and 10 mL of 20 mM ferric chloride solution under darkness (*see* **Note 6**).
    (a) 0.03 M acetate buffer solution (pH 3.6): Dissolve 2.46 g of sodium acetate with an approximate volume of 70 mL of distilled water in a 100 mL beaker. Adjust the pH to 3.6 using glacial acetic acid (with the help of a benchtop pH meter, previously calibrated with buffers 4 and 7). After adjusting the pH to 3.6, bring the volume to 100 mL.
    (b) 10 mM 2, 4, 6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution: Dissolve 0.0312 g of 2, 4, 6-tri(2-pyridyl)-1,3,5-triazine in 10 mL of 40 mM hydrochloric acid (measured with a 500–5000 μL micropipette).
    (c) 20 mM ferric chloride solution: Dissolve 0.0540 g of ferric chloride in 10 mL of distilled water (measured with a 500–5000 μL micropipette).
17. Ferrous sulfate stock solution (5 mM): Dissolve 0.0139 g of ferrous sulfate heptahydrate in 10 mL of 40 mM hydrochloric acid (*see* **Note 6**).

2.4 2,2-Diphenyl-1-Picrylhydrazyl Radical (DPPH) Scavenging Assay

1. Analytical balance, with a resolution of 0.0001 g, mod. ME 614S (Sartorius).
2. Borosilicate glass weighing funnels (*see* **Note 7**).
3. Volumetric flasks and beakers of borosilicate glass.
4. Aluminum foil (*see* **Note 4**).
5. Opaque glass test tubes of 12 mL (*see* **Note 4**).
6. Metal tube rack.
7. Magnetic stirrer plate and magnetic stirrer bars.
8. Temperature controlled water bath, mod. 6001197 (JP Selecta™).
9. Eppendorf Research Micropipettes of 20–200, 100–1000 and 500–5000 μL.
10. Quartzglass cuvettes of 3 mL (Hellema Analytics).
11. UV/Vis spectrophotometer, mod. UV-1800 (Shimadzu).
12. 2,2-Diphenyl-1-picrylhydrazyl reagent (60 μM): Dissolve 0.0059 g of 2,2-diphenyl-1-picrylhydrazyl in 250 mL of pure methanol under darkness (*see* **Notes 6–8**).

13. Trolox stock solution (1.2 mM): Dissolve 0.0030 g of trolox in 100 mL of pure methanol protected from light using a volumetric flask (*see* **Notes 6**, **7** and **9**).

2.5 2,2′-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) Radical Cation (ABTS) Scavenging Assay

1. Analytical balance, with a resolution of 0.0001 g, mod. ME 614S (Sartorius).
2. Borosilicate glass weighing funnels (*see* **Note 7**).
3. Volumetric flasks and beakers of borosilicate glass.
4. Aluminum foil (*see* **Note 4**).
5. Microtubes of 1.5 mL.
6. Opaque rack for microtubes.
7. Magnetic stirrer bars and magnetic stirrer plate.
8. Eppendorf Research Micropipette of 10–100, 20–200, and 100–1000 μL.
9. Quartzglass cuvettes of 1000 μL (Hellema Analytics).
10. UV/Vis spectrophotometer, mod. 115 VAC (Shimadzu).
11. 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) reagent: Generated from the dilution with distilled water of the initial mixture of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and potassium persulfate (initial concentrations for ABTS and potassium persulfate: 7 and 2.45 mM, respectively) (*see* **Notes 6** and **10**).
12. Ascorbic acid stock solution (1.7 mM): Dissolve 0.0030 g of ascorbic acid in 100 mL of distilled water under darkness (*see* **Notes 6** and **11**).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Sample Preparation for Analysis

The first step that we must take is to mince the whole sample and homogenize it correctly [19]. Firstly, we must cut the sample into small blocks and then, mince and homogenize them. Both minced and homogenization are carried out with a professional mill KN 295 Knifetec (Foss) or a Homogeniser HM 294 (Foss) depending on the type/size of the sample. This step is very important to achieve a good and representative result.

