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***Polycyclic Aromatic Hydrocarbons analysis
by acetonitrile-based extraction – Smoked
Salmon compliance with EU legislation***

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Few things in life are accomplished alone and I am grateful to everyone crossing my path in each stage. I would like to acknowledge:

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Abstract

Polycyclic Aromatic Hydrocarbons (PAHs) are ubiquitous compounds, unavoidably present in food. The European Commission sets maximum levels for benzo(a)pyrene (BaP) and for the sum of four substances – PAH4 (BaP, chrysene, benz(a)anthracene, benzo(b)fluoranthene) in a range of foodstuffs, considering this sum a suitable marker for the occurrence and carcinogenic potency of PAHs in food. Smoked and grilled/barbecued foods may contribute significantly to PAHs intake, if such foods comprise a large part of the usual diet. The European Regulation 836/2011 laid down criteria of performance for the methodologies intended to determine PAH4 in foods.

In this thesis, a simple, rapid and efficient methodology was developed, for the extraction and determination of 14 PAHs, including PAH4, in grilled and smoked meat and fish. The analytical strategy consisted in using an acetonitrile-based extraction coupled to high performance liquid chromatography with fluorescence detection. The optimal working conditions were 5 g of sample homogenised with 5 mL of water and 5mL of acetonitrile, followed by partitioning through the addition of 2.5 g of magnesium sulphate and 2.5 g of sodium chloride, determined through the (i) application of two-level full factorial design and (ii) implementation of a response surface methodology based on *Central Composite Design*. Matrix-matched calibration curves were built and an excellent linearity for all compounds were obtained, with correlation coefficients (R^2) varying from 0.9957 to 0.999. Limits of detection and quantification varied from 0.04-0.12 ng g⁻¹ and 0.12-0.39 ng g⁻¹, respectively. Generally, recoveries from 80 to 110% were attained and the relative standard deviations (RSDs) were lower than 13.3% for reproducibility and 8% for repeatability. The developed methodology was compliant with the European Regulation criteria.

A monitorization of PAHs in smoked salmon samples available in the Portuguese market was performed. Some variation in the samples composition in PAHs was found. However, all samples were compliant with the maximum levels established by the European Regulation 835/2011, for both BaP and PAH4.

Keywords

PAH4, BaP, processed muscle foods, acetonitrile-based extraction, HPLC-FLD.

Resumo

Os Hidrocarbonetos Aromáticos Policíclicos (PAHs) são compostos ubíquos, sendo inevitável a sua presença nos alimentos. A Comissão Europeia estabeleceu teores máximos para o benzo(a)pireno (BaP) e para a soma de quatro substâncias – PAH4 (BaP, criseno, benzo(a)antraceno e benzo(b)fluranteno), considerando esta soma como marcador relativamente à ocorrência e ao efeito de PAHs cancerígenos nos géneros alimentícios. Destes últimos, destacam-se os alimentos fumados e grelhados na grelha ou em «barbecue» pelo potencial contributo para a ingestão de PAHs quando o seu consumo faz parte da dieta habitual. Através do Regulamento Europeu 836/2011 foram também estabelecidos critérios de performance para as metodologias a visar a quantificação de PAH4 em alimentos.

Nesta tese foi desenvolvida uma metodologia simples, rápida e eficiente para a extração e determinação de 14 PAHs em carne e peixe grelhados e fumados, incluindo os PAH4, recorrendo a uma extração baseada em acetonitrilo acoplada a cromatografia líquida de alta performance com deteção de fluorescência. As condições ótimas consistiram em homogeneizar 5 g de amostra com 5 mL de água e 5 mL de acetonitrilo, seguidos de partição através da adição de 2,5 g de sulfato de magnésio e 2,5 g de cloreto de sódio, determinados recorrendo a (i) desenho fatorial de dois níveis e (ii) implementação de uma metodologia de superfície de resposta baseada no *Central Composite Design*. Curvas de calibração em matriz foram construídas, obtendo-se excelente linearidade para todos os compostos, com coeficientes de correlação (R^2) a variar entre 0.9957 e 0.999. Os limites de deteção e quantificação variaram entre 0.04-0.12 ng g⁻¹ e 0.12-0.39 ng g⁻¹, respetivamente. Em geral, as % de recuperação variaram entre 80 a 110 e os desvios padrão relativos (RSDs) foram inferiores a 13.3% para a reprodutibilidade e 8% para a repetibilidade. A metodologia obedeceu aos critérios da Comissão Europeia.

Efetuuou-se a monitorização de PAHs em amostras de salmão fumado disponíveis no mercado português. Verificou-se alguma variação na composição das amostras nos 14 PAHs avaliados, no entanto, todas as amostras estavam em conformidade com os valores máximos estabelecidos pelo Regulamento Europeu 835/2011, para o BaP e para os PAH4.

Palavras-Chave

PAH4, BaP, carne e peixe processados, extração baseada em acetonitrilo, HPLC-FLD.

Table of Contents

Acknowledgments.....	iii
Abstract.	iv
<i>Resumo</i>	v
List of Figures.....	viii
List of Tables.....	ix
Abbreviations.....	x
1. Motivation, Objectives and Outline.....	1
1.1. Motivation.....	1
1.2. Objectives.....	2
1.3. Outline.....	3
2. Introduction.....	5
2.1. Polycyclic Aromatic Hydrocarbons (PAHs).....	5
2.1.1. Formation, Metabolism, Exposure.....	10
2.1.2. Regulatory framework.....	14
2.1.3. Factors affecting the formation of PAHs in grilled and smoked muscle foods.....	19
2.1.4. Reduction of PAHs levels in food.....	23
2.2. Analytical methods to determine PAHs in foods.....	25
2.2.1. Sample Preparation – Extraction Methods.....	26
2.2.2. Separation and Detection Methods.....	32
2.2.3. Quantification Methods.....	33
3. Materials and Methods.....	35
3.1. Standards and Reagents.....	35
3.2. Sampling.....	35
3.2.1. Meat and fish samples used for method validation.....	35
3.2.2. Smoked salmon samples.....	36
3.3. PAHs Extraction.....	38
3.4. HPLC-FLD Conditions.....	39
3.4.1. Experimental Design.....	40
3.5. Quantification and Method Validation Parameters.....	42
4. Results and Discussion.....	43
4.1. Optimization of the Acetonitrile-Based Extraction Method.....	43

4.2. Method Validation and Compliance with European Commission Regulation No. 836/2011.....	45
4.2.1. Comparison with Performance of Other Analytical Methods.....	51
4.3. Analysis of Smoked Salmon Samples from the Portuguese Market.....	53
4.3.1. Compliance with European Commission Regulation No. 835/2011 and comparison with current literature.....	59
5. Conclusions.....	61
6. References.....	63

List of Figures

Figure 1 – Environmental and Anthropogenic sources of PAHs.....	8
Figure 2 – Major metabolic transformations of PAHs.....	14
Figure 3 – Representative scheme of the developed acetonitrile-based extraction with partitioning, coupled with HPLC-FLD.....	38
Figure 4 – CCD design for the optimization for two variables	40
Figure 5 –. Effect of different acetonitrile volumes (mL) in PAHs extraction (sum of peak areas from the 14 PAHs).....	43
Figure 6 – Contour plots for the interaction effects of anhydrous magnesium sulphate and sodium chloride amounts on PAHs extraction (2D model).....	44
Figure 7 – Contour plots for the interaction effects of anhydrous magnesium sulphate and sodium chloride amounts on PAHs extraction (3D model).....	44
Figure 8 – Representative chromatograms from grilled chicken, unspiked and spiked	48
Figure 9A and 9B – Recovery values (%) in muscle food matrices	49
Figure 10 – 2-Dimensional score plot of Principal Component 1 (PC1) (57%) vs PC2 (22%) for 23 commercial smoked salmon samples. The Table represents the correlation of the variable with the components.....	58

List of Tables

Table 1 – Relevant PAHs of the regulatory framework.....	6
Table 2 – Maximum levels of PAHs in some foodstuffs established in the Regulations (EU) No 1881/2006 (EU) No 835/2011 and (EU) No 1327/2014.....	17
Table 3 – Moisture and fat content of the analysed samples.....	36
Table 4 – Characterization of the analysed samples of smoked salmon.....	37
Table 5 – Fluorescence program used to separate PAHs	39
Table 6 – Mobile phase gradient program used to separate PAHs.....	40
Table 7 – Central composite design to evaluate the effects of anhydrous magnesium sulphate (X1) and sodium chloride (X2) amounts on PAHs extraction	41
Table 8 – Calibration parameters and related uncertainties.....	46
Table 9 – Repeatability and Reproducibility (RSD %) at three levels of spike in blank matrix.....	47
Table 10 – PAHs content in different cooked muscle food by acetonitrile-based extraction with partitioning.....	51
Table 11 – Comparison of several analytical methods for PAHs determination in grilled and smoked muscle foods.....	52
Table 12 – Recovery values (%) in the smoked salmon matrix.....	53
Table 13 – PAHs levels in smoked salmon samples determined by acetonitrile-based extraction with partitioning.....	55

Abbreviations

5mC – 5-Methylchrysene

A – Anthracene

AAE – Acetonitrile-Assisted Extraction

Ac – Acenaphthene

Ace – Acenaphthylene

AhR – Aryl Hydrocarbon Receptor

ALARA – As Low as Reasonably Achievable

ANOVA – Analysis of Variance

ARE – Antioxidant Response Element

ASE – Accelerated Solvent Extraction

BaA – Benzo[a]Anthracene

BaP – Benzo[a]Pyrene

BbF – Benzo[b]Fluoranthene

BcF – Benzo[c]Fluorene

BgP – Benzo[g,h,i]Perylene

BjF – Benzo[j]Fluoranthene

BkF – Benzo[k]Fluoranthene

BMD – Benchmark Doses

BMDL – Benchmark Dose Lower Confidence Limit

CAC – Codex Alimentarius Commission

CCD – Central Composite Design

CCP – Critical Control Points

Chr – Chrysene

CONTAM – Contaminants in the Food Chain

CpP – Cyclopenta[c,d]Pyrene

CRMs – Certified Reference Materials

CYPs – Cytochrome P450

DAD – Diode Array Detector

DeP – Dibenzo[a,e]Pyrene

DhA – Dibenzo[a,h]Anthracene

DhP – Dibenzo[a,h]Pyrene

DiP – Dibenzo[a,i]Pyrene

DLLME – Dispersive Liquid–Liquid Microextraction

DIP – Dibenzo[a,i]Pyrene

DNA – Deoxyribonucleic Acid

dSPE – Dispersed Solid Phase Extraction

EC – European Commission

EFSA – European Food Safety Authority

EH – Epoxide Hydrolase

EI-MS – Electron Impact Mass Spectrometry

EU – European Union

F – Fluorene

FAO – Food and Agriculture Organization

FID – Flame Ionization Detector

FI – Fluoranthene

FLD – Fluorescence Detection

GC – Gas Chromatography

GST – Glutathione S-transferases

HAAs - Heterocyclic Aromatic Amines

HACCP – Hazard Analysis and Critical Control Point

HF-LPME – Hollow-Fibre Liquid Phase Microextraction

HPLC – High Performance Liquid Chromatography

HS-SPME – Headspace solid-phase microextraction with Gas Chromatography

IARC – International Agency for Research on Cancer

ICH – International Conference on Harmonization

IP – Indeno[1,2,3-cd]Pyrene

IPCS – International Program on Chemical Safety

IS – Internal Standard

ISO – International Organization for Standardization

JECFA – Joint Expert Committee on Food Additives and Contaminants

LC – Liquid Chromatography

LLE – Liquid-Liquid Extraction

LOD – Limit of Detection

LOQ – Limit of Quantification

MAE – Microwave-Assisted Extraction

MCNTs – Magnetic Multi-Walled Carbon Nanotubes

MoE – Margin of Exposure

MPL – Maximum Permitted Level

MRL – Maximum Residue Levels

MS – Mass Spectrometry

MSPE – Magnetic Solid-Phase Extraction

Na – Naphthalene

ND – Not Detected

NOAEL – No-Observed-Adverse-Effect Level

NQ – Not Quantified

NQO1 – NAD(P)H:Quinone Oxidoreductase

Nrf2 – Nuclear Factor Erythroid 2 Related Factor 2

P – Pyrene

Pa – Phenanthrene

PAHs – Polycyclic Aromatic Hydrocarbons

PC – Principal Component

PCA – Principal Component Analysis

PID – Photoionization Detector

PLE – Pressurized Liquid Extraction

QuEChERS – Quick, Easy, Cheap, Effective, Rugged and Safe

RPF – Relative Potency Factor

RSD – Relative Standard Deviations

RSM – Response Surface Methodology

RWPS – Red Wine Pomace Seasoning

SCF – Scientific Committee on Food

SDME – Single Drop Microextraction

SFE – Supercritical-Fluid Extraction

SIM – Selected Ion Monitoring

SLE – Solid-Liquid Extraction

SPE – Solid Phase Extraction

SPME – Solid Phase Microextraction

SULTs – Sulfotransferases

TDS – Total Diet Study

TEF – Toxic Equivalent Factors

UGTs – UDP-glucuronosyltransferases

UPLC – Ultra-Performance Liquid Chromatography

US EPA – United States Environmental Protection Agency

USAEME – Ultrasound-Assisted Emulsification-Microextraction

UV – Ultraviolet

WHO – World Health Organization

1. MOTIVATION, OBJECTIVES AND OUTLINE

1.1. Motivation

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous carcinogenic agents, present in smoked, grilled and barbecued muscle foods such as meat and fish and have long been associated with increased risk of cancer. The European Food Safety Authority (EFSA) concluded that the sum of 4 PAHs (benz[a]anthracene, chrysene, benzo[b]fluoranthene and benzo[a]pyrene), denominated as PAH4, is the most suitable indicator for the occurrence and carcinogenicity potential of these compounds in food.¹ The European Commission (EC) established maximum levels for these compounds in foods² and also established criteria for their analysis.³ Several analytical methods for PAHs detection and quantification have been proposed, along with pre-concentration and clean-up steps due to the complexity of muscle foods.⁴ However, there is currently an increasing demand for fast and reliable analytical methodologies which are also more economic and environmentally friendly, since in routine analytical applications, sample throughput, time, cost and solvent consumption are important issues to consider in the choice of an analytical method.

The growing need of fast, reliable and environmentally friendlier methods for routine analyses of PAHs in processed muscle foods and its application to monitor the levels of PAHs in foods available in the market in order to check that PAHs are within the maximum allowed levels, motivated the following research work. Commercial smoked salmon was selected for this purpose.

1.2. Objectives

The main objectives of this dissertation were to develop a fast and reliable analytical methodology for PAHs analyses suitable for routine analyses of grilled and smoked muscle foods and monitor the PAHs content of smoked salmon available in Portuguese market. To achieve these main objectives, the following specific objectives were established:

- The optimization of a fast acetonitrile-based extraction method for PAHs analysis on grilled and smoked muscle foods aided by experimental design (Central Composite Design - CCD);
- Validation of the optimized method and compliance with the performance criteria established by EC 836/2011;
- Survey of smoked salmon samples commercially available in Portuguese market concerning PAHs levels and compliance with the levels established by European Regulation EC 835/2011.

1.3. Outline

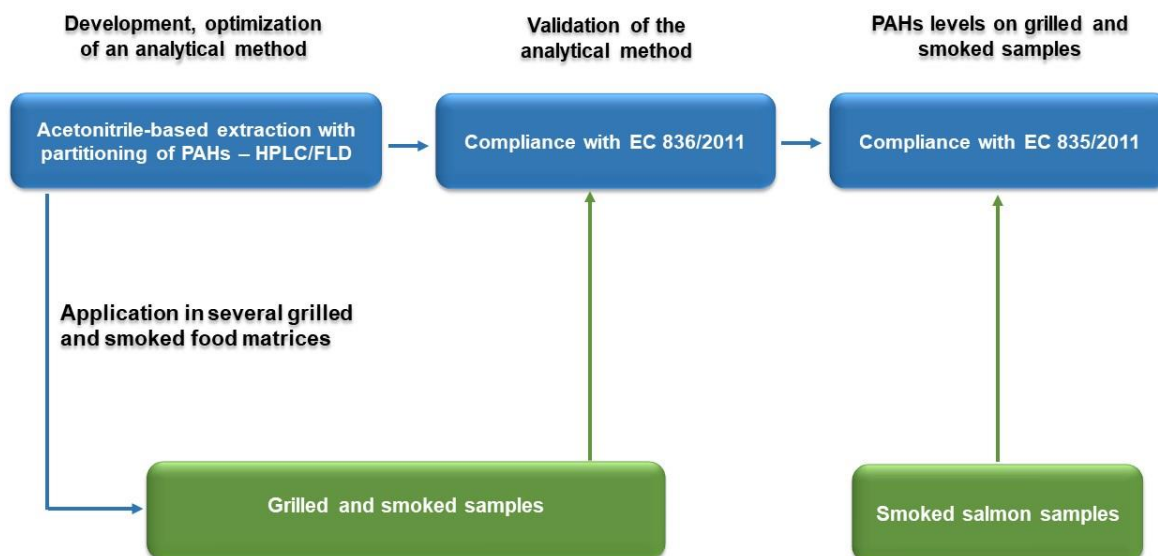
In the introduction, chapter 2, provides important information on PAHs, describing their formation mechanisms, sources, human exposure and metabolism, the factors affecting their formation on processed muscle foods, as well as ways to reduce their levels in foods, the regulatory framework and analytical methods to determine PAHs in foods.

In chapter 3, the materials and methods used are identified and the development of the technique is described, including PAHs extraction and purification method, high performance liquid chromatography and fluorescence detection (HPLC-FLD) conditions, quality control of the method and the experimental design.

In chapter 4, results and discussion of the experimental work are described together. This chapter is divided in three main sections:

- Section 4.1: the development and optimization of the acetonitrile-based extraction of PAHs from processed muscle foods is described;
- Section 4.2: the validation of the developed method is described and compliance with legislation – EC 836/2011 – is assessed. A comparison with other analytical methods to extract and quantify PAHs in grilled and smoked muscle foods is described. The developed method is applied to several grilled and smoked muscle foods and compliance with EC 835/2011 is assessed;
- Section 4.3: Different samples of smoked salmon from the Portuguese market are analysed using the developed method and compliance with EC 835/2011 is assessed.

In chapter 5, the conclusions of the present thesis are summarised, and future perspectives are proposed based on the questions raised with the present work.



