Methods and Protocols in Food Science

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Rodrigo Hoff
Luciano Molognoni *Editors*

Chemical Food Contaminants Analysis

Methods and Protocols in Food Science

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Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science.

Volumes and chapters will be organized by field and presented in such way that the readers will be able to reproduce the experiments in a step-by-step style. Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol will be clarified.

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Edited by

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Preface to the Series

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

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

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

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

Campinas, Brazil ***Anderson S. Sant'Ana***

Preface

The subject of chemical contaminants in food has always been a constant concern for society and has consistently played a significant role in news regarding physical health, environmental health, and consumer well-being. Despite still facing serious issues with the so-called classic contaminants, every year introduces new players to the headlines, bringing information about new chemical risks related to food. While in the 1980s, the major concern was, for example, organochlorine pesticides and other persistent organic pollutants in food; today, we see numerous news reports about micro and nanoplastics, PFAs, marine biotoxins, and recently introduced pesticides.

The issue of chemical residues in food extends beyond the academic sphere, as it is also a critically important regulatory matter, particularly a priority agenda in international negotiations, especially concerning commodities. International bodies like the Codex Alimentarius have established maximum limits for certain chemical contaminants in food residues, in addition to individual countries having their own regulations.

There are numerous publications in specialized literature increasingly focusing on the qualitative or quantitative determination of chemical contaminants in food. However, these published methods do not always constitute a sufficiently versatile tool in terms of cost, efficiency, and execution speed to become analytical tools in routine or regulatory laboratories, and even in research laboratories.

Protocols in Chemical Food Contaminants Analysis is a book that delves into the most recent and pertinent protocols essential for the accurate analysis of chemical contaminants in food. This book seamlessly integrates well-established methodologies and procedures commonly employed across various academic and industrial laboratories. It offers a comprehensive guide aimed at addressing the complex landscape of analyzing chemical contaminants in food.

In this book, we aim to provide a compilation of protocols encompassing different combinations of contaminants and matrices, representing protocols that share the common characteristic of ease or efficiency in sample preparation. Consequently, this book is divided into two parts, the first addressing more classical chemical contaminants or those previously unknown to the scientific community, yet bringing innovative elements to the sample preparation method for analysis. It also presents protocols for the determination of these known contaminants but in unconventional samples.

The second part of the book focuses more on recent concerns in the scientific community and food toxicology, particularly emphasizing the so-called emerging contaminants as well as micro and nanoplastics. We hope in this way to contribute to the arsenal of available analytical tools for researchers and laboratories aiming to implement reproducible methods with high throughput capable of analyzing numerous samples in a short amount of time.

So, for example, in the first part of the book, we have chapters dedicated to polycyclic aromatic hydrocarbons (Chap. 1) but introduce innovative elements in sample preparation. We have chapters dedicated to dioxins and furans (Chap. 2), as well as organochlorine pesticides (Chap. 3), toxic elements (Chap. 4), and mycotoxins (Chap. 8). Moreover, other chapters present different approaches than what is commonly seen for the determination of

mercury in food products (Chaps. 9 and 12), acrylamide (Chap. 13), and various drug residues, such as polypeptide antibiotics (Chap. 10), tetracyclines and its epimers (Chap. 11), coccidiostats (Chap. 14), beta-blockers and sedatives (Chap. 5), and glucocorticoids (Chap. 15) in food of animal and/or vegetal origin.

Additionally, in the first part of this book, we also include several screening assays applicable to a wide range of analytes. For instance, antimicrobials in muscle (Chap. 5), and pesticides and mycotoxins in brewery products (Chap. 6). Sample preparation methods suitable for a multitude of emerging contaminants in products like fish and determination of emerging contaminants in fish and milk as well.

In Section II, which deals with less conventional analytes and food matrices, we present a scope for determining palytoxin and palytoxin-like marine biotoxins in fish (Chap. 21), determining selected polar drugs and contaminants in animal feed (Chap. 18), analyzing mutagenic compounds formed by preservative interaction in meat products (Chap. 17), UV filters and related metabolites analysis in seafood (Chap. 20), screening and confirmation of micro and nanoplastics in seafood (Chap. 22), tetracyclines in vegetables (Chap. 24), determination of pyrrolizidine alkaloids in honey, plants, and pollen (Chap. 23), and MCPDEs and glycidyl esters in fish oil-based dietary supplements (Chap. 25). Regarding the determination of contaminants of emerging concern, at least three chapters are directly related with this subject, including a screening method for fish and milk (Chap. 16) and a miniaturized sample preparation protocol for 69 pharmaceuticals in seafood (Chap.19).