3.2 Extraction of Antioxidant Compounds

Prior to the determination of the meat antioxidant capacity, an extraction of the antioxidant compounds must be carried out. For this, a solid-liquid extraction was performed following the procedure of Santos et al. [20], with modifications. Figure 1 shows in a

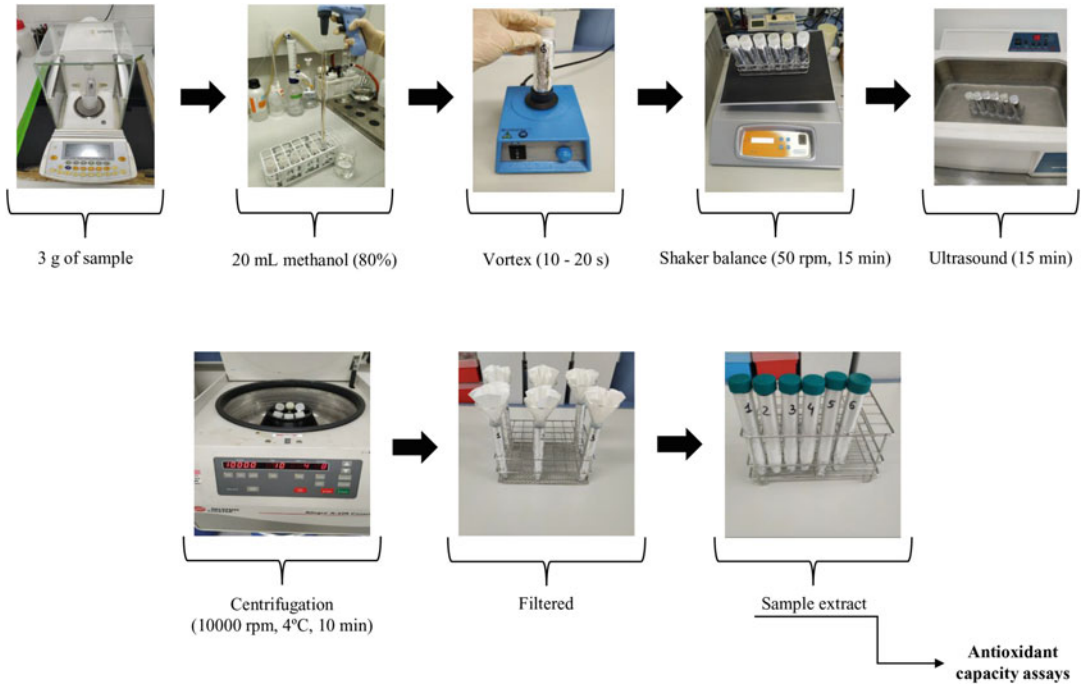

Fig. 1 Schematic representation of the steps of the antioxidant compounds extraction

simplified and schematic way the steps of the antioxidant's extraction.

1. Firstly, weigh 3.0000 ± 0.0100 g of sample (*see* **Note 12**) in 40 mL polypropylene tubes protected from light.
2. Then, add 20 mL of 80% methanol (*see* **Notes 13** and **14**) to the polypropylene tube, close it, and homogenize for 10–20 s in a vortex-type stirrer (*see* **Note 2**).
3. After initial vortex homogenization, place tubes on a balance shaker in a rack at 50 rpm, for 15 min.
4. Next, take the tubes in the same rack in an ultrasound water bath for 15 min, applying the sonic mode (*see* **Note 15**).
5. Following that, you must centrifuge the tubes at 10,500 × *g* for 10 min, maintaining a temperature of 4 ± 1 °C.
6. Subsequently, filter the supernatant through qualitative filter paper (*see* **Note 3**) on 20 mL polypropylene tubes under darkness (*see* **Note 16**).
7. Finally, store at refrigeration temperatures (3 ± 1 °C) if your analysis is going to be shortly, or on the contrary, store frozen at temperatures below −30 ± 1 °C.

3.3 Ferric Ion Reducing Antioxidant Power (FRAP) Assay

The FRAP method was carried out by spectrophotometry according to the procedure previously described by Benzie and Strain [14], with brief modifications. Figure 2 displays in a simplified and schematic way the steps of the FRAP determination.