This dissertation work resulted in the following publications and oral communications:

- Silva M, Viegas O, Finteiro D, Melo A, Pinho O, Ferreira I. *Fast and Reliable Extraction of Polycyclic Aromatic Hydrocarbons from Grilled and Smoked Muscle Foods*. Food Analytical Methods, 2018. <https://doi.org/10.1007/s12161-018-1325-8>
- Silva M, Viegas O, Cordeiro T, Pinho O, Ferreira I. *Application of an Acetonitrile Based-Extraction in Processed Muscle Foods for the Determination of 4 Polycyclic Aromatic Hydrocarbons* – XVIII Congresso de Nutrição e Alimentação, Associação Portuguesa dos Nutricionistas, Alfândega do Porto, 2019
- Silva M, Viegas O, Melo A, Pinho O, Ferreira I. *Optimized Acetonitrile Based-Extraction for Determination of Polycyclic Aromatic Hydrocarbons in Cooked Muscle Foods* – XXIV Encontro Luso Galego de Química, Faculdade de Ciências da Universidade do Porto, 2018

Furthermore, the methodology developed in the present thesis was also applied in the submitted paper:

- Cordeiro T, Viegas O, Silva M, Martins Z, Fernandes I, Ferreira I, Pinho O, Mateus N, Calhau C. *Effect of vinegars on the Formation of Polycyclic Aromatic Hydrocarbons in Charcoal-Grilled Pork* – Submitted

2. INTRODUCTION

2.1. Polycyclic Aromatic Hydrocarbons (PAHs)

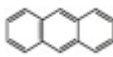
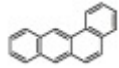
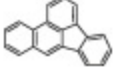
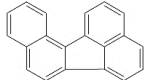
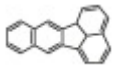
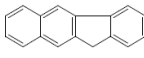
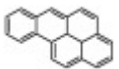
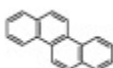
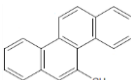
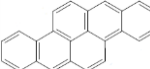
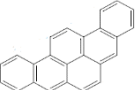
Polycyclic Aromatic Hydrocarbons (PAHs) are the most studied subgroup of the chemical group of polycyclic aromatic compounds, which also includes heterocyclic aromatic compounds.⁵ They are a class of organic compounds ubiquitous in nature, mostly colorless, white or pale-yellow solids, generated through natural or anthropogenic sources.^{6,7} PAHs have also been identified in meteorites and are thought to be the most plentiful and widespread organic compounds across the universe;⁶ they are made of carbon and hydrogen atoms bound in aromatic ring systems, containing at least two fused aromatic rings, each with different structural arrangements, presenting varied physical, chemical, and toxicological characteristics.^{1,6,8-10}

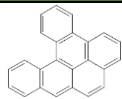
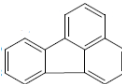
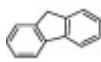
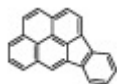
PAHs are semi- or non-volatile (presenting high melting and boiling points) and are all very persistent and bio accumulative compounds mainly due to their hydrophobicity and stability, which justifies the continuous study of their chemical structure, formation mechanisms, bioavailability, toxicity and health effects.^{8,11-13} PAHs degradation and resistance to oxidation increases with increasing mass.¹³ They are classified as light or heavy: light PAHs contain up to three rings and heavy PAHs contain four rings or more; heavy PAHs are more stable and toxic than light PAHs *per si*.¹⁴

PAHs have characteristic ultraviolet (UV) absorbance spectrums. Each ring structure generates a unique UV spectrum, therefore each isomer has its own spectrum, which is very useful in their identification.⁷ Most PAHs are fluorescent as well, emitting unique wavelengths of light after absorbing light.⁷

PAHs are the biggest class of carcinogenic substances comprising circa 10000 known compounds, although only 16 are commonly studied and have been extensively monitored and considered a priority by the United States Environmental Protection Agency (US EPA) based on their occurrence in environmental and food samples and potential carcinogenicity.¹⁵⁻¹⁸ The EU also classified 15+1 PAHs based on their genotoxic and carcinogenic potential. **Table 1** presents both PAH16 and PAH15+1, as well as the respective International Agency for Research on Cancer (IARC) classification.

Table 1. Relevant PAHs of the regulatory framework.

Compound	Chemical Structure	Molecular Weight (AMU)	US-EPA ¹⁹	EU ²⁰	IARC ¹⁰
Acenaphthene (Ac)		154	✓	---	3
Acenaphthylene (Ace)		152	✓	---	-
Anthracene (A)		178	✓	---	3
Benzo[a]Anthracene (BaA)		228	✓	✓	2B
Benzo[b]Fluoranthene (BbF)		252	✓	✓	2B
Benzo[j]fluoranthene (BjF)		252	---	---	2B
Benzo[k]Fluoranthene (BkF)		252	---	---	2B
Benzo[c]fluorene (BcF)		252	---	✓	3
Benzo[g,h,i]Perylene (BgP)		276	✓	✓	3
Benzo[a]Pyrene (BaP)		252	✓	✓	1
Chrysene (Chr)		228	✓	✓	2B
5-Methylchrysene (5MC)		242	---	✓	2B
Cyclopenta[c,d]pyrene (CPP)		226	---	✓	2A
Dibenzo[a,h]Anthracene (DhA)		278	✓	✓	2A
Dibenzo[a,e]pyrene (DeP)		302	---	✓	3
Dibenzo[a,h]pyrene (DhP)		302	---	✓	2B
Dibenzo[a,i]pyrene (DiP)		302	---	✓	2B

Compound	Chemical Structure	Molecular Weight (AMU)	US-EPA ¹⁹	EU ²⁰	IARC ¹⁰
Dibenzo[a,l]pyrene (DIP)		302	---	✓	2A
Fluoranthene (FI)		202	✓	---	3
Fluorene (F)		166	✓	---	3
Indeno[1,2,3-cd]Pyrene (IP)		276	✓	✓	2B
Naphthalene (Na)		128	✓	---	2B
Phenanthrene (Pa)		178	✓	---	3
Pyrene (P)		202	✓	---	3

US-EPA: 16 PAHs recognized as priority contaminants by the United States Environmental Protection Agency. EU: 15+1: 15 PAHs plus 5-methylchrysene recognized as priority PAHs by the European Union. IARC: classification of the carcinogenic potential by the International Agency for Research on Cancer.

It is generally accepted that PAHs are produced by pyrolysis or incomplete combustion of organic matter during natural and anthropogenic processes involving heat. Natural sources include wood forest fires, volcanoes, and hydrothermal processes, while anthropogenic sources comprise the processing of coal, crude oil and natural gas, petroleum refineries, deposition from sewage, and heating sources (both at home and power plants). Moreover, PAHs are formed during food processing (drying, smoking, roasting), cooking practices involving high temperatures (pan-frying, grilling and barbecuing) and are found in packaging material (treated with polluted oils, for example) and smoke flavoring products that are used to improve organoleptic characteristics, since they are produced from smoke condensates.^{1,14,20-24} Anthropogenic sources account for roughly 80% of total annual PAH emissions.¹ PAHs may also be produced biologically, by some plants and bacteria, as well as by degradation of vegetative matter.⁷

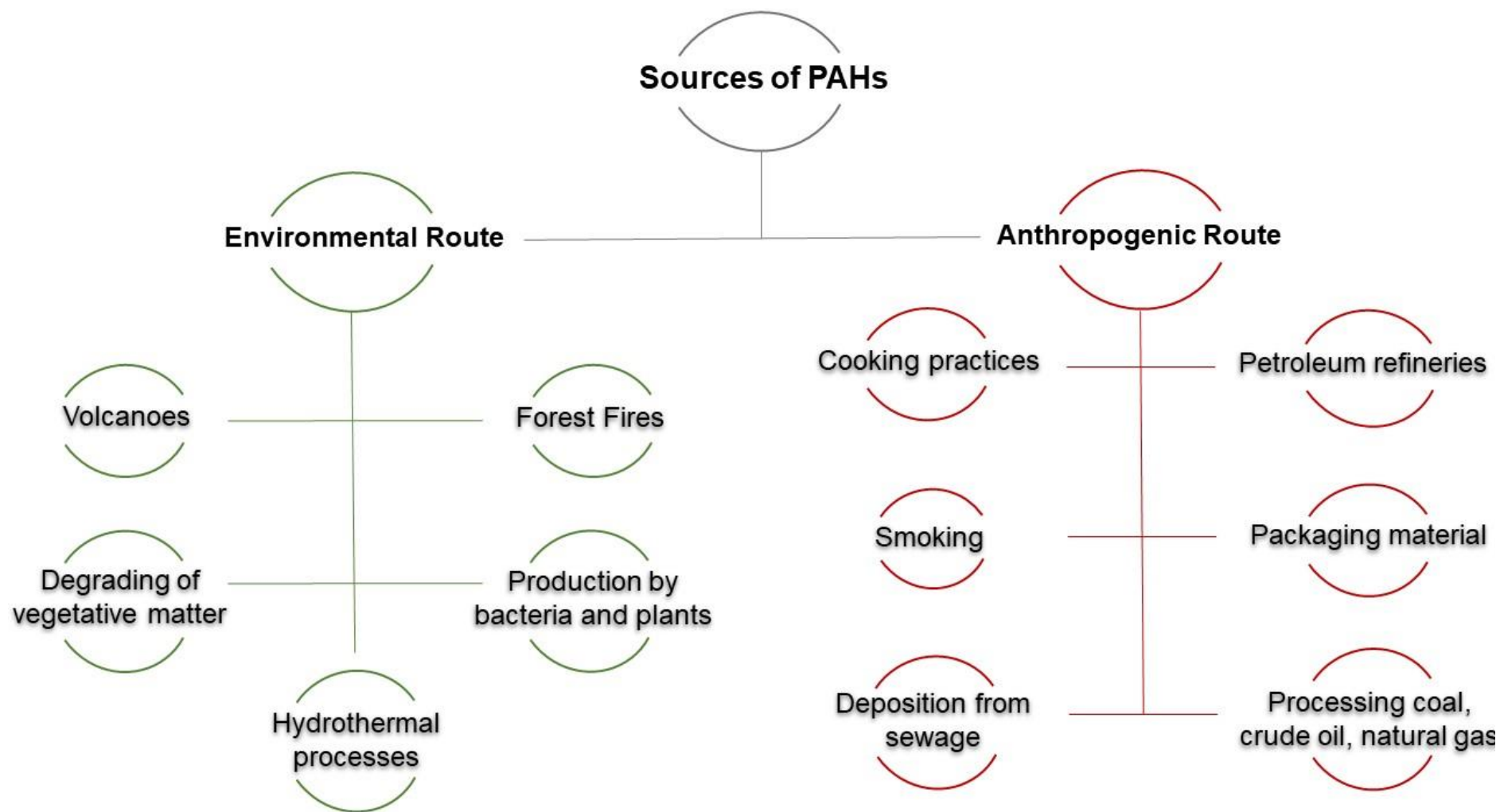


Figure 1. Environmental and Anthropogenic sources of PAHs.

PAHs ubiquity in the environment is a cause of concern, since human exposure occurs very easily: breathing contaminated air either in the street or work facilities (near intense traffic or exhaust from factories, breathing fumes from vehicle exhaust coal, wildfires, other wood fires, breathing the air of hazardous waste locations, among others); smoking (tobacco smoke is a significant source of PAHs); skin contact (e.g. roofers, road construction workers); or through the gastrointestinal track, ingesting naturally contaminated uncooked food (grains, seeds, and surfaces of fruits and vegetables, for example – PAHs have the ability to accumulate in plants, contaminating crops) or processed contaminated with PAHs (charred, smoked or grilled meat, for example).^{1,10,12,13,22,25,26} Relevant occupational exposure includes the conversion of coal to coke and coal tar, and processing and use of coal tar derived products (which may include coal gasification, coke production, coal-tar distillation, paving and roofing with coal tar, aluminum production, carbon electrode manufacture, calcium carbide production), occupation as a chimney sweep, jobs in kitchens, among others.^{10,27}

Regarding PAHs in food, the sources of contamination are numerous, and PAHs can be formed and/or accumulate in different food matrices, including fruits, vegetables and cereals, dairy products, meats and meat products, fish, beverages, infant formulations, among others.²⁸ Depending on each food matrix, the determinants of PAHs accumulation and concentration properties vary. Contamination of raw fruits and vegetables usually occurs through e.g. environmental pollution, deposition of contaminated air particles, transfer by contaminated air, soil and/or water and is most significant in crops growing next to industrial sites and traffic dense areas. In raw animal foods (such as meat and fish), the most important determinants for bioaccumulation are lipid tissue, skin, metabolism, among others. PAH-contamination of waters can also significantly contribute to the human ingestion of PAHs, mainly through the contamination of crops and the consumption of aquatic organisms (such as fish and seafood), as PAHs accumulate in them.^{4,22,28,29}

Excepting some shellfish (e.g. mussels and oysters) which are known to absorb and bioaccumulate PAHs from water, being a concern for human exposure especially in usually contaminated areas or after petroleum leakages and ship accidents, foods such as uncooked meat, poultry, milk, fish and eggs oxidize and

metabolize PAHs very fast, therefore before processing those foods PAHs values are usually low.³⁰⁻³² On the other hand, industrial processing and cooking practices involving intense thermal processing from the combustion of coal, wood or petroleum products, most of the referred foods are contaminated with PAHs mainly during processing.^{6,30} The highest PAH concentration in foods is usually described in grilled, smoked, roasted and/or barbecued food, mainly when charred,^{1,14,20} which contribute significantly to the daily intake of these compounds.

2.1.1. Formation, Metabolism, Exposure

Human exposure to PAHs generally occurs through three routes: respiratory tract, skin contact and ingestion. To non-smokers and non-occupationally exposed people, circa 70% of PAHs exposure may be diet-related, therefore the PAHs presence in food requires constant monitoring.^{13,33} PAHs formation in **processed foods**, as well as other carcinogenic compounds such as heterocyclic aromatic amines (HAAs), is strongly dependent on the chemical composition of food, the heating source (type of fuel and reaction conditions)^{6,28,34-36} and the cooking method, such as grilling, smoking, roasting, frying and baking.^{1,31,36-38}

The precise mechanism of PAHs formation in food is still unclear,^{14,15,38} although three of them have been proposed:

- a. **Incomplete Combustion** of the heat source (e.g. coal). The incomplete combustion of charcoal can generate PAHs, which are then transported by smoke and brought to the surface of the food;^{14,39}
- b. **Pyrolysis** of organic matter (protein, carbohydrates, fat).^{14,40} Pyrolysis is the chemical decomposition of organic matter through heat. It is the first step in combustion and gasification occurring in the absence or low quantity of oxygen (different from complete combustion, which happens only when enough oxygen is present).⁴¹ Many products can be formed in this process: biochar (a form of charcoal) by heating wood, and coke by heating coal.⁴¹ Pyrolysis is often used to treat sewage or other materials which need decontamination, since it removes contaminants by either destruction (breaking down contaminants into smaller molecules) or removal (separating contaminants from the material),⁴¹ but if PAHs can be broken due to the

presence of such high temperatures, in the other hand PAHs may also can be produced during this process, depending on the initial material. ^{40,42}

- c. **Pyrosynthesis.** Organic matter, when exposed to high temperature generates different hydrocarbon radicals, and intramolecular addition or polymerization of small molecules (such as alkenes, alkynes and aromatic rings) occurs. Then PAHs originated from these free radical takes place to form larger aromatic ring structures. ^{12,38,43} Supporting this hypothesis, Ledesma et al (2002) found a decreasing number of PAHs as the ring number increased. ^{15,38,40} Additionally, it has been suggested that the instable free radicals generated then reorganize to form polynuclear aromatic compounds, most likely light PAHs first and then heavy PAHs. ^{28,44}

In the European Food Safety Authority (EFSA) report from 2008, ¹ the food groups that most contributed to human dietary exposure were cereals and cereal products, most likely due to the frequently high daily consumption instead of their high PAHs content; seafood (mainly shellfish) and seafood products were also high contributors to exposure. Although the importance of high consumption of barbecued foods was mentioned in the referred report, little data was available regarding consumption of foods with higher PAHs content, such as smoked/grilled/barbecued/roasted muscle foods. ¹ However, several other works and reviews have shown the importance of this food group and the health risk it can represent, especially in high consumers. ^{21,33,45-51} Fish consumption has been increasing over the years, although meat consumption is still much higher. According to the latest report from Food and Agriculture Organization (FAO), fish consumption averages around 26.8 kg year⁻¹ in industrialized countries and 20 kg/year worldwide, while meat consumption is about 95.7 kg year⁻¹ in industrialized countries and 41.3 kg year⁻¹ worldwide. ^{52,53} Numerous studies have been assessing the possible connection between consumption of meat, particularly red or processed meat, and meat products, and cancer (such as colorectal cancer)/other chronic illnesses. ^{50,54} The consumption of processed meat *per si* (all meat transformed through salting, curing, fermentation, smoking or other processes, including grilled and barbecued meat) has been epidemiologically associated with high incidence of colorectal cancer. ^{50,55} In fact, as previously mentioned, in 2015 IARC classified

processed meat as carcinogenic to humans (Group 1),⁵⁰ being PAHs one of the implicated carcinogens.⁵⁰ Human exposure to PAHs, including occupational and dietary exposure, has already been associated with an increased risk of some human cancers such as skin, lungs and bladder cancer,^{12,22,56} thus, human exposure should be as low as possible and mitigation strategies are crucial.^{1,20}

Since PAHs occur in complex mixtures that present infinite possible combinations, interactions among them, as well as their effects and toxicity in humans, may be complex and unpredictable.^{57,58} PAHs are not toxic *per se* but are activated in the human body, which in attempting to eliminate them generally increases their toxicity via addition of polar groups.^{20,57} Generally, PAHs are metabolized by phase I enzymes and peroxidases, which produce deoxyribonucleic acid (DNA) reactive metabolites, and phase II enzymes, which form polar conjugates. They are toxic especially due to their interference with cellular membranes functioning, as well as with enzyme systems.⁷ The resulting health hazards in humans highly depend on the via and length of exposure, the amount of PAHs and their innate characteristics and toxicity, as well as the health status and age of the individual exposed.⁵⁹ PAHs capacity to cause short term health effects in humans is yet to be clarified, being that acute effects seemingly caused by PAHs include skin irritation and inflammation, possibly eye irritation, nausea, diarrhea and vomits.⁵⁹

The main biochemical mechanism of PAHs leading to adverse physiological outcomes is the mutagenicity through DNA attack.⁴⁹ They are considered mutagenic, genotoxic and carcinogenic contaminants, besides being linked to cytotoxicity and immunosuppression (affecting the host resistance, immune system development and humoral immunity), epigenetic mechanisms and possibly being neurotoxic, therefore continuously assessing the risk for health hazards after contact/ingestion of PAHs is essential.^{1,7,12,13,22,60}

Once ingested and absorbed, the bioavailable PAHs circulating in blood are distributed to several tissues, especially to those with a high lipid content,⁴⁹ and some can undergo metabolic activation to diol-epoxides (mutagen/genotoxic/carcinogenic compounds), which adhere to macromolecules such as genetic material DNA and other tissues, inducing mutations which may lead to carcinogenic processes.^{4,22,61} Metabolic activation of PAHs seems to occur

primarily in the liver but also in other tissues, including the epithelial barriers.¹⁰ A scheme of the main biotransformation pathways is provided in **Figure 2**. Briefly, PAHs are quickly transformed into their hydroxylated metabolites, thought to occur via cytochrome P450 (CYP) oxidase system, with a oxidation or hydroxylation as the first step.⁶² Then, the formed epoxides or phenols might be further metabolized producing glucuronides, sulfates or glutathione conjugates, increasing the polarity to facilitate their excretion.^{62,63} Also, PAHs are potent ligands for the aryl hydrocarbon receptor (AhR), a transcription factor; through this interaction PAHs activate the transcription of various genes encoding phase I and II xenobiotic metabolizing enzymes, which include CYP1A1, CYP1A2, CYP1B1, and also GST, UGT1A1 and NQO1.^{64,65} The transcription of Phase II genes is also regulated by Nrf2 (Nuclear factor erythroid 2 related factor 2) transcription factor which binds to the cis-acting element named the antioxidant response element (ARE). The Nrf2 regulates the genes that produce enzymes to detoxify electrophiles and reactive oxygen species, as well as the removal or repair of some of their damage products, which includes GSTs, UGTs and NQO1.⁶⁶

Some metabolites from PAHs commonly found in urine include 1-hydroxypyrene (1-HP), 1-naphthol, 9-phenanthrol, 2-hydroxyfluorene (2-HF), 9-hydroxyfluorene (9-HF), 2-naphthol, 3-hydroxyfluorene (3-HF).^{63,67} Other epoxides may be metabolized into dihydrodiols that can suffer conjugation and form soluble detoxification products, or oxidize to diol-epoxides.⁶² Despite this, PAHs have different molecular structures and intrinsic characteristics, therefore each compound presents different *in vivo* carcinogenicities.⁴⁹