São José, Brazil *Rodrigo Hoff*
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Part I

General Methods and Procedures for Classic Contaminants

Chapter 1

Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood by Matrix Solid-Phase Dispersion and Pressurized Liquid Extraction Followed by GC-MS/MS Analysis

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are hazardous organic contaminants that pose a significant chemical risk to consumers. One of the main sources of PAHs occurrence in food is the oil spills that can reach the seafood chain. The extraction of PAHs from such matrices can be challenging. Here, we describe the association of matrix solid-phase dispersion (MSPD) and pressurized liquid extraction (PLE) to extract and concentrate PAHs from several seafood matrices such as mussels, oysters, fish, and prawns, resulting in extracts that can be analyzed using GC-MS/MS.

Key words Energized dispersion guide extraction, Perlite, Benzo[*a*]pyrene, Mussels, Oyster, Fish, Prawns

1 Introduction

PAHs are a class of hundreds of potentially toxic, environmentally persistent organic compounds that often occur as complex mixtures composed of different congeners [1]. A substantial source of human exposure to PAH is attributed to the consumption of contaminated food (88–98%) [2]. Daily intake of food contaminated with PAH has been proposed as one of the causal sources of cancer in humans [3]. Despite the high risk attributed to the consumption of food contaminated with PAH and the known adverse effects on human health, several countries still do not have their own regulatory limits and often apply the European limits for monitoring PAH in food. Levels of concern for PAH in fish were established in

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emergency situations, such as the oil spill from the Deepwater Horizon oil rig in the Gulf of Mexico in 2010 and more recently, in 2019, in the Northeast and Southeast regions of Brazil, whose origin remains inconclusive [4]. The US regulatory authorities, Food and Drug Administration (FDA) and National Oceanic and Atmospheric Administration (NOAA), and the Brazilian National Health Surveillance Agency (ANVISA) established levels of concern used as a reference for assessing the risk to human health arising from the consumption of fish contaminated with PAH [5]. Fish, crustaceans, and mollusks can be contaminated with PAH due to industrial processing, culinary practices, and environmental contamination, especially oil spills. Analytical methods to monitor PAH levels in seafood are therefore mandatory to ensure food safety. However, the extraction of PAHs from food matrices can be challenging, especially considering the low levels of these contaminants.

The classic approach used in sample preparation for PAHs determination in food matrices is often based on Soxhlet extraction and saponification. These extraction techniques are generally followed by clean-up procedures such as solid-phase extraction (SPE), open-column chromatography, or gel permeation chromatography (GPC). Thus, there is a need for faster and easier sample preparation protocols for PAHs analysis.

Recently, MSPD and PLE have been associated to promote simultaneous analytes extraction, enrichment, and clean-up. In few words, MSPD is based on the matrix mechanical disruption promoted by the dispersion of the sample on a solid phase, which can be inert or can interact with the sample, generally with the aim to remove interfering compounds. Regarding PLE, this technique uses pressure and temperature to promote a solid-liquid extraction. Under high temperatures and pressure, aqueous solvents can exhibit the behavior of organic solvents such as acetonitrile and methanol, increasing analytes solubility.

Here, we present a general protocol based on MSPD and PLE to obtain enriched extracts from relatively low amounts of seafood samples (2.0 g). After concentration and clean-up, the extract is analyzed using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) with detection limits around 0.5 μg kg^{-1}.

2 Materials

All reagents are analytical grade unless otherwise specified. Use LC-MS or GC/MS grade solvents. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Reagents, Standards, and Apparatus

1. Acetonitrile.
2. Ethyl acetate.
3. Solid phases: perlite (*see* **Note 1**) and florisil.
4. Analytical standards: a mix containing 16 PAHs in acetonitrile (10 mg L^{-1}) was used, purchased from Sigma-Aldrich (CRM47940). The internal standard was an isotope-labeled chrysene-d12 (CAS n° 1719-03-5), purchased from Toronto Research Chemicals (Ontario, Canada).
5. PLE aluminum tube (model Q-Cup®, CEM Corporation, Matthews, USA).
6. C9 and M2 membrane filters (Q-Discs®, CEM Corporation).