1. Initially, you must prepare in microtubes the seven dilutions of the calibration curve (ranging from 0 to 2 mM) from the 5 mM ferrous sulfate stock solution, under darkness (*see* **Note 17**). You can see the volumes of 5 mM ferrous stock solution and distilled water needed to prepare the seven points of the calibration curve in Table 1.
2. You must transfer 30 μL of each point of the calibration curve of ferrous sulfate to a microtube, protected from light. Identically, pipet 30 μL of the sample extract into its respective microtube (*see* **Note 18**).
3. Next, add 90 μL of distilled water to each previous microtube.
4. Subsequently, you must add 900 μL of FRAP reagent.
5. Then, homogenize each microtube in a vortex.

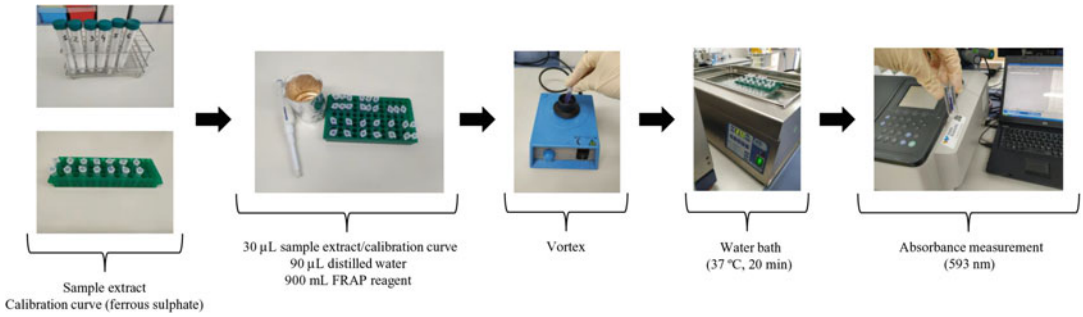

Fig. 2 Schematic representation of the steps of the FRAP determination

Table 1
Volumes required for the preparation of the ferrous sulfate calibration curve

Point	5 mM ferrous sulfate solution (μL)	Distilled water (μL)	Ferrous sulfate concentration (μM)
1	0	1000	0
2	25	975	125
3	50	950	250
4	100	900	500
5	200	800	1000
6	300	700	1500
7	400	600	2000

6. Afterwards, incubate all the microtubes in a water bath during 20 min at 37 ± 1 °C using a rack and protecting the microtubes from light.
7. Finally, you must measure the absorbance of each point of the calibration curve and sample extract in the spectrophotometer, at a wavelength of 593 nm (*see* **Note 19**).
8. Expression of results:

   The results of antioxidant capacity through the FRAP method can be expressed in μmol Fe^{2+}/100 g of sample following the next steps:

   (a) Calculation of the equation of the ferrous sulfate calibration curve (*see* **Note 20**), where the absorbance is related to the Fe^{2+} concentration as follows:

   $$\text{Absorbance} = a \cdot [Fe^{2+}] + b$$

   where: a is the slope value. $[Fe^{2+}]$ is the known concentration of Fe^{2+} (μM). b is the y-intercept value.

   (b) Next, the absorbance value obtained for the sample extracts is substituted in the previous equation and the Fe^{2+} concentration is calculated in μM as follows:

   $$[Fe^{2+}] = \frac{\text{Absorbance} - b}{a}$$

   where: a is the slope value, previously calculated. $[Fe^{2+}]$ is the concentration of Fe^{2+} calculated for the sample extract (μM). b is the y-intercept value, previously calculated.

   (c) Lastly, the results can be transformed into μmol Fe^{2+}/100 g of sample following the next formula:

   $$\frac{\mu\text{mol } Fe^{2+}}{100 \text{ g of sample}} = [Fe^{2+}] \cdot \frac{V}{g} \cdot 100$$

   where: $[Fe^{2+}]$ is the concentration of Fe^{2+} (μM), previously calculated. V is the volume used in the prior extraction of antioxidant compounds (L). g is the sample weight used in the extraction of antioxidant compounds (g).

3.4 2,2-Diphenyl-1-Picrylhydrazyl Radical (DPPH) Scavenging Assay

The DPPH assay was performed by spectrophotometry following the method previously reported by Brand-Williams [21], with slight changes. Figure 3 displays in a simplified and schematic way the steps of the DPPH determination.

1. Initially, you must prepare in test tubes the seven dilutions of the calibration curve (ranging from 0 to 1.2 mM) from the 1.2 mM trolox stock solution, protected from light (*see* **Note 17**). You can see the volumes of 1.2 mM trolox stock solution and methanol needed to prepare the seven points of the calibration curve in Table 2.