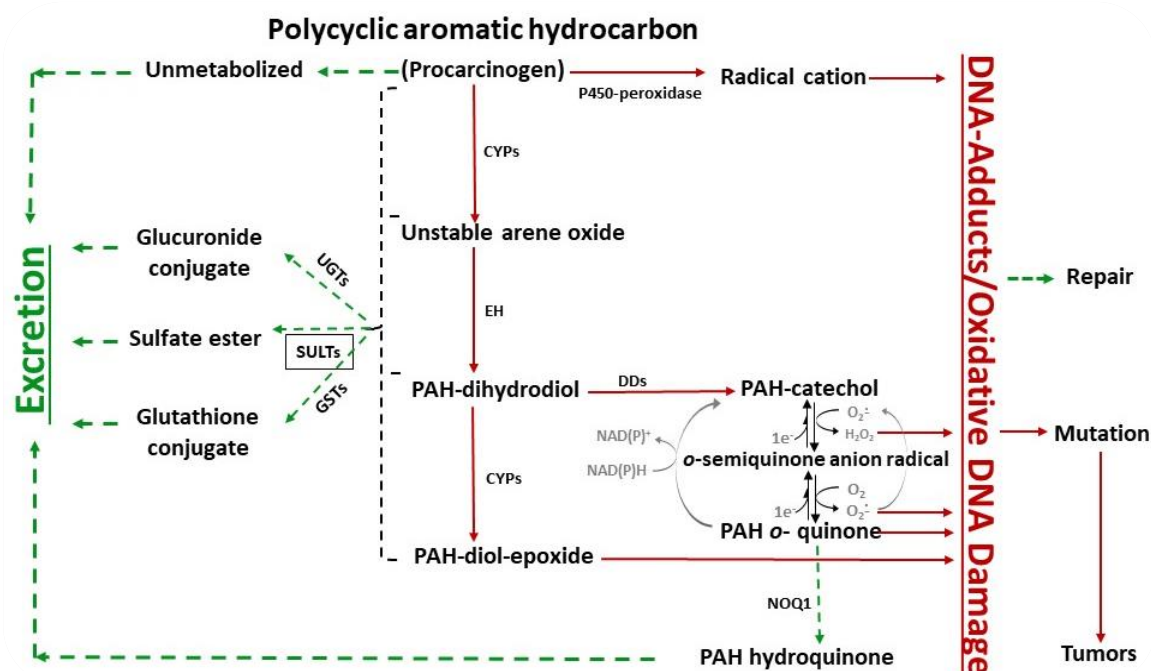


Figure 2. Major metabolic transformations of PAHs. Activation (green arrows) and detoxification (red arrows) routes. Abbreviations: CYPs, cytochromes P450 1A1, 1A2, 1B1, others; EH, epoxide hydrolase; NQO1, NAD(P)H:quinone oxidoreductase 1; UGTs, UDP-glucuronosyltransferases; SULTs, sulfotransferases; GSTs, glutathione S-transferases; DDs, dihydrodiol dehydrogenases. Adapted from Viegas et al 2019 ⁶⁶

2.1.2. Regulatory framework

PAHs have been regulated by numerous international organizations, who have expressed the carcinogenicity, mutagenicity and bioaccumulation abilities of these compounds, ⁶⁸ therefore expressing recommendations and/or reference values for PAHs exposure. The Regulatory Bodies include:

- European Commission ^{2,3,69-71}
- European Food Safety Authority (EFSA) ¹
- European Scientific Committee on Food (SCF) ⁶⁹
- US Environmental Protection Agency (US EPA) ^{19,25}
- International Agency for Research on Cancer (IARC), of the World Health Organization (WHO) ¹²
- WHO/Food and Agriculture Organization of the United Nations (FAO)'s Joint Expert Committee on Food Additives and Contaminants (JECFA) ⁷²

PAHs were firstly recognized as relevant contaminants in 1976 by the US-EPA,¹⁷ which proposed the monitorization of the 16 PAHs most frequently found in environmental samples, previously presented in **Table 1**.^{4,19} Two years earlier, a law was created to protect public health by regulating the country's public drinking water supply under the Safe Drinking Water Act (SDWA), where US-EPA determined the legal maximum levels for BaP in drinking water as 0.2 µg L⁻¹.⁷³ So far and to the best of our knowledge, no other US organization has yet established any maximum limits for PAHs content in foodstuffs.

In 2002 the European Commission Scientific Committee on Food (SCF) carried out a risk assessment on 33 PAHs, previously evaluated by the International Program on Chemical Safety (IPCS) in 1988⁷⁴ and released the Opinion on the risks to human health from PAHs in food, concluding that 15 PAHs out of the 33 initially evaluated showed clear mutagenicity and genotoxicity activity in animal somatic cells *in vivo*. The 15 PAHs are presented in **Table 1**.²⁰ It was also clear that the main route of exposure of PAHs to non-smoking and non-occupationally exposed individuals was food and this exposure should be as low as possible.²⁰ In 2005, after the SCF opinion, the European Commission established maximum levels for BaP in various foods, including smoked meat and fish.⁷⁵ In the same year, JEFCA re-evaluated PAHs, concluding that 13 PAHs were genotoxic and carcinogenic, and two other PAHs could contribute to the formation on lung cancer, which compose the PAH15 or PAH15+1 when 5-methylchrysene is included (presented in **Table 1**).⁷⁶

Four years later, in 2006, via Commission Regulation (EC) No 1881/2006, maximum levels for BaP (and other contaminants) in different foodstuffs, such as smoked meat and meat products, smoked fish and fish products, oils and fats (**Table 2**), were established.⁶⁹ The EU recommended the monitorization of the 15 priority PAHs established by JEFCA, including 8 heavy PAHs (BaA, Chr, BbF, BkF, BaP, IP, BgP, DhA), from which BaP was chosen as a marker of occurrence and carcinogenic/genotoxic potency of the entire class,⁶⁹ and for which analytical performance criteria was established in 2007, in the Commission Regulation (EC) No 333/2007.⁷⁷

In a screening of levels of PAHs in foods, a third of samples containing carcinogenic PAHs were negative for BaP.⁷⁵ In 2008, the EFSA CONTAM Panel,

the EU risk assessment authority for food and feed safety, published an Opinion on PAHs and stated that BaP alone was not a suitable marker for these contaminants' occurrence. Instead, the sum of four PAHs – PAH4 (BaP, BaA, Chr, BbF) – or the sum of eight PAHs – PAH8 (BaA, Chr, BbF, BkF, BaP, IP, BgP, DhA) – were better indicators of carcinogenic PAHs contamination and should be considered. ¹ The Panel also concluded that PAH8 did not provide additional information compared to PAH4. ¹ Consequently, in 2011, the Commission Regulation (EU) 835/2011 ² amended Commission Regulation (EC) No 1881/2006, acknowledging the importance of determining PAH4 instead of solely relying on BaP, therefore establishing maximum levels for the sum of the four PAHs – BaP, BaA, BbF and Chr. ² The maximum allowed levels for BaP and sum of PAH4 in smoked meats and smoked meat products, as well as for heat treated meat and heat treated meat products (grill and barbecue) established in the European Union (EU) Regulation No 835/2011, are 2.0 ng g⁻¹ and 12.0 ng g⁻¹, and 5.0 ng g⁻¹ and 30.0 ng g⁻¹ respectively. ² In the same year, along with the latter, the Commission Regulation (EU) No 836/2011 was published, amending Regulation (EC) No 337/2007, adding analytical performance criteria for the new three PAHs – BaA, BbF and Chr –, covering criteria for PAH4. ³

Since the established maximum values for smoked meat/fish and smoked meat/fishery products are hard to achieve despite the application of good practices, some traditionally smoked products from several small and medium size enterprises would disappear from the market, thus the Commission Regulation (EU) Regulation No 1327/2014 ⁷⁰ amended the Regulation (EC) No 1881/2006 ⁶⁹ about maximum levels of PAHs, excluding some requirements for traditionally smoked products (meat and meat products, fish and fishery products). Consequently, the maximum levels are currently 5.0 ng g⁻¹ for BaP and 30.0 ng g⁻¹ for PAH4, since 2014, as established in the Commission Regulation (EU) No 1327/2014. ⁷⁰ The member states should nonetheless continuously monitor the presence of PAHs in traditionally smoked meat/fish products, establishing good practices programs within what is possible without losing the organoleptic characteristics of such products, considering the economic conditions as well. ⁷⁰

Table 2: Maximum levels of PAHs in some foodstuffs established in the Regulations (EU) No 1881/2006,⁷¹ (EU) No 835/2011² and (EU) No 1327/2014⁷⁰

Food Product	Maximum Levels (ng g ⁻¹)	
	BaP	PAH4
Oils And Fats (Excluding Cocoa Butter) For Human Consumption	2.0	10.0
Smoked Meat And Smoked Meat Products	2.0	12.0
<i>Traditionally Smoked Meat And Smoked Meat Products ^A</i>	5.0	30.0
Heat Treated Meat And Heat Treated Meat Products (Grilled Or Barbecued)	5.0	30.0
Smoked Fish And Smoked Fishery Products	2.0	12.0
<i>Traditionally Smoked Fish And Smoked Fishery Products ^B</i>	5.0	30.0
Bivalve Molluscs (Smoked)	6.0	35.0
Processed Cereal-Based Foods And Baby Foods For Infants And Young Children	1.0	1.0
Infant Formulae And Follow-On Formulae, Including Infant Milk And Follow-On Milk	1.0	1.0

BaP = Benzo(a)pyrene. PAH4 = Sum of benzo(a)pyrene, chrysene, benz(a)anthracene, benzo(b)fluoranthene. ^A These levels apply to the following countries: Ireland, Latvia, Romania, Finland, Sweden and the United Kingdom; ^B These levels apply to the following countries: Ireland, Spain, Croatia, Cyprus, Latvia, Poland, Portugal, Romania, Slovak Republic, Finland, Sweden and the United Kingdom.

In 2015, both Commission Regulation (EC) 2015/1125 ⁷⁸ and Commission Regulation (EC) No 2015/1933 also amended Commission Regulation (EC) No 1881/2006 ⁶⁹ in terms of maximum levels for PAHs in other foods such as Katsuobushi/certain smoked Baltic herring, ⁷⁸ and cocoa fibre/banana chips/food supplements/dried herbs/dried spices, ⁷⁹ respectively.

Good practices programs should aim to lower PAHs dietary occurrence and, consequently, human exposure. When developing a food safety management system based on HACCP principles, food business operators must consider PAHs as hazardous contaminants and recognize the need to control their presence, therefore identifying any critical control points (CCP) the respective processes, especially those most likely linked to PAHs formation (drying, roasting, smoking,

grilling, among others). Accordingly, food business operatives must ensure that beyond their overall responsibility to supply and commercialize safe food, established by General Food Law (European Commission Regulation No 178/2002),⁸⁰ they must ensure their products are within the legislative limits for PAHs (Commission Regulations (EC) No 835/2011 and EC No 1327/2014)⁶⁹ and an HACCP plan including CCPs to control PAHs leads to their reduction to acceptable levels and/or elimination is established. To ease this process, the Codex Alimentarius Commission's Code of Practice for the Reduction of Contamination of Food with Polycyclic Aromatic Hydrocarbons (PAH) from Smoking and Direct Drying Processes CAC/RCP 68/2009 was created, describing 10 variables that should be controlled to reduce and prevent carcinogenic PAH formation in meat products during smoking and other drying methods.⁸¹ However, it does not include PAHs contamination from the use of herbs and spices, indirect drying, environmental contamination of raw materials and other food processes, including barbecuing or other domestic cooking processes.⁸¹

Whenever the current exposure of the population or specific vulnerable groups in the population is close to, or exceeds the tolerable intake of compounds such as PAHs (considered both genotoxic and carcinogenic), the maximum levels should be set at a level that is as low as reasonably achievable (ALARA), forcing food business operators to apply preventive to reduce contamination as much as possible.⁷¹ However, the referred regulatory maximum levels are only applied to food trade and are not applied to domestic cooked foods, reinforcing the need for good practices and sensibilization of the general population for the occurrence of PAHs and how to decrease them, applying the ALARA principle not only to food manufacturers, but also within households.

In 2015 IARC classified red meat as probably carcinogenic to humans (Group 2A), and processed meat as carcinogenic to humans (Group 1), being PAHs one of the implicated carcinogens.⁵⁰ IARC has also classified PAHs according to the scientific evidence of carcinogenicity, as presented in **Table 1**. This classification is defined by the IARC Working Group as (detailed in *IARC Monographs Preamble*):⁸² Group 1: Carcinogenic to humans; Group 2A: Probably carcinogenic to humans; Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as to its carcinogenicity to humans.

Among all the PAHs considered in foods, the priority PAHs from EU, PAH8, comprise the same eight carcinogenic heavy PAHs from US-EPA list. These PAH8 were classified by IARC as: BaP in Group 1, BaA, Chr, BbF, BkF, IP and DhA in Group 2B and BgP in group 3.¹⁰

2.1.3. Factors affecting the formation of PAHs in grilled and smoked muscle foods

PAHs are formed during cooking and other thermal treatments, and several factors can affect their formation in processed muscle foods. Consequently, it is crucial to understand which conditions promote higher levels of PAHs, to eventually control and minimize their creation. Model system studies are important to understand the mechanisms of PAHs formation and how to decrease or eliminate their formation. Min et al 2018 designed model systems to assess the influence of the heating conditions, water content, lipid precursors and antioxidants on the creation and inhibition of PAHs.³⁸ The authors observed that water had an inhibitory effect on PAHs formation due to the water as an oxygen source while heating, avoiding incomplete combustion, as previously shown by Olatunji et al 2014.^{38,83}

The effects of some of the most significant determinants of PAHs formation in food are briefly described below:

- **Composition of food: fat content.** The presence of PAHs, as well as other carcinogens, is strongly influenced by the chemical composition of food,⁵³ and although other parameters such as the cooking process are strong influencers as well, PAHs formation seems to depend more on each specific food item than the cooking process itself.^{84,85} PAHs formation appears to be more related to fat content than to protein or carbohydrates^{28,37,86} and higher fat levels usually lead to more PAHs formation.^{1,37,87-90} PAHs seem to arise from the pyrolysis of fat that melts and drips onto the heat source and leads to the direct deposition of PAHs from smoke produced through the incomplete combustion of charcoal.⁸⁶ PAHs formation seems to be related to the saturation degree of lipids as well, since the more unsaturated a lipid is, the

more formation increases, most likely because the unsaturated fatty acid is more susceptible to oxidation during thermal processes, among other factors such as the possibility of polymerization of polyunsaturated fatty acids to form cyclic monomers or dimers.³⁸

- **Cooking method.** PAHs are more likely formed during high temperature cooking methods (150 to 300 °C), as in smoking, barbecuing and grilling, which are accordingly known to be the main contributors to PAHs formation, especially when subjecting fish or meat directly over charcoal, open flames or direct smoke.^{44,53,91} Meat products grilled in electric ovens seems to produce lower PAHs than meat grilled in other ways such as barbecuing or pan-frying (even without oil).^{1,92}

In smoked foods, the amount of PAHs formed and transported to foods during smoking depends on various factors, including the smoking technology and smoke generation process, smoking method (direct or indirect), combustion temperature, composition of the smoke, washing or filtering the smoke, distance between the food and heat source, type/nature of wood, humidity of the wood, oxygen and smoke concentration, circulation rate and exposure of the edible parts to the smoke.^{81,93-95} Some authors suggest that PAHs levels might also be strongly influenced by the lignin content of the wood type used for smoking,⁹⁶ and higher fat content of meat usually translates into higher levels of PAHs.^{86,97}

The importance of the material used to generate smoke is highlighted by the European Union (EC Regulation 2065/2003, on smoke flavourings used or intended for use in or on foods), stating that the wood intended to produce smoking flavor agents shall not have been treated.⁹⁴ Softwoods should also be replaced by hardwoods to reduce the presence of PAHs in smoke and in smoked foods.⁹⁶

- **Cooking time and temperature.** The binomial time/temperature is critical and seems to be more important than temperature or time alone (although temperature remains the main influencer with a more pronounced effect),^{38,98} since PAHs can be formed not only at high temperatures (500-800 °C) but

also at temperatures as low as 100 °C if the organic matter is subjected to it for long periods of time.^{28,37,38,98} In general, cooking at shorter time and lower temperatures lead to lower amounts of PAHs.⁹⁰

The degree to which a food is cooked translates into different doneness levels: rare, medium, well done and very well done, and is essential to estimate PAHs exposure.⁹⁹ Well done and very well-done barbecued beef steak usually present higher PAH amounts, compared to rare and medium cooked beef steak, suggesting the increase of PAHs amounts when cooking meat for a longer time.^{35,87} However, “doneness” is a limited measure since it is quite subjective and can induce misclassification. For example, Sinha et al¹⁰⁰ defined beef doneness level in relation to the internal temperature: 60 °C as rare, 70 °C as medium, 80 °C as well done and 90 °C as very well done, but the classification is not uniform among studies. The quantitative binomial time and temperature itself is more objective and rigorous to assess the degree to which a food is cooked, and can be achieved using predictive models based on quantitative time and temperature as demonstrated by Puzou et al,⁹⁹ which makes it a clear and more rigorous method to estimate PAH intake and, consequently, to ensure consumers’ safety through objective harmonized recommendations, and harmonization with consumer’s food safety guidelines, which rely on internal temperature to assess doneness.