2.2 Equipments

1. GC-MS/MS: We use a gas chromatography system coupled to mass spectrometry with electron ionization source (GC-EI-MS/MS) from Agilent, composed of a GC model 7890A and a MS model GC-MS Triple Quad 7000.
2. PLE: The PLE system was an automated pressurized fluid extraction system EDGE® (CEM Corporation, Matthews, USA), with 12 positions. Alternatively, the PLE can be adapted to be done using a hard cap espresso machine (*see* **Note 2**).

2.3 Solutions

1. PAH fortification solution (100 ng mL^{-1}): dilute 0.1 mL of the mix of standards (10 μg mL^{-1}) with ethyl acetate and bring to volume in a 10 mL volumetric flask. Validity of 6 months stored in a freezer.
2. Chrysene-d12 stock solution 1000 μg mL^{-1} (internal standard): Weigh 10.00 mg of the standard and bring it to volume in a 10 mL volumetric flask, dissolving with acetone with the aid of an ultrasound bath. Shake before use. Validity of 12 months stored in a freezer.
3. Chrysene-d12 intermediate solution 10 μg mL^{-1}: Dilute 0.1 mL of the Crs-d_{12} stock solution with ethyl acetate and bring to volume in a 10 mL volumetric flask. Validity of 6 months stored in a freezer.
4. Chrysene-d12 working solution 200 ng mL^{-1}: Dilute 0.500 mL of the intermediate solution with ethyl acetate and bring to volume in a 25 mL volumetric flask. Validity of 3 months stored in a freezer.

3 Methods

3.1 PLE Procedure in EDGE® System

1. Extraction solvent is acetonitrile.
2. Samples were extracted in just one cycle, programmed as follows: 25 mL of acetonitrile added on top of the extraction cell;

Table 1
Instructions for preparing the calibration curve in a fortified blank sample before the extraction process

Calibration levels (μg kg^{-1})	PAHs working solution (μL)	IS (Crs-d12) working solution (μL)
0.000	0.00	50
0.750	12.5	50
1.25	25.0	50
2.50	50.0	50
5.00	100	50
7.50	150	50
10.0	200	50

10 mL of solvent added on the bottom of the extraction cell; temperature 70 °C, held for 3 min. The rinse solvent was 5 mL of acetonitrile.

3. After each extraction, run a wash method, as follows: solvent = purified water, volume = 10 mL, time = 30 s, temperature = 100 °C.

3.2 Standard Calibration Curve Preparation

1. A matrix-matched calibration curve is prepared using blank samples, which are selected based on the predominant matrix in the batch, such as fish, bivalve mollusks, or crustaceans.
2. Weigh 2.0 ± 0.1 g of blank sample for each point on the calibration curve. Prepare the calibration curve by fortifying blank samples according to the volumes of fortification solutions shown in Table 1.
3. Analytical Quality Control: run at least 1 double-blank sample, 1 blank sample, and 3 recovery samples. Recovery samples are blank samples fortified at the same conditions of the center point of the calibration curve (2.5 μg kg^{-1}).

3.3 PLE Extraction Tubes Assembling

1. The PLE tubes were assembled with a sandwich of membrane filters. To do this, remove the threaded bottom piece of the tube and position the filters in the following order: C9 + M2, as shown in Fig. 1. It is important to ensure that the textured face of the M2 filter is facing upward (in contact with the sample).

3.4 Sample Extraction

2. Weigh 2.0 ± 0.1 g of sample into a 50 mL polypropylene tube.
3. Fortify all samples with 50 μL of internal standard working solution (200 ng mL^{-1}).
4. Homogenize the samples in a vortex and stand for 1–2 min.

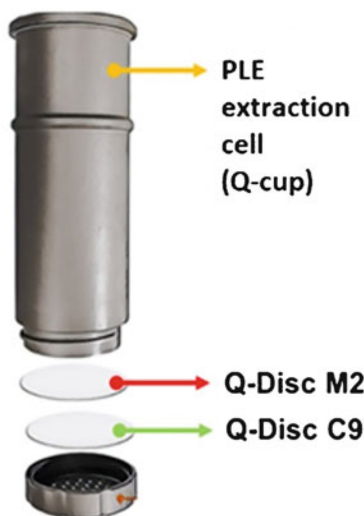

Fig. 1 Positioning of filters in the Q-Cup® tube for use in an EDGE pressurized liquid extraction system for extracting PAHs from fish