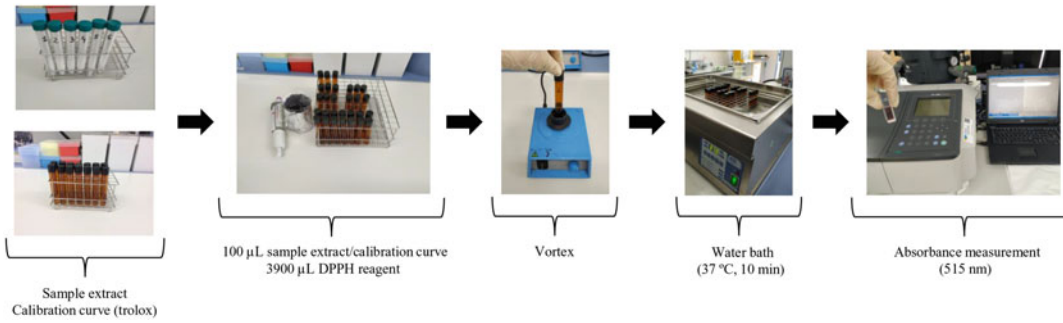

Fig. 3 Schematic representation of the steps of the DPPH determination

Table 2
Volumes required for the preparation of the trolox calibration curve

Point	1.2 mM trolox solution (mL)	Methanol (mL)	Trolox concentration (mM)
1	0	4.000	0
2	1.000	3.000	0.30
3	2.000	2.000	0.60
4	2.500	1.500	0.75
5	3.000	1.000	0.90
6	3.500	0.500	1.05
7	4.000	0	1.20

2. You must pipet 100 µL of each point of the calibration curve of trolox in its respective test tube, under darkness. Identically, transfer 100 µL of the sample extract into a test tube (*see* **Note 18**).
3. Next, you must add 3900 µL of DPPH to each previous test tube.
4. Then, homogenize the contents of the test tubes in a vortex.
5. Subsequently, incubate all the microtubes in a water bath at 37 ± 1 °C for 10 min, employing a rack and protecting the microtubes from light.
6. After the incubation, you must measure the absorbance of the DPPH reagent and the absorbance of each point of the calibration curve and sample extract in the spectrophotometer, at a wavelength of 515 nm (*see* **Note 21**).
7. Expression of results:

   The results of antioxidant capacity through the DPPH assay can be expressed in µg trolox/g of sample following the next steps:

(a) Calculation of the inhibition percentage of the calibration curve and the sample extract by the following formula:

$$\text{Inhibition (\%)} = \frac{\text{Abs(blank)} - \text{Abs(calibration curve/sample extract)}}{\text{Abs(blank)}} \cdot 100$$

where: Abs(blank) is the absorbance at 515 nm of the DPPH reagent. Abs(calibration curve/sample extract) is the absorbance at 515 nm of points 1–7 of the calibration curve (Table 2) and of the sample extracts, respectively.

(b) Calculation of the equation of the trolox calibration curve (*see* **Note 20**), where the percentage of inhibition calculated in the previous step is related with the trolox concentration as follows:

$$\text{Inhibition (\%)} = a \cdot [\text{Trolox}] + b$$

where: a is the slope value. [Trolox] is the known concentration of trolox (mM). b is the y-intercept value.

(c) Next, the percentage of inhibition calculated in the previous step for sample extracts is substituted in the trolox calibration curve equation and the trolox concentration is calculated in mM as follows:

$$[\text{Trolox}] = \frac{\text{Inhibition (\%)} - b}{a}$$

where: a is the slope value, previously calculated. [Trolox] is the concentration of trolox calculated for the sample extract (mM). b is the y-intercept value, previously calculated.

(d) Then, the concentration of trolox must be changed from mM to μg/L, using the molecular mass of trolox as follows:

$$\frac{\mu\text{g trolox}}{\text{L}} = \text{mM trolox} \cdot \text{PM} \cdot 10^3$$

where: PM is the molecular weight of trolox (250.29 g/mol).