- **Distance between the food and the heat source and equipment design.** Direct contact between the food and the heat source promotes PAHs formation, therefore should be avoided.^{31,101} Cooking over open flames also leads to higher levels of PAHs, compared to when the flames are extinct or do not exist.^{35,102} The geometry and design of the cooking equipment are very important as well and might prevent PAHs formation, which is mainly influenced by the formation of smoke and pyrolysis after fat drippings contact with the heating source.¹ When the heat source is vertical or above the food, instead of horizontal, or when an ice bucket is placed underneath food in a grill, no PAHs are detected in food, most likely because no fat drips onto the heat source.^{85,86,103,104}

- **Type of the heat source.** The fuels used to cook food differ with the cooking technique (electrical, wood, gas, charcoal or others) and are an important aspect regarding PAH contamination of food, which varies accordingly. ^{31,81,105} Different types of charcoal lead to different PAHs formation. Some authors reported that meat and fish grilled with coconut shell charcoal, labeled as flameless and smokeless, lead to less formation of PAHs compared to wood charcoal, these results were more prone in fattier foods. ³⁵ Wood pyrolysis is also linked to PAHs formation and different woods may significantly influence the PAH levels in foods. ^{1,36} Accordingly, it was also found that smoking with acacia charcoal (from *Acacia auriculiformis*), Neem woodchips (*Azadirachta indica*) or apple-tree and alder, lead to less PAHs in shrimps, sausages and other types of meat, respectively. ^{34,106,107}
Among charcoal, charcoal + wood chips, briquettes and gas, in different grilled foods, charcoal and woodchips produced the higher amounts of PAHs. For gas-fueled barbeque the main source of PAHs was not the pyrolysis of fat drips but the gas flames themselves or the contact between the surface of the food and the gas flames. ⁸⁵
- **Effect of added ingredients and pre-cooking procedures.** Different ingredients can be added prior the processing of muscle foods and some might have an inhibitory effect in thermally induced contamination. The oxidative status of food, namely meat, may have an impact on the formation of carcinogenic compounds including PAHs, since their formation involves radical reactions. ⁴⁴ Antioxidant agents (that can be of synthetic or natural origin, found especially in herbs, plant extracts, spices, seasonings, or others) interfere with radical reactions and may trap free radicals, including intermediates of PAHs, preventing their formation, ^{92,108} thus incorporating antioxidants is becoming a strategy to reduce the formation of PAHs in foods. Ingredients containing antioxidants may have an individual or a synergic effect, therefore many times the combination defines the strength of the final effect; the type of antioxidants may have different activities in different meat systems as well. ¹⁰⁸

Some ingredients with inhibitory effect in thermally induced PAHs contamination include: sage and thyme; ¹⁰⁹ garlic and onion; ⁹² red wine pomace seasoning; ^{44,110} spices (e.g. cumin, coriander, black pepper, rosemary, garlic, paprika, ginger). ^{108,111}

Marinades, a traditional cooking method in which the meat is soaked in a mixture of ingredients that improves tenderness, flavor, color, texture and moisture of the cooked foods. ^{112,113} Marinades can be of many types and compositions and are becoming a very popular precooking procedure for meat, due to their capacity in improving meat quality and decreasing the formation of harmful compounds. ¹¹⁴ Some marinades that decrease or inhibit the formation of PAHs include: marinades including onion and garlic, ⁹² pickle sauce (vegetable oil, vinegar, paprika, salt and other spices) ¹¹⁵ lemon juice, onion, garlic, turmeric, coriander, cinnamon, cumin, pepper powder; ^{90,111,116} beers (especially black beer); ^{92,117,118} teas (especially green tea); ¹¹⁴ marinades with pure compounds, such as polyphenols gallic, protocatechuic and ferulic acids; ^{119,120} antioxidants BHA, BHT, sesamol and α -tocopherol. ³⁸

2.1.4. Reduction of PAHs levels in food

The current exposure of the population/certain groups in the population to PAHs, exceeds many times the tolerable intake, therefore the exposure to these compounds should be at the lowest possible level – ALARA principle. ⁷¹ To prevent PAHs contamination of food and considering how PAHs contaminate processed muscle foods, several approaches have been made that show promising effects in reducing PAH levels.

The main route of PAHs formation and contamination in grilled foods seems to occur when fat drips onto the heat source, therefore preventing it is an effective method to reduce levels of carcinogenic PAHs ^{37,90} That can easily be accomplished by wrapping the food with e.g. aluminum paper or banana leaf ³⁷ or by placing a bowl of water/ice underneath the food to collect the fat drips. ⁸⁶ Removing the smoke generated by charcoal before it contacts with the food surface with a ventilation duct

also prevents the deposition of smoke in meat, lowering the amounts of formed PAHs as well is another effective method to reduce PAHs levels.⁸⁶

Preheating treatments, such as steaming or microwaving meat, before charcoal grilling are also good options to prevent PAHs formation, presenting maximum inhibitory effects (100% inhibition) for BaP and BbF in beef and chicken meat.³⁷

Different types of charcoal also generate PAHs differently. Coconut shell derived charcoal was suggested as a safer charcoal to human health, considering the reduction of PAHs formation in grilled muscle foods, especially in fatty ones, in comparison with wood charcoal. Authors justified that coconut charcoal was smokeless and flameless and absorb the fat that drips from the cooking food.³⁵ Also, it was observed that acacia charcoal (from *Acacia auriculiformis*) in a chorkor kiln to smoke shrimps,¹⁰⁶ or neem woodchips (*Azadirachta indica*) to smoke sausages,¹⁰⁷ decreased PAHs concentrations, respectively.

Marinades can also decrease PAHs levels in grilled and barbecued products for two mainly different reasons: prevention of the direct contact between the meat and the flame,^{109,117} and ingredients related to the inhibition or reduction of PAHs formation, as previously mentioned.¹¹⁴

In smoked meat and fish, different parameters should be monitored during smoking to provide food safety, including fuel type, pyrolysis temperature, smoke generator air flow, smoking chamber design, smoking type, drying type, food fat content, processing effect, closeness between food and heat source, smoking duration and cleanliness of the equipment.^{28,43,121} It has been proved that controlling these mechanisms leads to less formation of PAHs,⁸⁶ therefore the Codex Alimentarius Commission (CAC) recommends that the smoking process results in the lowest PAHs levels possible, encouraging on-going research to identify the optimal smoking conditions.⁸¹

Modern industrial ovens usually use an external chamber to generate smoke, cleaning it by different techniques including electrostatic filters or smoke washing and only then, directed to the smoking chamber, which all leads to the minimization of PAHs contamination.⁹⁵ A o-polyamide/low density polyethylene laminated film packaging has also been proved to reduce PAH4 in smoked sausages, most likely because the superficial PAHs migrated to the packaging.¹²²

In general, different strategies reduce PAHs in smoked foods, such as: indirect and cold smoking;^{32,68,93,123,124} filtering the smoke (especially with zeolite, followed by granular activated carbon and gravel filters);^{125,126} not consume the casing (especially in freshly smoked products); use liquid smoke flavoring products instead of real smoking techniques.^{94,127}

2.2. Analytical Methods to Determine PAHs in Foods

Analytical methods to monitor PAHs in food depend on intrinsic and extrinsic factors such as type of product, fat composition, extraction methods, instrumentation and solvent used to extract PAHs.¹²⁸ The robustness of the method and potential for automatization are important factors as well.⁶ The high complexity of food matrixes such as fish and meat (with high doses of protein and fat and other organic components that are co-extracted from the matrix with PAHs), together with the low concentration of PAHs (ppb concentrations) compared to other components and their structural similarity among themselves, makes the selective separation and the identification of individual compounds very difficult, therefore usually requires rigorous and sometimes extensive sample extraction and preparation steps.^{22,32,88,129}

The number of analytical methods to determine PAHs in food increased significantly after the publication of the US EPA's priority PAHs list and the number of published methods is vast.⁶ Only two methods (a general method and a specific method for coconut oil and vegetable oils with short-chain fatty acids) for the determination of 15 PAHs in animal and vegetable fats and oils have been described by the International Organization for Standardization (ISO 2016).¹³⁰ For fish and meat and fish and meat products, no standardized procedure has been published so far.

2.2.1. Sample Preparation – Extraction Methods

The first stages of identification and quantification of PAHs are sample collection and preparation, which are essential to separate PAHs from the matrix and obtain an extract sufficiently pure and with enough amounts to be detected and quantifiable. These first steps should therefore be optimal in order to reduce analysis time, sources of error and lead to a more precise identification and accurate quantification; sampling methods are established according to the nature and complexity of the food matrices.^{1,131} To obtain reliable results and avoid variations in the composition of the sample in terms of PAHs, some precautions should be taken, such as protecting the sample from tobacco smoke, light and high temperatures.^{1,23,131-133}

Considering the high lipophilicity of these compounds, they are associated with the lipid constituents when extracted and therefore need to be removed by cleaning steps,^{67,134} that are still significantly time-consuming and often laborious, especially in fat rich matrices such as meat and fish.^{129,131} The removal of lipids is central to obtain high sensitivity (reach low detection limits (LODs); which is important due to the low PAHs concentrations in food and the maximum permitted levels in the current legislation^{2,70}) but also to minimize the maintenance of the chromatographic system.^{23,129}

Extraction is the process in which the analytes present in a sample are dissolved and transferred to a solvent or a mixture of solvents, suitable for the analysis process. It is the isolation of the compounds of interest by removing the interfering substances, which generally involves concentration enrichment of the compounds to a level that allows their analytical determination.¹³⁵

The extraction and isolation of PAHs from different food matrices usually starts with a homogenization step, followed by the extraction (that is often non-selective, thus usually includes one or more clean up steps to remove the undesirable compounds that were extracted along with the analytes). Several preparation methods have been proposed and extensively used to extract and isolate PAHs from different food matrices, traditionally starting with the saponification of lipids with KOH-methanol,⁴ followed by Soxhlet extraction or sonication,¹³⁶ and then purification/clean-up with liquid-liquid extraction (LLE),^{158,159}

solid-liquid extraction (SLE), solid phase extraction (SPE),^{31,166,167} dispersed solid phase extraction (dSPE), solid phase microextraction (SPME),^{137,138} supercritical-fluid extraction (SFE),^{139,140} microwave-assisted extraction (MAE),¹²³ pressurized liquid extraction (PLE), dispersive liquid–liquid microextraction (DLLME),^{141,142} accelerated solvent extraction (ASE),^{136,143} ultrasound-assisted emulsification-microextraction (USAEME) and solvent centrifugal extraction.^{4,31,131,134,144-146} The use of rapid, efficient and environmentally friendly methodologies such as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) for extraction and purification¹⁴⁷ and other extraction methods coupled with DLLME^{148,149} have received increasing attention.

In addition to the selection of the extraction method, its optimization is of major importance as well. The Response Surface Methodology (RSM) has been designated a valuable tool to optimize extraction methods.^{150,151}

Below is a brief description of some of the most used extraction and clean-up methods for PAHs analysis in meat and fish matrices:

- **Saponification of lipids with KOH-methanol** – The alkaline saponification of lipids is traditionally the first step of PAHs extraction^{129,136} and has been vastly performed in foods rich in fat, including meat⁴⁵⁻⁴⁷ and fish.^{85,152} It is usually performed prior to the extraction process itself in order to ease the extraction of PAHs by eliminating/decreasing fat, pigments and other organic contaminants that might interfere with the further analytical determination¹³⁶ and can provide high recovery values (>120%).²³ It is a time and high solvent consumption process.¹³⁶
- **Soxhlet extraction and Sonication** – The Soxhlet extraction includes a device that consists of a flask placed at the bottom that gathers the extract, and a condenser at the top. It has the advantage of extracting the analytes of interest from a solid sample, using an adequate organic solvent, enabling the preparation of the extract without emulsification, eliminating the insoluble impurities.¹³⁶ Despite the major disadvantages being that it is a non-selective, time and solvent consuming process, requiring 6 to 24h of extraction, its efficiency still makes it widely used to analyse PAHs.^{23,36,108,153-155}

A modern and much faster alternative to Soxhlet extraction is the sonication/ultrasonic extraction, which applies sound energy at ultrasonic frequencies to extract various compounds from food, ^{123,136} and has been widely used to ease the extraction of PAHs from meat and fish. ^{4,36,48,121,123,156}

- **LLE and LLE microextraction techniques** – The principle of LLE, also called solvent extraction, is transferring the analyte from an aqueous layer into an organic layer. Initially, this method required a great amount of organic pollution solvents, but due to environmental concerns and optimization of processes, solvents amounts and toxicity decreased, with a more efficient partition of the matrix compounds to the extraction solvent, leading to sufficiently purified and concentrated extracts to chromatographic analysis. ¹³⁵ Water can be added to compensate the low water content of some samples. Solvents that are miscible with water, such as methanol, acetonitrile and acetone, and partially miscible ethyl acetate, are the most common solvents; they intrinsically merge with the sample, leading to an efficient extraction. ¹⁵⁷ Acetonitrile might be more interesting and selective, since it does not extract components from the food matrix such as lipids, proteins, sugars and minerals. ^{158,159}

In the constant attempt of improving the expensive and intensive labour LLE, there was a miniaturization of the technique, which drastically reduced the extractant volumes and the number of steps. Based on this, three new techniques were created: single drop microextraction (**SDME**), hollow-fibre liquid phase microextraction (**HF-LPME**) and dispersive liquid-liquid microextraction (**DLLME**). ¹⁴²

SDME is a simple preconcentration technique that uses a micro drop of extractant, not miscible with water, exposed to the sample solution. The exposition can occur in three forms: complete immersion in the solution; placement in the space right above the solution (headspace) without touching it, to extract volatile or semi-volatile compounds; utilization of another solvent less dense than water, where the drop is only immersed in that solvent to perform the extraction. ^{142,160} It is a non-expensive, quick and free of solvents technique, based on the great reduction of the ratio extractant/sample volume. Only a small number of analytes are extracted or pre-concentrated to analysis. The main disadvantages of SDME are the instability

of the drop when agitation or temperature are high, and the impossibility of using solvents miscible with water and with a low ebullition point.¹⁴²

HF-LPME is a two-phase sampling mode, where a hydrophobic hollowfibre, made of propylene and supported by a syringe, is used to protect and expose a certain volume of extractant to the sample. The extraction process happens in the pores of the hollow fibre, where the solvent is immobilised. In the three-phase sampling mode (hollow fibre liquid–liquid–liquid microextraction, HF-LLLME), the analytes are extracted to an organic solvent that fills the pores of the hollow fibre and then re-extracted into an aqueous phase placed inside the fibre.¹⁴²

DLLME is based on the formation of a cloudy solution, that is formed when a suitable mixture of two solvents, an extraction solvent and a disperser solvent, are quickly injected into the aqueous sample. The water-immiscible extractant solvent should have higher density than water (e.g. chloroform, carbon tetrachloride, carbon disulphide, nitrobenzene, bromobenzene, chlorobenzene or 1,2-dichlorobenzene) while the disperser solvent (e.g. methanol, ethanol, acetone or acetonitrile) must be miscible in the extractant solvent and the aqueous sample. The droplets of extraction solvent are dispersed throughout the aqueous sample, allowing its interaction with the analyte.^{142,161} The surface area between the extractant solvent and the aqueous sample is vast, therefore the equilibrium state is reached fast, thus the extraction is very quick; which is the greatest advantage of this technique. After centrifugation, the inferior sedimented part is collected and further analysed.¹⁶¹ The efficiency of DLLME depends on several factors, including the extraction's types and volumes, disperser solvents, pH, sample amount, extraction time and salt addition.

¹⁶²

- **SPE** – a sample preparation technique that enables the extraction, clean-up and concentration of analytes prior to their quantification, in which substances dissolved/suspended in a liquid solvent go through a solid (stationary phase) that retains either the analytes of interest or the undesired impurities; the portion that passes through the stationary phase is either collected or rejected, as well as the portion retained on the stationary phase (in case the compounds of interest are there, which can be removed with an appropriate eluent).^{163,164} SPE cartridges and disks are compact, disposable, easy to use, relatively selective and versatile, making this method an attractive option

compared to traditional approaches, especially due to the low solvent consumption, possibility of automatization and ability to concentrate the analytes of interest.^{135,165} SPE has been widely used in the PAHs determination in meat.^{31,166,167}

- **SPME** – a solvent free method that uses a fused silica fibre coated with an appropriate stationary phase attached to a modified micro syringe. In general, it is not an exhaustive extraction technique, nonetheless matrix effects can be an issue, and quantification normally requires matrix matched standards or the method of standard additions. It can be performed in various ways, including direct immersion into a liquid sample, or headspace (HS) sampling. HS sampling which is the most used method in solid food but can present some limitations, such as e.g. the damaging of the fibre, or irreversible absorption of proteins to the fibre.¹⁶⁸ Another alternative would be to homogenise the solid sample with a liquid solution.²³

SPME is habitually used in liquid samples, although it has already been applied to analyse PAHs in meat, using a direct extraction device (SPME-DED), which contains a gelatine support for the SPME fibre that allows the interaction between the fibre and the solid sample.¹⁶⁹

- **SFE** – this technique uses supercritical fluids to extract the compounds of interest from solid matrices. A supercritical fluid (commonly a gas) is produced by heating the fluid above its critical temperature and pressure; the extraction fluid is usually carbon dioxide (CO₂), mainly due to its mild supercritical condition ($t_c = 31.1\text{ }^{\circ}\text{C}$, $p_c = 7.38\text{ MPa}$), its low cost and environmentally safe.^{136,168} The supercritical fluid is easily removed after extraction, decreasing temperature or pressure, so it becomes a gas again, and the compounds of interest form a liquid or a solid.¹⁶⁸ SFE is effective in shortening extraction times with high efficiency and low usage of organic solvents.^{136,168} However, SFE is lengthier than solvent extraction processes, requires expensive instrumentation and often suffers matrix effects.¹⁷⁰ It is vastly used in environmental PAHs analysis, although it has also been applied to determine PAHs processed fish and meat.^{171,172}
- **MAE** – in this method the samples are extracted with organic solvents and irradiated with microwaves, that heat the both the solvent and the sample

without getting to the boiling point. Irradiation can be repeated as many times as needed to extract the maximum of the analytes of interest.¹⁷⁰ Thus, this method increases the transfer rate of the analytes of interest from the food matrix to the extraction solvent.¹⁷³ However, this technique also has some disadvantages, including the fact that it might not be as selective and cause the loss of volatile compounds, the need of different extraction containers to each sample and the difficulty of automatization.¹⁶⁸

The MAE technique has been largely used in food samples. Meat and fish usually require an additional clean-up step such as e.g. SPE purification or DLLME.²³

- **QuEChERS** – The constant search for techniques that do not require numerous extraction solvents, is less time consuming, does not involve expensive equipment and presents high efficiency of extraction lead Anastassiades et. al¹⁵⁹ to develop the sample preparation method QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) which, as the name implies, is a simple, fast and inexpensive method attempting to overcome these challenges. It was firstly developed to determine pesticide residues in fruits and vegetables¹⁵⁹ but is also able to determine PAHs in different foods, including meat^{129,174,175} and fish.¹⁷⁶⁻¹⁷⁸ Besides being simple to perform, fast and cheap, the accuracy of the performance and the recovery ranges are also usually high, being associated with low limits of detection that can be in the threshold of $\mu\text{g kg}^{-1}$ or μL^{-1} as well.¹⁴⁴

QuEChERS is a SPE method with a dispersive SPE (d-SPE);¹⁷⁹ it is based on the extraction with acetonitrile (or other organic solvents) partitioned from the aqueous phase by the addition of the anhydrous salts MgSO_4 and NaCl . The residual water removal and the clean-up are simultaneously performed by the d-SPE. The most used sorbent is primary–secondary amine (PSA),¹⁵⁹ although other sorbents (e.g. gCB, C_{18} , OASIS HLB and activated carbon) can also be used.¹²⁹ The d-SPE with PSA effectively removes some polar matrix components such as sugars, organic acids and other polar pigments. After the clean-up, the extract is centrifuged and the supernatant can either be directly analysed or further concentrated.¹⁵⁹

Some changes have been applied to the original method, according to each type of sample or target analytes. For example, some authors remove the PSA since no differences are found in the LC-FLD analysis, although they usually add an additional clean-up step, such as an LLE or an SPE cartridge.¹⁸⁰ On the other hand, some authors add PSA and C₁₈ simultaneously, to clean-up some samples better, especially the fatty ones such as olive oil.¹⁸¹ Other modification was combining QuEChERS with DLMLE (to extract pesticide residues, for example), using a DLLME with a dispersive solvent, replacing the d-SPE phase; assuring a high enrichment factor and good LODs.¹⁴¹

2.2.2. Separation and Detection Methods

There is an ongoing demand to develop techniques that reduce solvents, are less time-consuming and environmentally friendly, as well as techniques presenting high sensitivity/low detection limits (LODs), since PAHs maximum levels allowed in foodstuffs established by the current legislation are low as well.^{2,23} Both liquid and gas chromatography are suitable to analyze PAHs, with no difference in accuracy of results and therefore roughly equivalent in quality criteria.¹⁸² They can be coupled with different detection systems, most commonly, LC coupled to fluorescence (FLD), diode array detection (DAD) or ultraviolet-visible (UV) detection, and GC coupled to mass spectrometry (MS), operated in selected ion monitoring (SIM).^{23,134,139}