5. Add perlite in a volume approximately equal to that of the sample (*see* **Note 3**).
6. Disperse the matrix in the solid phase (*see* **Note 4**).
7. After the complete dispersion of the matrix on the adsorbent, the mixture is carefully transferred to the PLE extraction tube (Q-Cup®) previously prepared (*see* **Note 5**).
8. Insert the PLE extraction tubes in the 12-position rack of the EDGE system.
9. Samples must be extracted using a previously configured method on the EDGE system according to the parameters described in **step 3.1**.
10. Turn on the PLE system, checking if there is a sufficient volume of extraction solution in the reservoir.
11. Collect the extract in a previously identified 50 mL polypropylene conical centrifuge tube.
12. Concentrate the extract to dryness in a water bath at 40 ± 2 °C with the aid of nitrogen flow (*see* **Note 6**).
13. Redissolve the dry extract in 500 μL of ethyl acetate.
14. Shake vigorously the tubes on an orbital shaker for 20 min (*see* **Note** 7).
15. Transfer the supernatant to a clean microcentrifuge tube (capacity 1.5–2.0 mL).
16. Add approximately 20 mg of florisil (*see* **Note 8**).
17. Homogenize vigorously in a vortex for 5–10 s.

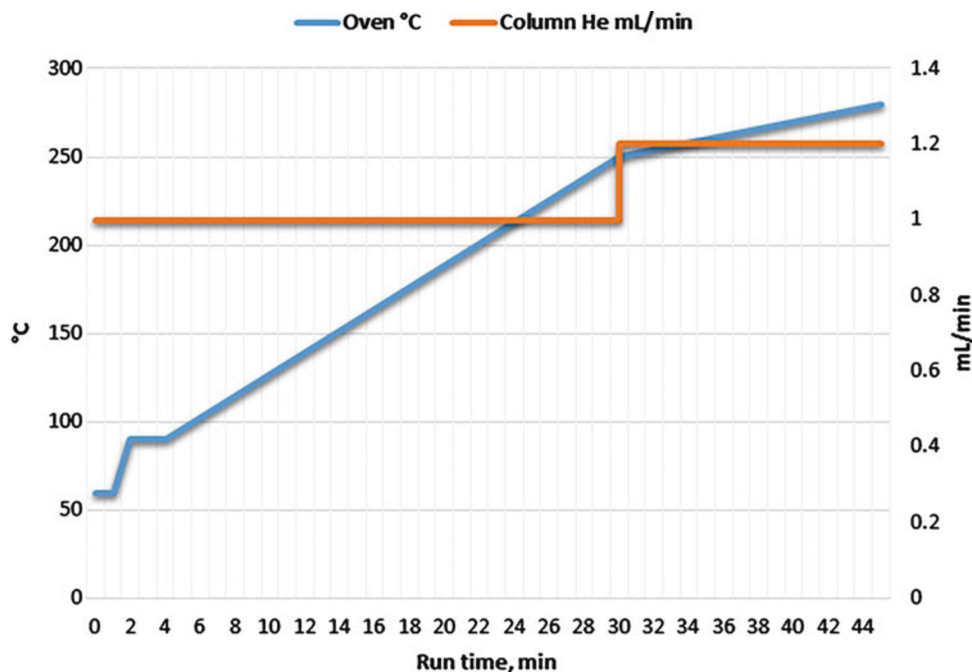

Fig. 2 Mobile phase gradient flow and oven temperature gradient

18. Centrifuge the tubes at 17,000 g-force for 10 min at 4 °C.
19. Transfer an aliquot of the supernatant (± 200 μL) to a vial with an insert (*see* **Note 9**).
20. Analyze in GC-MS/MS.

3.5 Analytical Parameters for GC-MS/MS

1. The chromatographic column was a HP-5MS (60 m × 0.25 mM (i.d.), film thickness 0.25 μm) analytical column from Agilent.
2. The helium flow (Fig. 2) is 1.0 mL min^{-1} for 30 min, then increased to 1.2 mL min^{-1} at a rate of 1.0 mL min^{-1} and held in this condition until the end of the run (45.33 min).
3. The oven temperature program is as follows: 60 °C (held for 1 min), then 90 °C at 45 °C min^{-1} (held for 2 min), followed by 250 °C at 6 °C min^{-1} and finally 280 °C at 2 °C min^{-1}.
4. The injection volume is 1.0 μL. The GC–MS/MS transfer line temperature is 280 °C. Gas saver is set to 20 mL min^{-1} after 3 min. Source temperature = 300 °C, quadrupoles temperature = 180 °C.
5. The instrumental parameters of the multimode intel (MMI) are 280 °C (heater), 8.23 psi (pressure), 3 mL min^{-1} (septum purge flow), split mode with 2.5: 1.0 split ratio, and 25 mL min^{-1} (post run total flow).
6. In the MS unit, electron ionization at 70 eV is used.