(e) Lastly, the results can be transformed into μg trolox/g of sample following the next formula:

$$\frac{\mu\text{g trolox}}{\text{g of sample}} = [\text{Trolox}] \cdot \frac{V}{g} \cdot 100$$

where: [Trolox] is the concentration of trolox (μg/L), previously calculated. V is the volume used in the prior extraction of antioxidant compounds (L). g is the sample weight used in the extraction of antioxidant compounds (g).

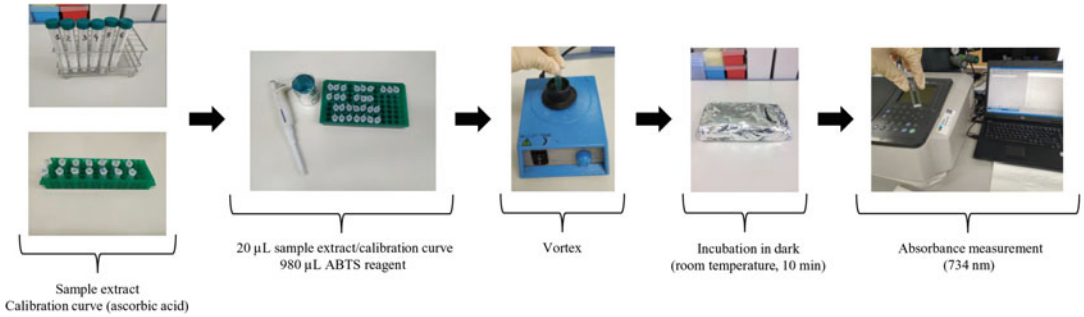

Fig. 4 Schematic representation of the steps of the ABTS determination

Table 3
Volumes required for the preparation of the ascorbic acid calibration curve

Point	1.7 mM ascorbic acid solution (µL)	Distilled water (µL)	Ascorbic acid concentration (µM)
1	0	1000	0
2	100	900	170.34
3	200	800	340.68
4	300	700	511.02
5	400	600	681.35
6	500	500	851.69

3.5 2,2′-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) Radical Cation (ABTS) Scavenging Assay

The ABTS method was carried out by spectrophotometry following the procedure previously described by Re et al. [22], with some modifications. Figure 4 displays in a simplified and schematic way the steps of the ABTS assay.

1. Firstly, you must prepare in microtubes the six dilutions of the calibration curve (between 0 and 851.69 µM) from the 1.7 mM ascorbic acid stock solution, protected from light (*see* **Note 17**). You can see the volumes of 1.7 mM ascorbic acid stock solution and distiller water needed to prepare the six points of the calibration curve in Table 3.
2. Then, you must transfer 20 µL of each point of the calibration curve to a microtube, protected from light. Identically, pipet 20 µL of the sample extract into its respective microtube (*see* **Note 18**).
3. Next, you must add 980 µL of the ABTS solution to each previous microtube.
4. Immediately, homogenize the content in a vortex.
5. Then, incubate all the microtubes protected from light at room temperature (21 ± 1 °C) during 10 min.

6. After the incubation, you must measure the absorbance of each point of the calibration curve and sample extract in the spectrophotometer, at a wavelength of 734 nm (*see* **Note 22**).
7. Expression of results:

   The results of antioxidant capacity through the ABTS assay can be expressed in mg ascorbic acid/100 g of sample following the next steps:

   (a) Calculation of the equation of the ascorbic acid calibration curve (*see* **Note 20**), where the absorbance is related to the ascorbic acid concentration as follows:

$$\text{Absorbance} = a \cdot [\text{AA}] + b$$

   where: a is the slope value. [AA] is the known concentration of ascorbic acid (μM). b is the y-intercept value.

   (b) Next, the absorbance value obtained for the sample extracts is substituted in the previous equation and the ascorbic acid concentration is calculated in μM as follows:

$$[\text{AA}] = \frac{\text{Absorbance} - b}{a}$$

   where: a is the slope value, previously calculated. [AA] is the concentration of ascorbic acid calculated for the sample extract (μM). b is the y-intercept value, previously calculated.

   (c) Then, the concentration of ascorbic acid must be changed from μM to mg/L, using the molecular mass of ascorbic acid as follows:

$$\frac{\text{mg ascorbic acid}}{\text{L}} = \mu\text{M ascorbic acic} \cdot \frac{\text{PM}}{10^3} \cdot 100$$

   where: PM is the molecular weight of ascorbic acid (176.12 g/mol).