Regarding HPLC columns, reverse-phase columns are the most frequently used due to their high resolution efficiency; with this method, the molecular sizes of PAHs can be estimated based on their retention times on the HPLC charts.¹³⁶ The most commonly used eluent for chromatographic separation is acetonitrile-water in isocratic conditions¹⁶⁶ or gradient elution.^{123,183} Water-methanol-acetonitrile¹⁸⁴ is also used for PAHs separation by HPLC/FLD. Other elution solvents such as methanol-water can also be used, turning PAHs analysis cheaper, although it generally generates poor peak resolution.⁴ HPLC with fluorescence detection is the most frequently used analytical method to determine carcinogenic priority PAHs in food samples,^{23,29} although Acenaphthylene does not give rise to fluorescence and must be determined otherwise, for example by LC/UV or GC/MS.^{6,23}

PAHs have also been analyzed by GC techniques coupled with different detection methods. Some typical detectors equipped in GC are Flame Ionization Detector (FID), photoionization detector (PID) and quadrupole electron impact mass spectrometry (EI-MS).¹³⁶ FID has a broad linearity and is generally non-selective; the response is directly proportional to the number of carbons. PID is more selective for aromatic hydrocarbons but is not responsive enough to some alkylbenzenes.¹³⁶ Therefore, PAHs detection is usually performed by MS analysis in the selected ion monitoring (SIM) mode. GC-MS is one of the most used methods due to its selectivity and sensitivity; PAHs identification is based on the characteristics of the mass spectrum of each compound with relative abundance of the ions selected for each PAH.¹³⁶

The most used methods are single quadrupole GC-MS and HPLC-FLD to determine PAHs in food.⁶ The advances of mass spectrometry and the increasing affordability of instruments led to the large application of tandem mass spectrometers combined with both GC (GC-MS/MS) and ultra-performance LC (UPLC-MS/MS).¹⁸⁵ GC-MS instruments nowadays are sensitive enough to implement the current legislation on PAHs contents of food, thus improving the detectability of PAHs is a low priority.⁶ Fast non-chromatographic methodologies for PAHs analysis in food have not yet been described.⁶

2.2.3. Quantification Methods

The external calibration curve is the most common method to quantify PAHs in several food matrices, including processed meat and fish.^{31,37,92,137,138,166,184} Despite the advantages, analyte losses can take place in sample preparation due to incomplete extraction, therefore causing underestimated results. On the other hand, overestimated results can also occur due to matrix effects, if the chromatographic separation of PAHs and co-extractive compounds is inadequate. This method, for the quantitative determination of PAHs, does not consider the effect of systematic errors arising from either sample preparation and/or chromatographic separation and detection.⁴

The standard addition is not commonly used to determine PAHs in food samples, most likely because it requires the addition of the analytes under study in

different concentrations and therefore a higher number of sample preparations and analyses are required. However the effect of systematic errors is diminished and can be a better choice when low levels of analyte are determined. ⁴ Viegas et al ⁴ showed that the standard addition method was the most adequate method (vs external calibration curve method) to quantify PAHs levels in processed meat and fish, when PAH8 are present in low amounts, since this method compensates PAHs losses in sample preparation due to incomplete extraction.

3. Materials and Methods

3.1. Standards and Reagents

The standard mixture (PAH Calibration Mix) prepared in acetonitrile, contained 10 $\mu\text{g mL}^{-1}$ of naphthalene (Na), acenaphthene (Ac), acenaphthylene (Ace), fluorene (F), phenanthrene (Pa), anthracene (A), fluoranthene (Fl), pyrene (P), BaA, Chr, BbF, BkF, BaP, DhA, IP and BgP. It was supplied by Supelco (47940-U, Bellefonte, PA, USA). The working solution was prepared from this one, diluted ten times (1 $\mu\text{g mL}^{-1}$).

The internal standard (IS) used was tryphenylene (purity $\geq 98\%$) and was supplied by Sigma. The working solution (10 ng mL^{-1}) was prepared in acetonitrile.

PAHs standard solutions were stored in amber flasks at $-20\text{ }^{\circ}\text{C}$.

All the solvents used for PAHs analysis – acetonitrile, methanol and ethyl acetate – were of HPLC grade (Merck, Darmstadt, Germany) and water was purified using a Milli-Q System (Millipore, Bedford, MA, USA). The glassware, mostly amber, was carefully washed and rinsed with HPLC grade acetone and hexane before use.

3.2. Sampling

3.2.1. Meat and fish samples used for method validation

A blank matrix was used with different purposes: to optimize PAHs extraction; to prepare matrix-standard calibration solutions and precision assays. The blank matrix consisted of fresh pork spare ribs (2 kg) purchased from a local butchery in Porto and immediately cooked. The blank matrix was carefully cooked in order to avoid PAHs formation, therefore the meat was slowly cooked in an oven at low temperature (120 $^{\circ}\text{C}$ – 2h), covered by an aluminium foil to avoid roasting and with a tray full of water below, to collect eventual meat drips. No seasonings or other ingredients were added to the meat. After cooking, the meat cooled down at room temperature. Afterwards, it was minced with a knife mill (Grindomix GM 200, Retsh, Hann, Germany) and kept frozen ($-20\text{ }^{\circ}\text{C}$) until further analyses. This matrix presented $17.1 \pm 0.2\%$ of fat and $47.8 \pm 0.2\%$ of moisture.

For recovery studies different types of charcoal grilled muscle samples (chicken breast, salmon, pork loin and ribs and beef steaks; $n = 3$ for each sample)

were purchased in a local barbecue grill restaurant cooked as usual, whereas smoked muscle samples (chorizo, n=3) were purchased in supermarket. All the samples were individually minced with a knife mill and frozen until PAHs analyses. They presented an average of 50% of moisture, ranging from 43% (chicken) to 61% (beef). Regarding fat content, different levels were found: in average, around 5% in pork, beef steaks and chicken, and 21% in grilled salmon and (smoked) chorizo. Detailed moisture and fat content of the samples used in the present thesis are displayed in **Table 3**. To determine moisture content, an infrared Scaltec-SMO 01 scale (100 °C) was used, and to determine fat content, a Soxhlet device (Extraction system Büchi B-811, Flawil, Switzerland) was used.

Table 3. Moisture and fat content of the analysed samples.

Moisture and Fat Content of the Analysed Samples			
SAMPLE		Fat (%)	Moisture (%)
GRILLED	Chicken Breast	4.0 ± 0.1	43.0 ± 2.0
	Pork Loin	5.5 ± 0.1	56.1 ± 1.0
	Pork Ribs	17.1 ± 0.2	47.8 ± 0.2
	Beef Steaks	4.6 ± 0.2	61.0 ± 0.1
	Salmon	21.0 ± 0.1	47.0 ± 0.1
SMOKED	Chorizo	20.5 ± 0.6	46.0 ± 0.4

3.2.2. Smoked salmon samples

Twenty-three different smoked salmon samples available on the Portuguese market (n= 23), purchased in supermarkets were selected for PAHs monitorization. Detailed characterization of the salmon smoked samples used in the present thesis are displayed in **Table 4**. The smoked salmon samples were classified in five groups, according to the origin and type of sample, being: A – simple smoked salmon; B – cold smoked salmon; C – hot smoked salmon, with skin; D – biological origin smoked salmon; E – non-smoked salmon; F – marinated smoked salmon.

Table 4. Characterization of the analysed samples of smoked salmon.

Group	Sample	Ingredients	Fat (%) *	Moisture (%) **	Other information
A	A1	<i>Salmo salar</i> , salt, sugar.	9.2	54.35±0.71	Norway.
	A2	<i>Salmo salar</i> fed w/o GMO 97%, salt 3%.	11	62.24±1.31	Smoked with beech wood. Norway.
	A3	<i>Salmo salar</i> , salt.	13	57.51±2.14	Norway.
	A4	<i>Salmo salar</i> , salt.	13	61.39±0.36	Smoked with fine woods. Norway.
	A5	<i>Salmo salar</i> , salt.	13	59.12±1.11	Smoked with fine woods. Scotland.
	A6	<i>Salmo salar</i> , salt, sugar.	9.2	58.94±0.02	Smoked with traditional oven.
	A7	<i>Salmo salar</i> fed w/o GMO 97%, salt 3%.	10	60.89±0.5	Smoked with beech wood. Scotland.
	A8	<i>Salmo salar</i> , salt.	14	58.79±0.26	Scotland.
	A9	<i>Salmo salar</i> , salt.	13	62.78±0.22	Smoked with beech wood. Ireland.
B	B1	<i>Salmo salar</i> , salt.	9.6	61.59±0.81	Cold smoked. Norway.
	B2	<i>Salmo salar</i> , salt, sugar, white pepper, dill.	13	54.29±0.5	Cold smoked. Norway.
	B3	<i>Salmo salar</i> , salt.	13	47.28±1.46	Cold smoked. Norway.
C	C	<i>Salmo salar</i> , salt, black pepper.	8.7	50.06±0.52	Hot smoked. Norway.
D	D1	<i>Oncorhynchus Nerka</i> , salt.	13	64.19±1.28	Wild salmon, Norwest Pacific. Alaska.
	D2	Organic <i>Salmo salar</i> , salt.	6.4	57.02±1.02	Smoked with fine woods.
	D3	Organic <i>Salmo salar</i> , salt.	13	60.41±0.22	Smoked with beech wood.
E	E	<i>Nature Salmo salar</i> , salt.	12	55.98±0.40	Non-smoked. Norway.
F	F1	<i>Salmo salar</i> , salt, dill.	13	55.56±0.65	Smoked with fine woods. Norway.
	F2	Marinated <i>Salmo salar</i> , salt, sugar, dill.	17	54.24±0.17	Norway.
	F3	<i>Salmo salar</i> , salt, spices.	13	55.86±0.12	Carpaccio. Norway.
	F4	Marinated <i>Salmo salar</i> , salt, sunflower oil, dill, spices.	14	53.17±1.48	Norway.
	F5	<i>Salmo salar</i> , salt.	13.4	59.38±1.17	Carpaccio. Norway.
	F6	<i>Salmo salar</i> , salt, herbs, curry 0.10%.	13	56.45±0.03	Smoked with fine woods. Norway.

*fat content according with the label.

**moisture content, determined using an infrared Scaltec-SMO 01 scale (100 °C).

GMO: Genetically Modified Organism

3.3. PAHs Extraction

An acetonitrile-based extraction with partitioning of PAHs was based on the technique developed by Anastassiades et al ¹⁵⁹ and changed to perform the analysis on muscled processed foods. The extraction was performed in 50 mL centrifuge tubes: 5 g of each sample (weighed in a Mettler Toledo AG04 (Urdof, Switzerland)) were spiked with standard (20 μ L of working solution) and/or internal standard (20 μ L of working solution); 5 mL of deionized water were added, and the tube was vortexed for 1 minute (with vortex from VELP Scientifica (Usmate, Italy)). Afterwards, 5 mL of acetonitrile were added and shaken vigorously by hand. Then, 2.5 g anhydrous magnesium sulphate and 2.5 g sodium chloride were added, followed by vigorous shaking (to avoid formation of oversized magnesium sulphate agglomerates) and centrifugation at 4000 rpm for 5 min (with Thermo Scientific Heraeus Megafuge 16 centrifuge). Afterwards, 2 mL of the upper layer of the acetonitrile extract were evaporated to dryness under nitrogen stream at room temperature. The residue was dissolved in 100 μ L of acetonitrile and injected into the HPLC-FLD.

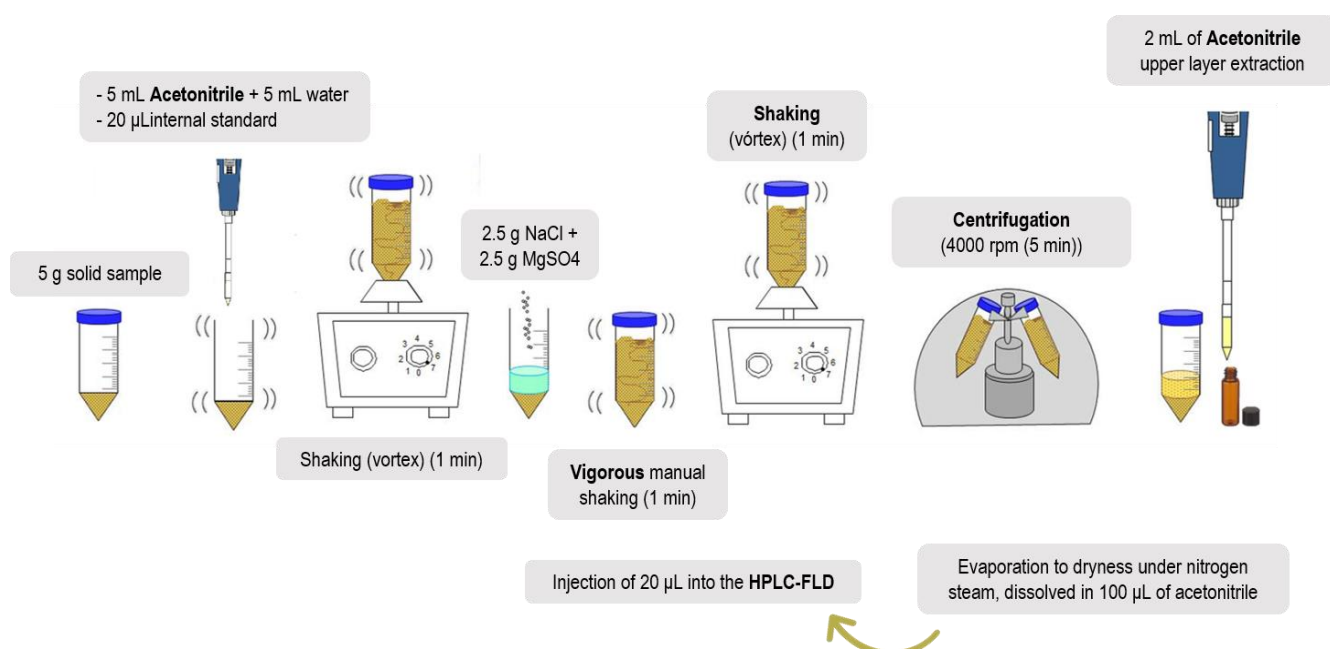


Figure 3. Representative scheme of the developed acetonitrile-based extraction with partitioning, coupled with HPLC-FLD.

3.4. HPLC-FLD Conditions

Gradient elution and fluorescence detection excitation/emission program were set up according to the method described by Viegas et al.³⁵ Regarding the equipment, the HPLC unit (Jasco, Japan) was equipped with one HPLC pump (PU-1580), an auto sampler (AS-950) with a 20- μ L loop. A C18 Supelcosil LC-PAH (25 cm length; 4.6 mm internal diameter, 5- μ m particle size) (Supelco, Bellefonte, PA, USA) column thermostated at 32 °C (with a Column Heater Model 7981 Jones Chromatography oven) was used, along with a fluorescence detector (FP-2020 Plus). The Fluorescence program used to separate PAHs is shown in **Table 5**.

The mobile phase gradient program used 3 solvents: A – methanol:water (75:25); B – methanol (100%); C - Ethyl Acetate (100%), with 1 mL min⁻¹ flow. The gradient program is shown in **Table 6**. Mobile phases degasification was performed using a magnetic stirrer coupled with a vacuum system, ESP Stirrer from VELP Scientifica (Usmate, Italy).

The system was controlled by Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France). The detection for IP had a gain tenfold higher than for the other PAHs.

Table 5. Fluorescence program used to separate PAHs.

PAHs	λ_{Ex} (nm)	λ_{Em} (nm)
Na, Ac, F	276	330
Pa	250	336
A	250	402
Fl	270	460
P, BaA, Chr	270	390
BbF	260	430
BkF, BaP, DhA, BgP	290	410
IP	290	470

Table 6. Mobile phase gradient program used to separate PAHs.

Time (min)	% Eluent A	% Eluent B	% Eluent C
0	100	0	0
18	20	80	0
19	0	100	0
20	0	90	10
28.5	0	82	18
37.5	0	80	20
40	0	100	0
45	100	0	0

3.4.1. Experimental Design

The amount of sample and acetonitrile were optimized by univariate analysis, whereas experimental design methodology (through a full factorial Central Composite Design (CCD)) was used to optimize the amount of salts, one of the most influential parameters on this extraction, and interact between them can occur.

Optimization of conditions for PAHs extraction from processed muscle foods was carried out using Response Surface Methodology (RSM). Experiments with two significant variables, magnesium sulphate amount (X_1) and sodium chloride amount (X_2), were conducted following the experimental design statistical analysis obtained by the full factorial central CCD. CCD consisted of n factorial points (quadratic points) (**Figure 4**), where each point has a distance of -1 or +1 from the centre of response. To each factorial point, axial points are added from the centre of the axial point, which is at a distance of $-\alpha$ or $+\alpha$. The α value is dependent on the number of variables included in the experimental design. This value can be obtained through the equation $\alpha = \sqrt[4]{2^k}$ and is usually within the interval $1-\sqrt{k}$.

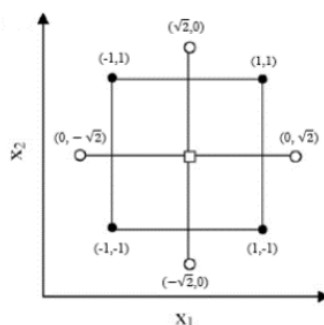


Figure 4. CCD design for the optimization for two variables. Adapted from Bezerra et al¹⁸⁶

In this work, the full CCD consisted of a complete 2^2 factorial design; C_0 , centre point ($C_0 > 1$); and four axial points on the axis of each variable at a distance of $\alpha = 1.414$ from the design centre. The total number of design points of $N = 2^k + 2k + C_0$ was used, meaning a complete 2^2 -factorial design as cubic points was used, with four axial points at a distance of $\alpha = 1.414$ from the design centre, and five centre points, leading to a total of 13 independent combinations randomly performed, which results are displayed in **Table 7**. The sum of peak area for PAHs represented the responses (Y).

The analysis of the experimental design was executed using the software Design Expert trial version 7 (Stat-Ease Inc., Minneapolis, MN, USA). The other analytical results were statistically analysed with IBM SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) of data was performed and differences were considered significant at $p < 0.05$.

Table 7. Central composite design to evaluate the effects of anhydrous magnesium sulphate (X1) and sodium chloride (X2) amounts on PAHs extraction.

Standard order	Independent variables		Response (sum of the peak areas, Y)
	X ₁ (g)	X ₂ (g)	
1	4	4	2885784.0
2	2.5	2.5	3598400.0
3	1	1	2139540.0
4	4.621	2.5	3274546.8
5	0.3787	2.5	2753370.0
6	2.5	2.5	3978790.0
7	1	4	2779614.8
8	2.5	2.5	4144248.6
9	4	1	3001334.1
10	2.5	0.3787	2564140.0
11	2.5	4.621	2963082.9
12	2.5	2.5	3829920.0
13	2.5	2.5	3810947.9

3.5. Quantification and Method Validation Parameters

The quality control of the extraction method was achieved through the analysis of blank samples and grilled or smoked samples with and without spiking. The identification criteria for PAHs were based on retention times and on co-elution with standards. Matrix-match calibration curves were prepared to study linearity, get regression equations and correlation coefficients (R^2). For that, 5 g of blank samples were spiked with appropriate amounts of PAH calibration mix and internal standard (40 ng g^{-1}) and extracted using the established conditions. Calibration curves with nine concentration levels were prepared in the interval between 0.2 and 60 ng g^{-1} . The weighted linear regression model was selected as the calibration model, to minimize the errors at the lower end of the calibration line that coexist with acceptable correlation coefficients (R^2) and are underestimated in analysing the dispersion of the regression parameters of least squares linear regression.¹⁸⁷ Thus, the study of the linearity involved the calculation of the standard deviations of slope ($S(b)_w$) and intercept ($S(a)_w$) as well as the random errors in the y-direction ($S(y/x)_w$), ($S(b)_w/bw\%$) and the sum of absolute % RE values, which are sensitive indicators of the quality of fit.¹⁸⁸

4. Results and Discussion

4.1. Optimization of the Acetonitrile-based Extraction Method

In order to optimize PAHs extraction, different variables were controlled, namely the amount of sample, reagents and salts added and used in the extraction. At the beginning, two different quantities of sample were tested (5 and 10 g); the amount of sample used was 5 g, since the results were much better compared to using 10 g (results not shown).