Table 2
Analyte-dependent mass spectrometry parameters

Analyte	Q1 (*m/z*, Da)	Q3 (*m/z*, Da)	CE (volts)
Benzo[*a*]anthracene	**228** 228	**226** 224	38 60
Benzo[*a*]pyrene	**252** 252 252	**250** 248 126	40 40 40
Benzo[*b*]fluoranthene	**252** 252 252	**250** 248 126	42 60 15
Benzo[*ghi*]perylene	**276** 274 274	**274** 272 272	42 60 42
Benzo[*k*]fluoranthene	**252** 252 252	**250** 248 126	35 15 50
Chrysene	**228** 228	**226** 224	38 42
Dibenzo[*a,h*]anthracene	**278** 276	**276** 274	42 42
Indeno[1,2,3-*cd*]pyrene	**278** 276	**276** 274	42 42
Chrysene-d12 (Crs-D12)	**240**	**236**	25

CE collision energy; m/z transitions in bold correspond to the transition used for quantification; the other transitions are qualifiers

7. Data acquisition is obtained using multiple reaction monitoring (MRM) analysis mode. Quantifier and qualifier ions are used for target analytes. The selected quantification m/z ions were typically base peaks or molecular ion peaks (Table 2).

3.6 Quantitative Analysis

1. The concentration of PAHs in each sample is determined through matrix-matched calibration with internal standardization.
2. The peak area ratio of each analyte to Crs-d_{12} is plotted against the concentration ratio of each analyte to Crs-d_{12}.
3. Sample concentrations are calculated directly using the calibration curve equation.
4. Since a matrix-matched calibration curve is employed, there is no requirement for recovery correction.
5. Samples with results exceeding the working range should be diluted and reinjected (*see* **Note 10**).

4 Notes

1. Perlite is easily found in garden and flower stores as an adsorbent to plants. In order to use, the material must be previously washed and dried. Place the perlite in a glass column and wash at least three times with hexane followed by acetonitrile. Dried the perlite in an incubation oven at 35–40 °C overnight with forced air passage.
2. In previous reports, we describe the use of a hard cap espresso machine as a cheap and easy way to perform PLE. For more information, see the references [6–8].
3. The perlite can be measured using a spatula/spoon (approximately 2.0–3.0 g). Adjustments in the perlite amount can be made based on the sample water content: the mixture of sample and perlite must result in a dry, powdered material. If humidity is still apparent, add more perlite.
4. The matrix dispersion on perlite can be achieved more easily using a glass stick or a double-ended stainless steel spatula/spoon. Alternatively, we have used an adapted manual cake mixer with dough hooks (Fig. 3). Between each sample, the hooks and/or spatula are cleaned in a methanol solution followed by ultrapure water and dried using a clean paper towel.
5. Taking care to assure that all mixture was transferred to the PLE tube: sample residues adhered to the walls of the tube can be transferred with the aid of stainless steel spatula/spoon.
6. Depending on the sample fat content, complete dryness cannot be achieved, due to the presence of residual fat (e.g., tuna). In these cases, avoid excessive dryness: that can result in low recoveries. When just fat remains in the bottom of the tube, remove from the water bath and follow with the solvent reconstitution.

Fig. 3 Utilization of an adapted manual cake mixer with dough hooks to perform the matrix solid-phase dispersion of perlite over a mussel sample

7. Place the tubes in a vertical position to avoid spills.
8. Similarly, to perlite, florisil can be added using a small spoon-type spatula previously selected for transfer of the adsorbent amount near 20 mg. Moreover, florisil can be added to the microcentrifuge tubes before the addition of the supernatant.
9. We use conical glass inserts with 200 μL of volume.
10. Dilute the samples using blank sample extract in order to maintain the matrix effects. Dilution with pure solvent will likely result in higher responses, with the potential to alter the ratio between the analyte and internal standard. This assumes different matrix effects responses for each analyte, with the exception of chrysene.

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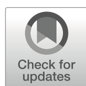

Chapter 2

Determination of Polychlorinated Dibenzo-p-Dioxins (PCDDs), Polychlorinated Dibenzofurans (PCDFs), and Dioxin-Like Polychlorinated Biphenyls (dl-PCBs) in Food by GC-MS/MS

Rafael Pissinatti, Matheus M. M. F. Gloria, Rafael F. Mota, Christiane R. Rocha, and Raquel Nogueira

Abstract

PCDD, PCDF, and DL-PCB compounds are classified as persistent organic pollutants (POPs) and are recognized as food contaminants. Therefore, monitoring these substances in food is imperative for safeguarding public health. This chapter outlines a modern analytical methodology, embracing both automated and manual clean-up approaches to the determination of such contaminants. Isotope dilution GC-MS/MS is used for accurate and reliable quantification of these contaminants across various food matrices.