   (d) Lastly, the results can be transformed into mg ascorbic acid/100 g of sample following the next formula:

$$\frac{\text{mg ascorbic acid}}{\text{100 g of sample}} = [\text{AA}] \cdot \frac{V}{g} \cdot 100$$

   where: [AA] is the concentration of ascorbic acid (mg/L), previously calculated. V is the volume used in the prior extraction of antioxidant compounds (L). g is the sample weight used in the extraction of antioxidant compounds (g).

4 Notes

1. All laboratory waste must be eliminated according to its nature following current regulations, differentiating at least acids, bases, organic solvents, halogenated solvents, and non-halogenated solvents.
2. Instead of vortex-type stirrers, dispersion devices can also be used in order to homogenize the sample.
3. Different filters can also be used at this stage according to sample needs.
4. Aluminum foil is not required if opaque material is used and vice versa.
5. The opaque rack will avoid the need to use aluminum foil or opaque microtubes, which can be more expensive.
6. The solution must be prepared daily and for each batch of samples to be determined.
7. Due to the very small mass that we have to use to prepare the solution, weighing must be performed in weighing funnels.
8. The DPPH reagent should be weighed on weighing funnels and then transferred to a beaker by dragging with methanol extra pure. After constant stirring until complete dissolution with a volume of methanol of approximately 220 mL, the volume of the solution will be adjusted to 250 mL in a volumetric flask and stored under darkness.
9. The trolox stock solution should be weighed on weighing funnels and then transferred to a beaker by dragging with methanol extra pure. After constant stirring until complete dissolution with a volume of methanol of approximately 80 mL, the volume of the standard solution will be adjusted to 100 mL in a volumetric flask and stored protected from light.
10. 0.0970 g of ABTS and 0.165 g of potassium persulfate are weighed separately on weighing funnels and transferred to a beaker by dragging with distilled water. After constant stirring until complete dissolution with a volume of distilled water of approximately 20 mL, the volume of the solution will be adjusted to 25 mL in a volumetric flask and stored protected from light during almost 16 h. After this period, the reagent is diluted with distilled water until its absorbance is adjusted to 0.700 ± 0.020 at 734 nm (with a previous autozero made with distilled water). It is possible to start with an illustrative quantity of 540 μL of ABTS and 50 mL of distilled water and gradually add more ABTS or distilled water if the absorbance is less or greater than 0.700 ± 0.020, respectively.

11. The ascorbic acid stock solution should be weighed on weighing funnels and then transferred to a beaker by dragging with distilled water. After constant stirring until complete dissolution with a volume of distilled water of approximately 80 mL, the volume of the solution will be adjusted to 100 mL in a volumetric flask and stored under darkness.
12. Sometimes and because meat normally has low antioxidant capacity, instead of being used fresh, the sample is employed after being lyophilized, so that the antioxidant compounds are concentrated [12].
13. The solvent used for extraction may vary according to the study in question, the usual solvents being ethanol, chloroform, phosphate buffer, and water [10–13], apart from 80% methanol [2].
14. It should be noted that different solvents will extract different antioxidant compounds.
15. Ultrasound treatment is done to help release antioxidant compounds from cells, increasing extraction performance [4, 10].
16. This liquid will be the extract of the sample that you will analyze through the different antioxidant capacity assays.
17. The calibration curve should be prepared immediately before adding the reagents, to avoid losing antioxidant capacity.
18. Depending on the antioxidant capacity of the sample extract, it may require prior dilution.
19. Before measuring absorbances at 593 nm, an autozero should be performed with distilled water.
20. The value of r^2 obtained for the equation of the straight line must be greater than 0.900.
21. Before measuring absorbances at 515 nm, an autozero should be performed with methanol.
22. Before measuring absorbances at 734 nm, an autozero should be performed with distilled water.

Acknowledgments

This work was supported by Centro Tecnolóxico de la Carne de Galicia. Thanks to GAIN (Axencia Galega de Innovación) for supporting this book chapter (grant number IN607A2019/01). Authors are members of the Healthy Meat network, funded by CYTED (ref. 119RT0568). Noemí Echegaray acknowledges to Consellería de Cultura, Educación e Ordenación Universitaria (Xunta de Galicia) for granting with a predoctoral scholarship (Grant number IN606A-2018/002).

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