The volume of acetonitrile used in the extraction was determined through preliminary experiments by univariate method. Different amounts of acetonitrile were tested: 5 mL, 7.5 mL and 10 mL. **Figure 5** presents the effect of acetonitrile amount on PAHs extraction, evaluated by the sum of PAHs peak areas. The experiments pointed out that 5 mL of acetonitrile promoted the best condition for PAHs extraction without losing sensitivity.

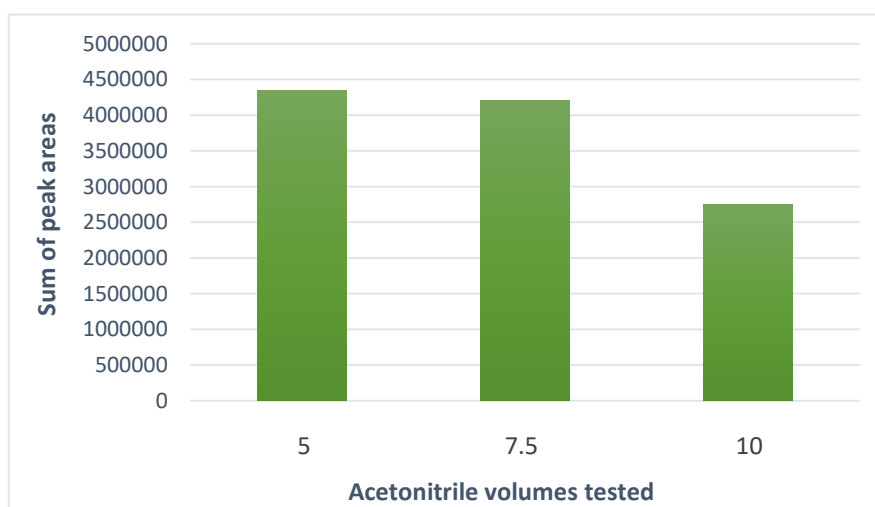


Figure 5. Effect of different acetonitrile volumes (mL) in PAHs extraction (sum of peak areas from the 14 PAHs).

After the preliminary assays for determination of the sample and acetonitrile amounts to be used, the acetonitrile-based extraction with partitioning was optimized using magnesium sulphate and sodium chloride, which are critical to promote phase separation of the acetonitrile extract containing PAHs from the water phase. As previously mentioned, the experiments were performed randomly at

different combinations of the parameters using statistically designed experiments of CCD.

Regression analyses were performed to fit the response functions, and the final model was obtained to PAHs compounds. Models presented a good suitability for quadratic interaction, with R^2 predicted value of 0.8220, which agrees with the R^2 adjusted value of 0.9081. Adequacy and significance of the models were evaluated by ANOVA through Fisher's F test, with an F value of 24.72, which implies the model is significant to the two variables with only 0.03% possibility that a large model F value could occur due to noise. The significant parameters obtained were the independent variable X1, the quadratic terms for X1 and for X2 had a significant effect on the response, whereas the independent variable X2 and the interactions between X1.X2 were not significant ($p < 0.05$). Thus, this model presented a quadratic response for the studied response. The “lack of fit value” for response was also obtained, with the value of Prob > F of 0.6388, demonstrating non-significant lack of fit ($p > 0.05$), which means that the model is valid for the present study and does not need reformulation.

Contour plot for experimental design permitted the prediction of response function (PAHs peak area) by the effects of the two variables under study, salts magnesium sulphate (X1) and sodium chloride (X2). From the analysis of contour plot (**Figure 6** and **Figure 7**), it is observable that the maximum of extraction was achieved in the middle range of the plot, so a higher level of response was obtained for an addition of approximately 2.5 g of each salt.

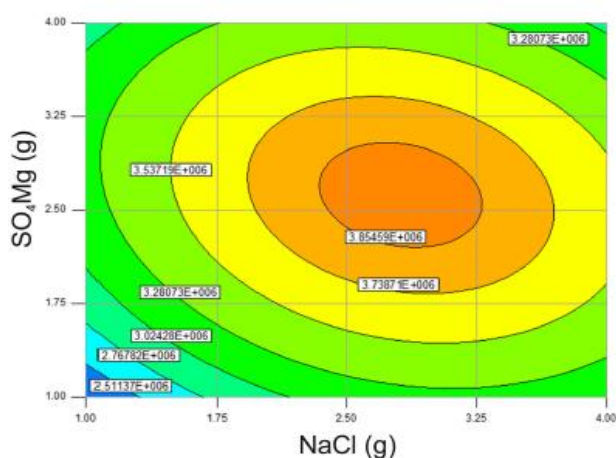


Figure 6. Contour plots for the interaction effects of anhydrous magnesium sulphate and sodium chloride amounts on PAHs extraction (2D model).

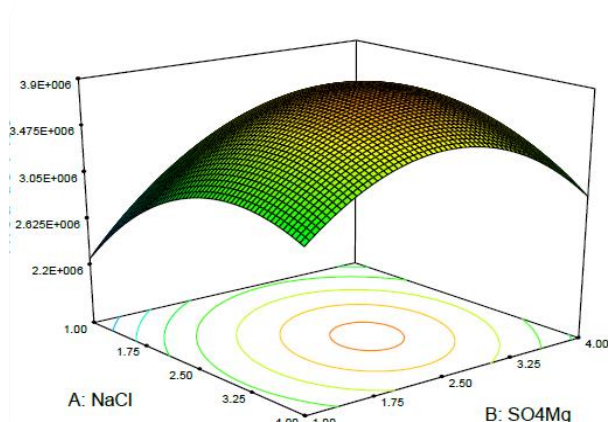


Figure 7. Contour plots for the interaction effects of anhydrous magnesium sulphate and sodium chloride amounts on PAHs extraction

4.2. Method Validation and Compliance with European Commission Regulation No. 836/2011

The analytical performance of the extraction method developed for reliable chromatographic quantification of PAHs in processed muscle foods was evaluated. Linearity, evaluated by the correlation coefficient (r_w) applying weighed least squares linear regression, was observed using matrix-calibration standards in the tested range, between 0.2 and 30 ng g⁻¹ for PAH8 and 0.2 and 60 ng g⁻¹ for other PAHs, usually found in grilled and smoked muscle foods in higher amounts.³⁵ The calibration parameters are summarized in **Table 7** and were calculated according to Miller and Miller.¹⁸⁸ Good linear responses were obtained for all compounds with coefficient correlations (R^2) above 0.99. The percentages of $S_{(b)w}/b_w$ were less than 0.14%, thus lower than 5%, which satisfies the validation conditions of the calibration curves. Relative errors ranged between 0.00 and 14.87%, therefore lower than the acceptable limits (20% for the lower concentration and 15% for the other standards of the calibration line).¹⁸⁸ For the 14 PAHs and under the experimental conditions, the LOD ranged from 0.04 to 0.12 ng g⁻¹, while the limit of quantification (LOQ) ranged between 0.12 and 0.39 ng g⁻¹. These values were similar to those found in the literature for grilled and smoked muscle foods using different extraction procedures and chromatographic methods. The uncertainties associated with the calibration curves (U) were calculated at two concentration levels of 0.2 and 12 ng g⁻¹ and in general, for higher concentrations, lower uncertainties were observed (**Table 8**).

Table 8. Calibration parameters and related uncertainties.

PAHs	b_w	a_w	r_w	%ER	$\sum\%ER$	LOD (ng g ⁻¹)	LOQ (ng g ⁻¹)	$S(y/x)_w$	$S(b)_w$	$S(a)_w$	$S(b)w/b_w\%$	% $U_{0.2 \text{ ng g}^{-1}}$	% $U_{12 \text{ ng g}^{-1}}$
Ac	0.1555	0.0492	0.9974	0.04-14.87	41.0	0.05	0.16	0.0024	0.00009	0.00109	0.0586	4.0	7.3
F	0.1616	0.0283	0.9934	1.41-14.04	54.3	0.05	0.18	0.0029	0.0001	0.0011	0.0561	4.4	9.5
Pa	0.2333	0.1488	0.9984	0.17-6.53	18.1	0.11	0.36	0.0084	0.00031	0.00373	0.1337	26.7	1.6
A	1.0481	0.0750	0.9988	0.00-7.82	21.4	0.05	0.17	0.0181	0.00057	0.00683	0.0545	4.4	4.9
Fl	0.0813	0.0271	0.9994	0.05-3.50	14.6	0.10	0.33	0.0027	0.00008	0.00102	0.1045	9.0	4.0
P	0.1930	0.0101	0.9999	0.00-3.78	7.8	0.04	0.12	0.0024	0.00009	0.00107	0.0463	4.8	0.5
BaA	0.3280	0.0168	0.9997	0.01-8.01	18.4	0.09	0.31	0.0102	0.00032	0.00386	0.0983	10.5	1.7
Chr	0.1846	0.0256	0.9983	0.13-12.39	26.9	0.09	0.31	0.0057	0.00021	0.00253	0.1144	14.1	3.3
BbF	0.1596	-0.0161	0.9991	0.09-5.81	21.5	0.11	0.38	0.0060	0.00022	0.00269	0.1406	14.6	5.9
BkF	0.6551	-0.0283	0.9996	0.24-2.47	10.0	0.06	0.18	0.0121	0.00045	0.00541	0.0690	16.1	2.3
BaP	0.2304	-0.0161	0.9990	0.35-5.57	20.5	0.06	0.21	0.0048	0.00015	0.00183	0.0663	5.5	3.1
DhA	0.1143	-0.0089	0.9957	1.07-10.89	34.1	0.07	0.22	0.0026	0.00008	0.00097	0.0711	5.7	2.5
BgP	0.0610	0.0030	0.9992	0.19-9.02	20.9	0.09	0.30	0.0018	0.00007	0.00080	0.1102	7.5	1.5
IP	0.1041	-0.0106	0.9970	0.76-9.09	29.3	0.12	0.39	0.0041	0.00013	0.00154	0.1238	12.1	21.5

Linear range: 0.2 – 60.0 ng g⁻¹ for Ac, F, Pa, A, Fl and P; 0.2 – 30.0 ng g⁻¹ for BaA, Chr, BbF, BkF, BaP, DhA, BgP and IP. b_w – weighted slope, a_w – weighted intercept, r_w – weighted correlation coefficient; $\sum\%ER$ – Sum of relative errors; LOD – limit of detection; LOQ – limit of quantification; $S(y/x)_w$ – standard deviation of y-residuals of weighted regression line, $S(b)_w$ and $S(a)_w$ – standard deviations of the weighted slope and weighted intercept, $S(b)w/b_w\%$ – Percentage of standard deviation of weighted slope of weighted slope, U - uncertainties associated with calibration curve

The validation of the method was performed in accordance with the guidelines established by ICH (International Conference on Harmonization) 2005¹⁸⁹ for the 14 PAHs under study. Results from repeatability and reproducibility are presented in **Table 9**; the relative standard deviations (RSD) were lower than 13.3% for reproducibility and 8% for repeatability, with respect to the lower PAHs concentration (0.4 ng g⁻¹).

Table 9. Repeatability and Reproducibility (RSD %) at three levels of spike (0.4; 2; 12 ng g⁻¹) in blank matrix.

Spike	Repeatability (n=3)			Reproducibility (n=3)		
	0.4 ng g ⁻¹	2 ng g ⁻¹	12 ng g ⁻¹	0.4 ng g ⁻¹	2 ng g ⁻¹	12 ng g ⁻¹
PAH	RSD (%)			RSD (%)		
Ac	7.64	3.31	2.96	9.03	6.20	4.26
F	3.11	4.58	1.35	5.88	7.26	3.00
Pa	5.32	3.44	0.82	13.30	6.64	1.83
A	5.98	0.37	2.73	11.34	4.81	3.68
Fl	2.41	2.00	0.51	11.65	7.19	1.56
P	5.86	4.73	1.10	5.68	7.02	1.20
BaA	1.62	2.41	2.26	1.61	4.54	2.92
Chr	6.26	2.52	0.53	11.87	3.12	2.29
BbF	6.30	2.68	1.99	6.43	4.13	3.59
BkF	1.37	1.78	1.00	3.88	2.84	1.28
BaP	6.34	2.25	1.11	10.64	4.57	2.11
DhA	3.66	3.27	0.13	7.38	3.35	2.09
BgP	8.01	3.73	0.31	12.51	4.38	0.76
IP	7.63	1.40	1.77	11.55	5.15	2.55

European Commission Regulation No. 836/2011³ establishes, as previously mentioned, performance criteria for the analysis of PAH4 in foods and the proposed methodology complies with the required parameters. **Figure 8** (see upper right side for PAH4) shows there are no coeluting interferences from the matrix for each compound, proving the specificity of the method. Regarding repeatability and

reproducibility for PAH4, when expressed as relative standard deviation were lower than 7 and 12%, respectively; and when expressed by the Horwitz ratio, $HORRAT_r$ was lower than 0.32, and $HORRAT_R$ was lower than 0.55, both lower than 2, which is the maximum value set by the European Commission.

Recovery is another parameter to meet and according to the European Commission,³ should be within the range of 50 – 120% to be acceptable. The recovery values observed in the proposed method were, for BaA and Chr between 80 and 105%, for BbF 72 and 107%, and for BaP 86 and 118%, respectively, showing all the results in agreement with the regulation criteria.

Regarding LODs and LOQs, the EC states that it should be lower than 0.30 and 0.9 ng g⁻¹, respectively,³ were very well accomplished for each of the four PAHs in the present method, being lower than 0.12 and 0.39 ng g⁻¹, respectively.

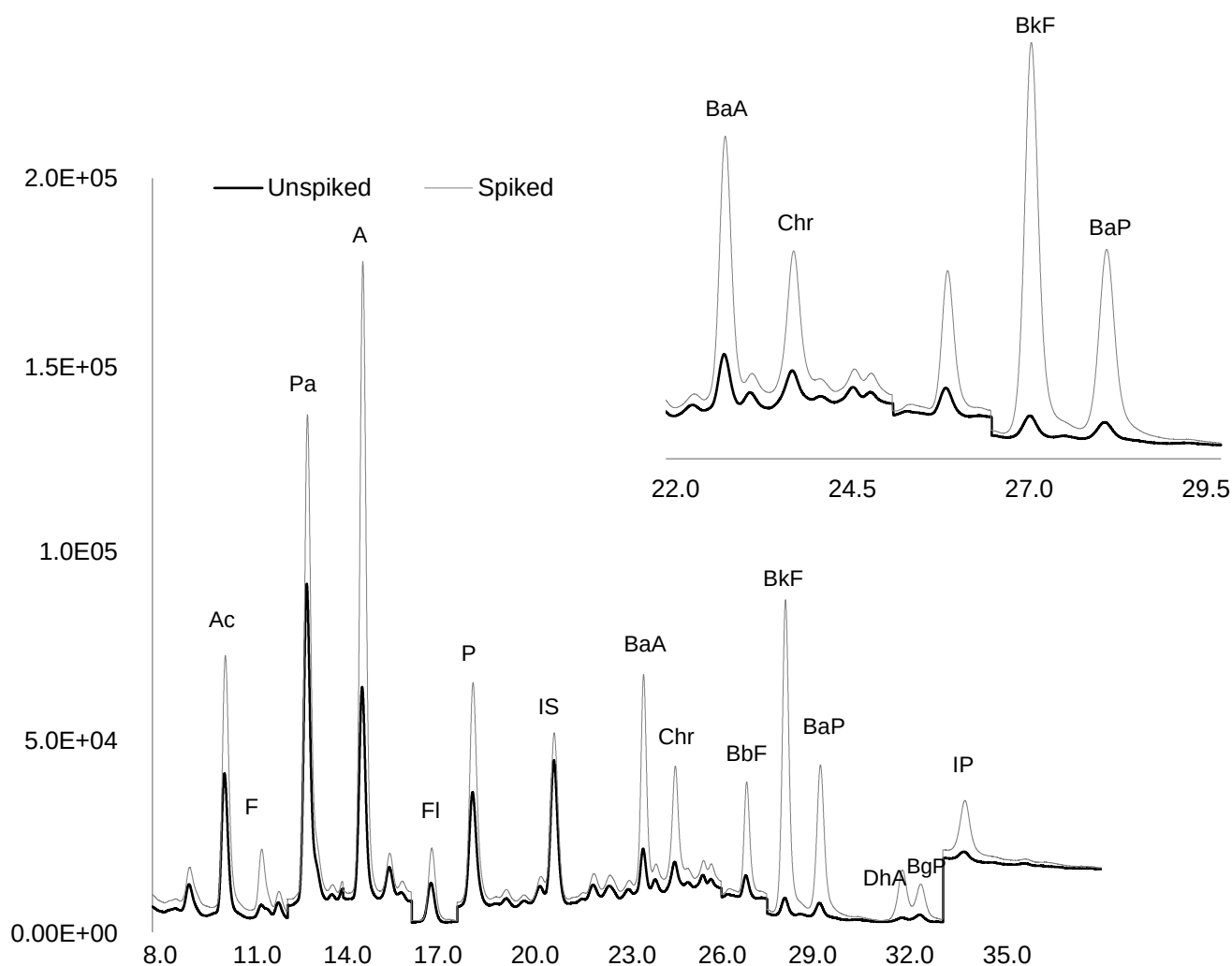


Figure 8. Representative chromatograms from grilled chicken, unspiked (black line) and spiked (grey line) with the 14 PAHs at the 2 ng g⁻¹ level and International Standard (IS). Detailed PAH4 is presented in upper right side.

There is a lack of certified reference materials (CRMs) for PAHs analysis in processed muscle foods, therefore to demonstrate the reliability of the results, the accuracy of the method was verified via spiking experiments at two levels, in different types of processed muscle foods; **Figure 8** displays representative chromatograms from grilled chicken, spiked and unspiked with 14 PAHs at the level of 2 ng g^{-1} . The recovery values were obtained for different grilled or smoked muscle food matrices, namely: grilled pork meat, beef, chicken, smoked chorizo, and grilled salmon. The recoveries, summarized in **Figure 9 A and B**, were within the control limits established by ICH guidelines (between 70 and 120%), being most of the recoveries in the range 80–110%. In accordance with European Commission Regulation No. 836/2011 ³ a recovery range of 50–120% was established to be acceptable. Considering the present method, BaA and Chr presented recovery values between 80 and 105%, and higher dispersion was found to the recoveries of BbF (72 and 107%) and BaP (86 and 118%), being all the results in good accordance with the Regulation.

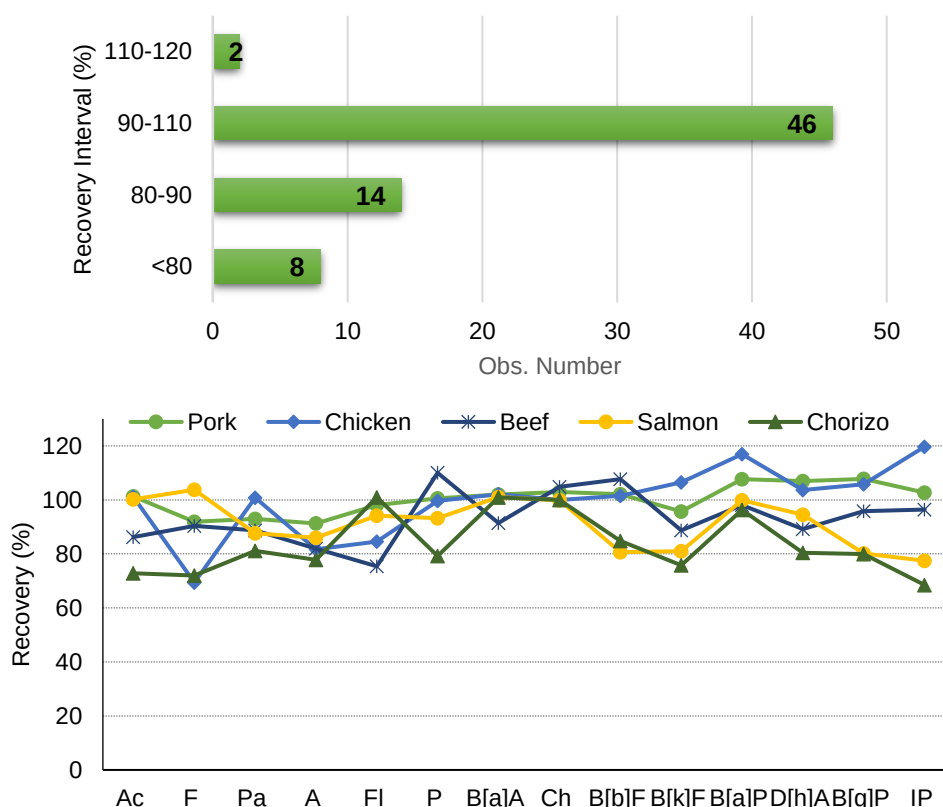


Figure 9 A. Recovery values (%) of 2 ng g^{-1} for Ac, F, Pa, A, Fl, and P and 10 ng g^{-1} for BaA, Chr, BbF, BkF, BaP, DhA, BgP and IP from muscle food matrices (pork, chicken, beef, chorizo, and salmon),. Histogram represents the recovery intervals (low spiked levels).

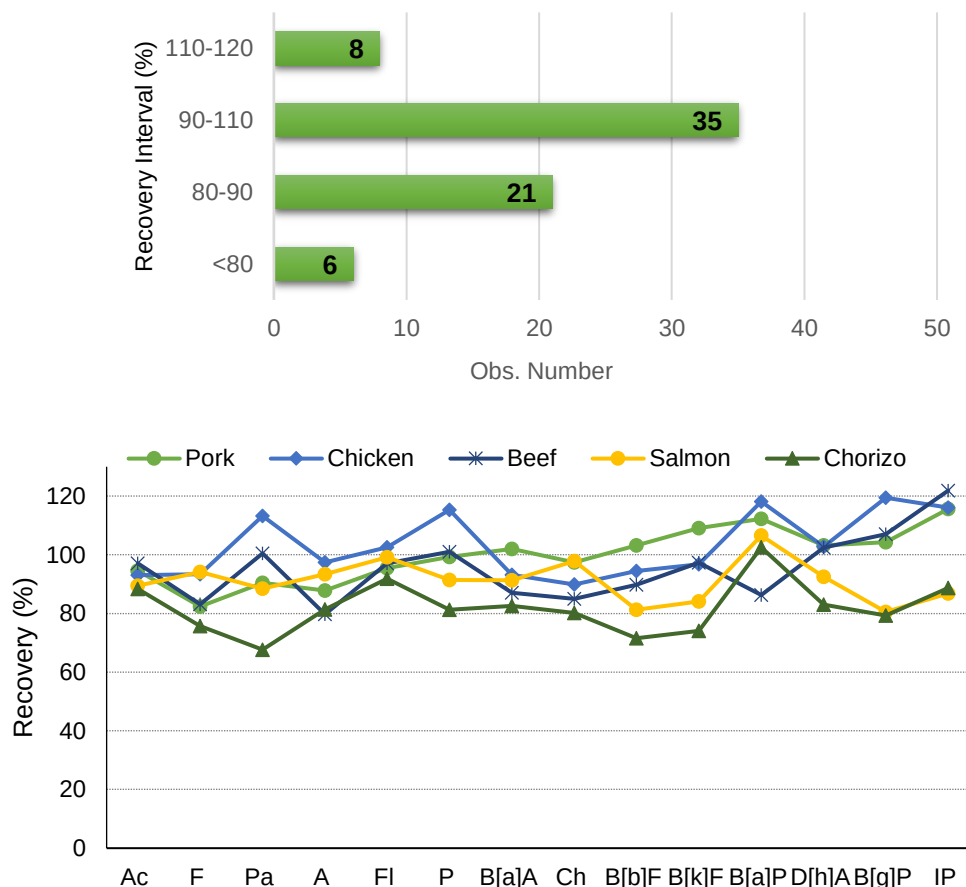


Figure 9 B. Recovery values (%) of 10 ng g⁻¹ for Ac, F, Pa, A, Fl, and P and 20 ng g⁻¹ for BaA, Chr, BbF, BkF, BaP, DhA, BgP, and IP from muscle food matrices (pork, chicken, beef, chorizo, and salmon). Histogram represents the recovery intervals (high spiked levels).

Grilled and smoked muscle foods were analysed in order to check the efficiency of the proposed method (**Table 10**). As previously mentioned, EC 835/2011 establishes the maximum levels of 5 and 30 ng g⁻¹ for BaP and PAH4 respectively in heat-treated meat, and 2 and 12 ng g⁻¹ for smoked meat.² All grilled samples presented BaP levels lower than the maximum allowed. Among these, grilled salmon was the sample that presented the highest content of BaP (2.12 ng g⁻¹), while other samples contained less than 1 ng g⁻¹. Concerning the PAH4 sum, salmon also presented the highest content (21.54 ng g⁻¹), followed by chorizo (7.30 ng g⁻¹), chicken (3.88 ng g⁻¹), pork (3.13 ng g⁻¹), and beef (2.61 ng g⁻¹), being all below the maximum values established by the regulation.

Table 10. PAHs content in different cooked muscle food by acetonitrile-based extraction with partitioning (results presented as mean $\pm$ standard deviation, n = 3).

PAHs	Charcoal-grilled (ng g ⁻¹)			Smoked (ng g ⁻¹)	
	Pork	Chicken	Beef	Salmon	Chorizo
Ac	13.99 $\pm$ 0.08	5.96 $\pm$ 0.07	26.17 $\pm$ 5.17	153.47 $\pm$ 12.10	24.36 $\pm$ 0.25
F	1.14 $\pm$ 0.06	0.52 $\pm$ 0.07	8.07 $\pm$ 0.52	59.02 $\pm$ 5.09	6.10 $\pm$ 0.23
Pa	15.28 $\pm$ 0.27	8.37 $\pm$ 0.37	43.54 $\pm$ 0.05	229.08 $\pm$ 7.30	39.92 $\pm$ 0.79
A	1.44 $\pm$ 0.00	1.33 $\pm$ 0.10	< 0.17	49.47 $\pm$ 0.76	8.56 $\pm$ 0.44
Fl	4.16 $\pm$ 0.11	3.30 $\pm$ 0.05	8.42 $\pm$ 0.09	47.35 $\pm$ 3.70	27.92 $\pm$ 4.42
P	5.19 $\pm$ 0.53	4.51 $\pm$ 0.19	4.25 $\pm$ 0.05	36.97 $\pm$ 0.85	16.02 $\pm$ 0.88
BaA	0.65 $\pm$ 0.01	0.84 $\pm$ 0.05	< 0.31	6.53 $\pm$ 0.31	2.51 $\pm$ 0.35
Chr	0.99 $\pm$ 0.14	1.16 $\pm$ 0.04	1.34 $\pm$ 0.03	9.98 $\pm$ 0.49	2.83 $\pm$ 0.18
BbF	1.03 $\pm$ 0.03	1.12 $\pm$ 0.10	0.82 $\pm$ 0.06	2.90 $\pm$ 0.16	0.98 $\pm$ 0.05
BkF	0.31 $\pm$ 0.02	0.35 $\pm$ 0.05	0.30 $\pm$ 0.03	0.79 $\pm$ 0.13	0.44 $\pm$ 0.03
BaP	0.46 $\pm$ 0.05	0.75 $\pm$ 0.11	0.29 $\pm$ 0.02	2.12 $\pm$ 0.02	0.95 $\pm$ 0.04
DhA	1.01 $\pm$ 0.09	0.78 $\pm$ 0.12	0.59 $\pm$ 0.09	1.18 $\pm$ 0.09	0.50 $\pm$ 0.09
BgP	0.97 $\pm$ 0.07	1.48 $\pm$ 0.19	0.87 $\pm$ 0.08	1.79 $\pm$ 0.02	0.63 $\pm$ 0.07
IP	0.68 $\pm$ 0.04	0.98 $\pm$ 0.18	0.78 $\pm$ 0.11	1.99 $\pm$ 0.21	0.72 $\pm$ 0.14
ΣPAH4	3.13 $\pm$ 0.07	3.88 $\pm$ 0.30	2.61 $\pm$ 0.11	21.54 $\pm$ 0.01	7.30 $\pm$ 0.05

4.2.1. Comparison with Performance of Other Analytical Methods

A previous study by Viegas et al ³⁵ concerning PAHs determination on charcoal-grilled meat, compared two different extraction methods followed by HPLC-FLD, the RSD ranged from 3 to 20% for PAHs amounts between 8 and 20 ng g⁻¹. In this work, the precision parameters were lower than 5% for similar PAHs amounts (**Table 10**). Although similar chromatographic conditions were used, the previous extraction methods involved several steps, including clean-up with silica cartridges, thus the present method is less laborious and more economic.

The RSD for other extraction procedures using GC-MS applied to smoked fish ranged between 4 – 15% ¹⁹⁰ and 3 – 14% ¹²⁶ as described by Lund et al and Jelena et al, respectively. For grilled meat, Lee et al ⁸⁶ and Rascón et al ¹⁵⁶ reported RSD values of 1.2 – 14.6 % and below 7.5%, respectively.

A brief comparison with other published methods for the determination of PAHs in grilled and smoked muscle foods is displayed on **Table 11**.

Table 11. Comparison of several analytical methods for PAHs determination in grilled and smoked muscle foods.

Method	Matrix	LOQ (ng g ⁻¹)	Repeatability RSD (%)	Recovery (%)	Ref.
ABE-HPLC/FLD	Grilled and smoked fish and meat	0.12-0.39	0.37-7.64	80-110	Present method
UAE-SPE-GC/MS	Grilled meat and fish, smoked fish	0.009-0.21	<7.5	85-105	156
PLE-GPC-SPE-GC/MS	Grilled meat	---	<23	82-111	191
MAE-DLLME-GC/MS	Smoked fish	0.36-1.6	2.8–8.9	82–106	148
PLE-GPC-SPE-GC/MS	Smoked fish	---	6-17	73-106	93
PLE-GC/MS	Smoked fish	0.9-14.6	4-15	53-108	190
MAE-DLLME-GC/MS	Grilled meat	0.48-1	<9	80-104	149
ASE-HPLC/FLD	Smoked meat	0.31-0.43	< 20.1	74–109	143
UAE-SPE-HPLC/FLD	Grilled meat	0.02-0.10	3–20	69–103	35

ABE acetonitrile-based extraction, ASE accelerated solvent extraction, DLLME dispersive liquid-liquid microextraction, GPC gel permeation chromatography, MAE microwave-assisted extraction, PLE pressurized liquid extraction; QuEChERS quick, easy, cheap, effective, rugged, and safe, SPE solid phase extraction, UAE ultrasonic-assisted extraction.

The present method gave similar or lower LOQs and RSDs (%) than previous methods described in literature, demonstrating its good performance. The recoveries were also similar to those found in the literature for cooked and smoked muscle foods using different extraction procedures. Also, the total analysis time (including sample preparation and instrumental analysis) is significantly shorter, and sample preparation and extraction is more economic, since it reduces the cost of reagents and other materials, such as expensive sorbents, and does not require specific equipment.

4.3. Analysis of Smoked Salmon Samples from the Portuguese Market

To verify the suitability of the proposed method to analyse PAHs in smoked salmon, recovery assays were performed. For that purpose, a salmon smoked sample was spiked with different levels of PAHS (0.5; 2; 10; 100 ng g⁻¹). The recoveries, summarized in **Table 12**, were within the control limits established by ICH guidelines (between 70 and 120%), and in accordance with European Commission Regulation No. 836/2011 ³ that established 50–120% as acceptable. Furthermore, all the results were in agreement with the recoveries observed for other processed samples (**Figure 9A and B**) using the present method.

Table 12. Recovery values (%) for different concentration levels (0.5; 2; 10; 100 ng g⁻¹) in the smoked salmon matrix (results presented as mean ± standard deviation, n = 3).

PAHs	0.5 ng g ⁻¹	2 ng g ⁻¹	10 ng g ⁻¹	100 ng g ⁻¹
Ac	106.84±0.61	101.10±1.16	106.56±4.17	94.99±3.00
F	97.88±7.04	97.73±2.03	94.77±0.57	91.39±2.33
Pa	100.79±0.98	79.66±4.58	86.84±1.56	79.89±1.96
A	103.59±5.41	109.82±2.66	108.08±1.63	--*
Fl	98.57±2.60	104.37±1.33	100.47±2.81	98.04±2.02
P	84.97±3.33	100.49±9.46	85.98±1.19	79.86±1.76
BaA	89.49±3.12	98.41±3.82	91.24±0.80	89.97±1.49
Chr	86.81±1.32	94.52±1.86	87.00±1.17	83.97±1.80
BbF	75.93±1.08	103.95±1.80	86.04±5.32	95.67±1.57
BkF	75.78±1.64	100.51±3.63	93.67±1.58	90.81±2.90
BaP	95.79±2.30	110.87±3.36	109.50±0.43	117.34±2.16
DhA	70.78±1.55	102.30±0.70	87.85±0.38	101.42±2.58
BgP	76.60±5.44	96.40±5.60	95.62±2.24	100.90±4.34
IP	80.16±1.66	108.98±4.97	97.26±1.43	100.70±0.26
Recovery Interval (%)	71-107	80-111	86-110	80-117

*exceed the linear range

The developed, optimized and validated method was applied in the quality control of 23 different samples (detailed in **Table 4** from section 3.2.2) of smoked salmon from the Portuguese market. As previously mentioned, the smoked salmon samples were classified in five groups, according to the origin and type of sample, being: A – simple smoked salmon; B – cold smoked salmon; C – hot smoked salmon, with skin; D – biological origin smoked salmon; E – non-smoked salmon; F – marinated smoked salmon. Groups A, D and F do not discriminate on the label the type of smoking process used. Except for the D group, all the samples comprise aquaculture salmon. The amounts of PAHs found in the analysed smoked samples by the developed method are depicted in **Table 13**.

Statistically significant difference between groups for all PAHs were observed, as demonstrated by one-way ANOVA: Ac ($F(22,45) = 454.90, p = .000$); F ($F(22, 45) = 54.57, p = .000$); Pa ($F(22, 45) = 1945.66, p = .000$); A ($F(22, 45) = 373.90, p = .000$); Fl ($F(22, 45) = 124.46, p = .000$); P ($F(22, 45) = 370.87, p = .000$); BaA ($F(22, 45) = 179.41, p = .000$); Chr ($F(22, 45) = 59.50, p = .000$); BbF ($F(22, 45) = 9.93, p = .000$); BkF ($F(22, 45) = 2.76, p = .002$); BaP ($F(22, 45) = 4.47, p = .000$); DhA ($F(22, 45) = 7.41, p = .000$); BgP ($F(22, 45) = 6.37, p = .000$); IP ($F(22, 45) = 4.89, p = .000$).

Tukey post hoc test was applied, because statistically significant results were found. The Tukey test showed that the C sample was the most different from the rest, except for PAH4, DhA, BgP and IP. The groups A, D, E and F were similar, but Group B presented most heterogeneous composition. More specifically regarding PAHs, for F and P only the sample C was statistically different; for Fl, B3 and C were the only statistically different samples and for DhA only A3 and B1 were statistically different.

Table 13. PAHs levels in smoked salmon samples determined by acetonitrile-based extraction with partitioning (results are presented as mean, $\pm$ the standard deviation below each mean value, n = 3).

	Ac	F	Pa	A	Fl	P	BaA	Ch	BbF	BkF	BaP	DhA	BgP	IP	Σ Total PAHs	Σ PAH8	Σ PAH4
ng g ⁻¹																	
A1	18.46 ^a	11.19 ^a	2.71 ^a	0.81 ^{a,b}	0.29 ^a	<0.09 ^a	<0.23 ^a	<0.06 ^a	<0.11 ^a	<0.18 ^{a,b}	<0.21^a	<0.22 ^b	0.14 ^a	<0.39 ^{a,b}	34.92	1.36	0.58
$\pm$sd	1.68	0.31	0.14	0.05	0.32								0.11		2.98	0.44	0.12
A2	10.33 ^a	2.63 ^a	2.01 ^a	0.37 ^a	<0.07 ^a	0.09 ^a	0.31 ^{a,b}	<0.31 ^a	<0.11 ^a	<0.18 ^{a,b}	<0.21^a	<0.22 ^b	0.14 ^a	<0.39 ^{a,b}	17.05	1.55	0.66
$\pm$sd	0.38	1.67	0.31	0.01		0.04	0.00						0.11		2.59	0.18	0.00
A3	8.83 ^a	2.11 ^a	3.35 ^a	0.66 ^a	0.16 ^a	0.61 ^a	0.06 ^a	<0.06 ^a	<0.11 ^a	0.04 ^a	<0.21^a	<0.22 ^a	0.06 ^a	<0.12 ^a	16.43	0.71	0.36
$\pm$sd	0.90	0.14	0.18	0.18	0.12	0.20	0.00			0.00			0.00		1.88	0.16	0.08
A4	5.01 ^a	1.26 ^a	1.33 ^a	0.42 ^a	<0.07 ^a	0.17 ^a	0.06 ^a	<0.06 ^a	<0.11 ^a	0.04 ^a	<0.21^a	<0.22 ^b	0.06 ^a	<0.12 ^a	9.07	0.83	0.42
$\pm$sd	1.10	0.23	0.12	0.05		0.10	0.00			0.00			0.00		1.60	0.00	0.00
A5	4.18 ^a	2.72 ^a	1.43 ^a	0.35 ^a	0.47 ^a	0.17 ^a	0.23 ^a	<0.06 ^a	<0.11 ^a	0.04 ^a	<0.21^a	<0.22 ^b	0.06 ^a	<0.39 ^a	10.41	1.09	0.58
$\pm$sd	0.39	0.08	0.22	0.03	0.05	0.00	0.12			0.00			0.00		1.05	0.26	0.12
A6	12.49 ^a	4.26 ^a	4.18 ^a	1.41 ^{a,b}	<0.07 ^a	0.70 ^a	0.17 ^a	<0.06 ^a	<0.38 ^{b,c}	0.04 ^a	<0.21^a	<0.22 ^b	0.06 ^a	<0.12 ^a	24.24	1.13	0.72
$\pm$sd	1.66	0.33	0.38	0.10		0.47	0.14			0.00			0.00		3.22	0.29	0.20
A7	12.11 ^a	20.39 ^a	2.34 ^a	0.50 ^a	0.24 ^a	0.12 ^a	0.06 ^a	0.31 ^{a,b}	<0.11 ^a	<0.18 ^{a,b}	<0.21^a	<0.22 ^b	0.06 ^a	<0.12 ^a	36.81	1.12	0.66
$\pm$sd	0.64	1.40	0.15	0.05	0.12	0.00	0.00	0.00					0.00		2.43	0.06	0.00
A8	10.98 ^a	27.76 ^a	4.11 ^a	1.07 ^{a,b}	0.21 ^a	0.23 ^a	0.06 ^a	<0.06 ^a	<0.11 ^a	<0.18 ^{a,b}	<0.21^a	<0.22 ^b	0.06 ^a	<0.12 ^a	45.27	0.92	0.42
$\pm$sd	0.94	1.67	0.38	0.08	0.20	0.03	0.00						0.00		3.37	0.07	0.00
A9	7.09 ^a	3.55 ^a	2.13 ^a	0.71 ^{a,b}	0.59 ^a	0.27 ^a	0.06 ^a	<0.06 ^a	<0.11 ^a	<0.18 ^{a,b}	<0.21^a	<0.22 ^b	0.06 ^a	<0.12 ^a	15.26	0.92	0.42
$\pm$sd	1.70	0.65	0.29	0.28	0.39	0.34	0.00						0.00		3.71	0.06	0.00

	Ac	F	Pa	A	Fl	P	BaA	Ch	BbF	BkF	BaP	DhA	BgP	IP	Σ Total PAHs	Σ PAH8	Σ PAH4
ng g ⁻¹																	
B1	38.64 ^b	13.93 ^a	14.09 ^b	2.10 ^b	1.08 ^a	0.58 ^a	0.55 ^b	0.55 ^b	<0.38 ^c	0.19 ^{a,b}	0.40^c	0.53 ^c	0.64 ^c	0.59 ^b	74.25	3.84	1.88
±sd	5.20	1.03	2.27	0.11	0.12	0.17	0.04	0.17		0.01	0.07	0.12	0.20	0.17	9.68	0.78	0.18
B2	19.59 ^{a,b}	76.57 ^a	4.85	1.16 ^{a,b}	0.35 ^a	<0.03 ^a	0.31 ^{a,b}	0.38 ^b	<0.38 ^c	<0.18 ^{a,b}	<0.21^a	0.25 ^b	0.40 ^{b,c}	<0.39 ^{a,b}	104.93	2.40	1.28
±sd	0.76	3.13	0.16	0.05	0.02		0.00	0.06				0.04	0.12		4.40	0.28	0.06
B3	174.09 ^c	30.54 ^a	16.81 ^b	5.28 ^c	3.46 ^b	0.40 ^a	0.31 ^{a,b}	<0.06 ^a	<0.38 ^{a,b}	<0.18 ^{a,b}	<0.21^a	<0.22 ^b	<0.09 ^a	<0.39 ^{a,b}	232.00	1.42	0.76
±sd	14.89	3.71	2.83	0.98	1.33	0.38	0.00								24.48	0.35	0.14
C	343.41 ^d	1157.95 ^b	124.83 ^c	24.33 ^d	13.16 ^c	14.57 ^b	3.49 ^c	1.67 ^c	<0.11 ^a	<0.18 ^{a,b}	0.36^{b,c}	0.26 ^b	<0.30 ^{a,b}	<0.39 ^a	1684.59	6.34	5.59
±sd	16.67	217.38	6.09	1.31	0.65	0.30	0.24	0.24			0.10	0.06			243.40	1.00	0.36
D1	8.34 ^a	4.11 ^a	3.06 ^a	0.87 ^{a,b}	<0.07 ^a	<0.03 ^a	0.06 ^a	0.34 ^b	<0.11 ^a	<0.18 ^{a,b}	<0.21^a	0.24 ^b	0.33 ^{a,b}	<0.12 ^a	17.96	1.48	0.69
±sd	0.72	0.18	1.18	0.11			0.00	0.03				0.03	0.04		2.36	0.17	0.03
D2	9.07 ^a	5.14 ^a	2.81 ^a	0.63 ^a	<0.07 ^a	0.47 ^a	0.06 ^a	<0.06 ^a	<0.11 ^a	0.18 ^{a,b}	0.26^{a,b}	0.28 ^b	<0.30 ^{a,b}	<0.39 ^{a,b}	19.58	1.40	0.46
±sd	2.54	1.85	0.29	0.05		0.39	0.00			0.00	0.07	0.08			5.63	1.85	0.29
D3	14.76 ^a	5.61 ^a	4.93 ^a	0.99 ^{a,b}	<0.07 ^a	0.16 ^a	0.06 ^a	<0.06 ^a	<0.11 ^a	0.04 ^a	<0.21^a	<0.22 ^b	<0.30 ^{a,b}	<0.12 ^a	27.43	0.90	0.42
±sd	5.05	2.44	1.76	0.32		0.03	0.00			0.00					9.72	0.11	0.00
E	2.08 ^a	1.02 ^a	2.12 ^a	0.17 ^a	<0.07 ^a	<0.03 ^a	0.08 ^a	0.08 ^a	<0.11 ^a	0.27 ^b	<0.21^a	<0.07 ^{a,b}	<0.09 ^a	<0.12 ^a	6.47	0.96	0.46
±sd	0.00	0.00	0.00	0.00			0.01	0.01		0.00					0.10	0.08	0.02
F1	5.99 ^a	3.16 ^a	2.10 ^a	0.56 ^a	0.39 ^a	0.15 ^a	0.06 ^a	<0.31 ^{a,b}	<0.11 ^a	0.18 ^{a,b}	<0.21^a	0.23 ^b	<0.30 ^{a,b}	0.40 ^{a,b}	14.04	1.69	0.66
±sd	0.61	0.33	0.41	0.02	0.01	0.02	0.00			0.00		0.01		0.02	1.54	0.14	0.00
F2	8.24 ^a	3.27 ^a	2.85 ^a	0.65 ^a	0.16 ^a	0.12 ^a	0.06 ^a	<0.06 ^a	<0.11 ^a	0.18 ^{a,b}	<0.21^a	<0.22 ^b	<0.09 ^a	<0.39 ^{a,b}	16.56	1.27	0.42
±sd	0.97	1.05	0.64	0.06	0.12	0.06	0.00			0.00					2.91	0.00	0.00

	Ac	F	Pa	A	Fl	P	BaA	Ch	BbF	BkF	BaP	DhA	BgP	IP	Σ Total PAHs	Σ PAH8	Σ PAH4
	ng g ⁻¹																
F3	4.40 ^a	9.49 ^a	2.22 ^a	0.54 ^a	0.38 ^a	0.33 ^a	0.23 ^a	0.06 ^a	<0.08 ^a	0.13 ^{a,b}	<0.21^a	0.22 ^b	<0.09 ^a	<0.39 ^{a,b}	18.65	1.29	0.58
±sd	0.58	1.51	0.29	0.07	0.44	0.10	0.12	0.00		0.06		0.00			3.32	0.32	0.12
F4	9.08 ^a	7.22 ^a	4.57 ^a	1.03 ^{a,b}	<0.07 ^a	0.70 ^a	0.34 ^{a,b}	0.06 ^a	<0.08 ^a	0.18 ^{a,b}	<0.21^a	0.22 ^b	<0.09 ^a	<0.12 ^a	23.91	1.24	0.69
±sd	0.96	0.84	1.08	0.19		0.50	0.05	0.00		0.00		0.00			3.62	0.05	0.05
F5	3.03 ^a	1.78 ^a	1.85 ^a	0.46 ^a	<0.07 ^a	0.13 ^a	0.31 ^{a,b}	0.06 ^a	<0.08 ^a	0.09 ^{a,b}	<0.21^a	0.22 ^b	<0.09 ^a	<0.12 ^a	8.43	1.12	0.66
±sd	0.12	0.46	0.28	0.01		0.07	0.00	0.00		0.06		0.00			1.01	0.06	0.00
F6	4.83 ^a	15.50 ^a	1.91 ^a	0.37 ^a	<0.07 ^a	0.32 ^a	0.33 ^{a,b}	0.06 ^a	<0.08 ^a	0.13 ^{a,b}	<0.21^a	0.22 ^b	<0.30 ^{a,b}	<0.12 ^a	24.35	1.35	0.69
±sd	1.04	0.69	0.51	0.03		0.06	0.02	0.00		0.06		0.00			2.53	0.19	0.02

In each column, a, b, c, d letters point the statistical significant differences ($p < 0.05$); sd: standard deviation; Total PAHs: Σ Ac, F, Pa, A, Fl, P, BaA, Chr, BbF, BkF, BaP, DhA, BgP, IP; Σ PAH8: BaA, Chr, BbF, BkF, BaP, DhA, BgP, IP; Σ PAH4: BaA, BbF, BaP, Chr. PAHs levels below the limits of detection (LOD) and limits of quantification (LOQ) are presented as < *value* of LOD or LOQ, respectively (see **Table 8**).

Principal Component Analysis (PCA) using PAHs levels as variables was applied in order to reduce the dimensionality of data and better understand how the samples are distributed regarding PAHs profile (**Figure 10**). Three components were extracted. However, the samples were organized in two components that justify 80% of data variation: Component 1 is associated with PAHs that present up to 4 aromatic rings (Ac, F, Pa, A, Fl, P, BaA, Ch), and Component 2 is associated with PAHs that contain 5 or more aromatic rings (BbF, BkF, BaP, DhA, BgP, IP). The number of aromatic rings of PAHs explained most of the variation between samples. Component 1 explained more than half of the total variation, while Component 3 had very low weigh.

As illustrated in **Figure 10** most of the samples (A, B3; D, E, F) clustered together, presenting similar PAHs profile. The C sample is clearly separated from the others mostly explained by Component 1, i.e., significantly contains more PAHs with up to 4 aromatic rings, and lower levels of PAHs with 5 or more aromatic rings. The B1 sample is also separated from the others, mostly explained by Component 2, i.e., significantly contains more PAHs with 5 or more aromatic rings.

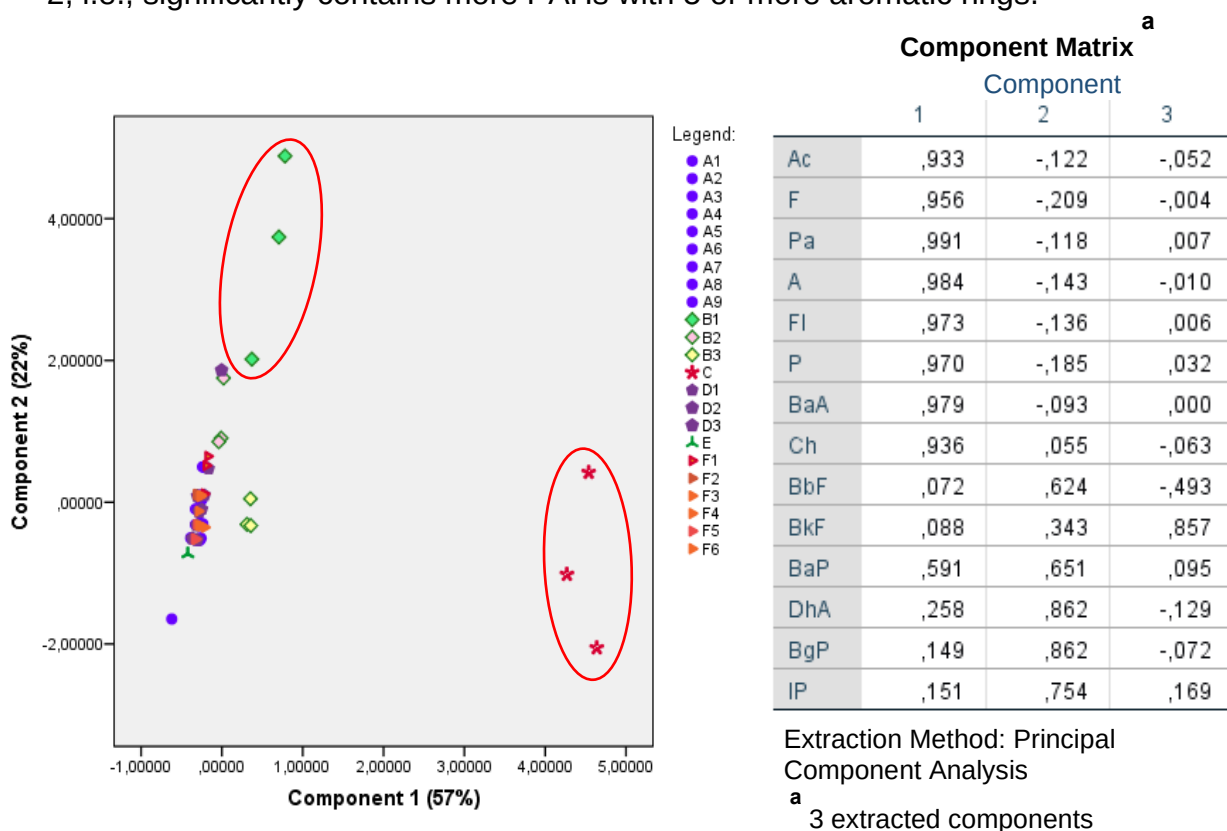


Figure 10. Plot representing the PCA from PAHs in 23 commercial smoked salmon samples: Component 1 (57%) vs Component 2 (22%) and on the right side the Table that summarises PCA Component Matrix.

4.3.1. Compliance with European Commission Regulation No. 835/2011 and comparison with current literature

Regarding the current legislation, EC Regulation 835/2011 ² establishes the maximum levels for BaP and PAH4 in smoked fish of 5 and 30 ng g⁻¹, respectively. Taken together, these results demonstrate all the samples were within the maximum permitted levels. Although BaP was detected, only two samples – B1 and C – presented levels above quantification limits, 0.40 and 0.36 ng g⁻¹ respectively.

The level of PAHs considered less carcinogenic (Σ Ac, F, Pa, A, Fl, P) ^{1,18} was highest in sample C (1678.25 ng g⁻¹), followed by samples B3, B2, and B1, with 230.58, 102.54, and 70.41 ng g⁻¹, respectively. Among these, the C sample was hot smoked, whereas samples B were described in the label as cold smoked. Hot and cold smoking are defined according to the temperature of the smoke: in hot smoking, temperature varies between 23-45 °C and in cold smoking, temperatures can vary between 12-25 °C or be below 20 °C or below 30 °C. Hot smoking is usually associated with higher levels of PAHs than cold smoking. ^{68,93,123,124} The values found in C sample were high compared with the other samples, however, similar and higher values have been described in the literature for less carcinogenic PAHs in smoked fish. ^{154,192} Accordingly, all of the analysed PAHs, including total PAHs and PAH8 levels are higher in the hot smoked sample – C (1684.59 and 6.34 ng g⁻¹, respectively) – than in the cold smoked samples – B3 (232.00 and 1.42 ng g⁻¹, respectively), followed by B2 (104.93 and 2.40 ng g⁻¹, respectively) and B1 (74.25 and 3.84 ng g⁻¹, respectively).

All the samples presented higher levels of the less carcinogenic PAHs than PAH8, which is in accordance to the literature about smoked fish and meat. ^{93,95,126,192} The smoke (produced by the incomplete combustion of the heat source) seems to boost the generation of less carcinogenic PAHs, whereas the fat that melts and drips from the food to the heat source generates more of the carcinogenic PAHs, namely PAH8. ^{35,44} Thus, it makes sense that amongst the same sample, grilled meat and fish usually present higher levels of PAHs than smoked meat and fish, especially of PAH8. ^{14,156} Comparing the grilled salmon described in **Table 10** with the smoked salmon C (**Table 13**), the grilled salmon presented higher levels of PAH8 than the C smoked salmon sample (28.62 vs 6.34 ng g⁻¹, respectively), and

lower levels of less carcinogenic PAHs of (575.36 vs 1678.25 ng g⁻¹, respectively) as well as of total PAHs (1684.59 vs 602.64 ng g⁻¹, respectively).

The levels of PAHs and respective profile in smoked samples, greatly depends on the smoking conditions, sometimes more significantly than in the process itself, namely the temperatures attained, technology of the smoking, location in the smoking room, fat content of the food, type of heating material used, closeness to the heating source.^{70,95,193}

The addition of ingredients in the analysed samples (either black pepper, white pepper, dill, curry and/or other herbs and spices) – F group – had no significant effect in PAHs levels, since they were very similar to the groups with no additional ingredients besides salt (common to all samples).

The lowest amounts for total and less carcinogenic PAHs were found in the non-smoked, natural dry-salmon (E), of 6.47 and 5.51 ng g⁻¹, respectively, which is in agreement with the literature, since smoked fish present considerably higher PAHs levels than non-smoked fish.^{156,194} PAHs occur in non-smoked salmon since they can accumulate in raw seafood; it is most relevant in contaminated areas, after petroleum leakages and ship accidents, for example; and PAHs levels in raw fish are considered a good marker for PAHs contamination of waters, e.g. petroleum leakages.^{6,29,30}

Several parameters are known to influence PAHs levels in smoked meat and fish and should always be monitored, specifically the fuel type, wood type, pyrolysis temperature, smoke generator air flow, smoking chamber design, smoking type, drying type, food fat content, processing effect, closeness among food and heat source, smoking duration and cleanliness of the equipment.^{28,43,121} The control of these parameters leads to less formation of PAHs.⁸⁶ The Codex Alimentarius Commission (CAC) recommends that the smoking process results in the lowest PAHs levels possible, encouraging on-going research to identify the optimal smoking conditions.⁸¹ All the analysed samples presented low levels of PAH₄, which shows that good practices are being applied in the industry, to assure that PAHs formation is low.

5. Conclusions

PAHs are ubiquitous and carcinogenic compounds, being food contamination unavoidable; however, high PAHs formation can result from industrial and domestic thermal processes. Thus, monitoring their levels is required to ensure low dietary exposition.

The search for rapid, reliable and environmentally friendly quantification methods is always going on for routine analysis. In this way, a method based on an acetonitrile extraction with partitioning coupled with HPLC/FLD to extract and quantify PAHs from processed muscle foods was successfully developed, optimized and validated, in accordance with the EC 836/2011 performance criteria.

The proposed method is a robust tool that can be an effective to determine and control marker PAHs in monitoring studies of grilled and smoked muscle foods.

Regarding the analysed smoked salmon samples, they were all compliant with the European Regulation 835/2011. In general, the samples were very similar to each other, with the hot smoked salmon sample being the most different; the latter presented the highest levels of PAHs, namely light PAHs. Nowadays, light PAHs that are less relevant from the toxicological point of view, are the most abundant in commercialized foods, the results found in the analysed smoked salmon samples showed a similar pattern. This shows the positive effects of regulations and the need to insist in good practices, on reinforcing the surveillance systems in industries and food production units/restaurants, as well as emphasizing the importance of the existing regulations.

As a future perspective, it would be interesting to understand if other smoked fish and meat products in the Portuguese market are within the maximum allowed limits. Also, a monitorization of PAHs levels in Portuguese traditionally smoked fish and meat could be done to assess their compliance with the most recent regulation – EC 1327/2014, regarding traditionally smoked products –, as well as the monitorization of PAHs in fish and meat domestically smoked in Portugal, since regulatory limits and measures do not apply to food produced and cooked domestically. Such foods are likely to contain PAHs and exceed the established regulatory limits, highlighting the importance of monitorization in order to raise awareness to the population on the hazards they may bring, determining health and

longevity, in addition to how to prevent or diminish PAHs ingestion while cooking at home, as well as implement mitigation/preventive strategies to decrease PAHs formation whenever and wherever necessary.

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