Key words Dioxins, PCDD, PCDF, PCB, Isotope dilution, Gas chromatography/tandem mass spectrometry (GC-MS/MS), Automated clean-up

1 Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) are a group of well-known persistent organic pollutants (POPs), listed in the Annex C of the Stockholm Convention (2001). Due to their lipophilic nature, persistence, and harmful effects, they are recognized as food contaminants [1, 2].

Differing in the number and in the position of the chlorine atoms, a total of 75 PCDDs, 135 PCDFs, and 209 PCBs can be generated. Nevertheless, only 7 PCDDs and 10 PCDFs, which have 2,3,7,8-chlorine substituted, raise toxicological concern. The most toxic PCBs adopt a coplanar configuration due to the absence of chlorine substitution in the *ortho* position [3]. The presence of one *ortho*-chloro substituent reduces the planarity of the rings, but some congeners can still assume a planar configuration [4]. For this

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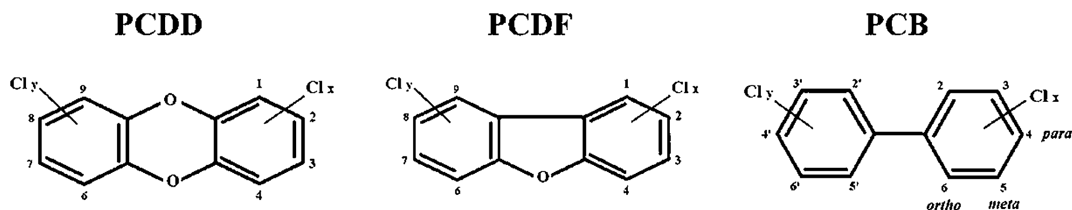

Fig. 1 Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (dl-PCBs)

reason, a group of 12 *non-ortho* and *mono-ortho* congeners induce a common spectrum of toxic responses, and have a common mechanism of toxicity, being defined as "dioxin-like" PCBs [5, 6]. Figure 1 shows the structural representation of each class of these compounds.

PCDD/Fs are unintentionally produced through different processes. They can be by-products of thermal processes, results of incomplete combustion, or by-products in some industrial processes [7–9].

PCBs were intentionally manufactured since 1929 to be used in numerous industrial applications due to their appealing physical and chemical properties: chemical stability, high boiling point, low heat conductivity, and high dielectric constant [6]. In 1980, after the biological effects were reported, PCB production was discontinued [1, 6]. Nonetheless, a significant quantity of these compounds has either entered the environment or is still in use, primarily in electronic and electrical equipment [10].

Harmful effects of those substances include carcinogenicity, teratogenicity, mutation, neurotoxicity, reproductive toxicity, endocrine disruption, immunotoxicity, and chloracne [7, 11].

Dietary intake is considered as the main pathway of PCDD/Fs and PCBs to human beings with more than 80% of total exposure [6].

Comprehensive monitoring programs for these contaminants in food have been conducted globally over the last decades. Substantial efforts have been made to safeguard consumer health. Maximum limits of dioxins, furans, and PCBs in food are restricted based on international regulations, stemming from the European Union. Table 1 provides an overview of regulatory levels for PCDD/Fs and PCBs for some food categories in accordance with the current regulation [11], which is followed by many countries.

In recent years, gas chromatography (GC) in combination with tandem mass spectrometry (MS/MS) has been commonly used for the identification and quantification of PCDD/Fs and dl-PCBs [13–15]. This chapter outlines a GC-MS/MS isotope dilution method using two different clean-up approaches: manual and auto-

Table 1
Regulatory levels for PCDD/Fs and PCBs in food [12]

Matrix	Sum of dioxins (WHO-PCDD/F) (pg TEQ g^{-1} fat, except for fish: pg TEQ g^{-1} wet weight)	Sum of dioxins and dioxin-like PCBs (WHO-PCDD/F-PCB)(pg TEQ g^{-1} fat, except for fish: pg TEQ g^{-1} wet weight)
Bovine meat/fat	2.5	4.0
Poultry meat/fat	1.75	3.0
Pork meat/fat	1.0	1.25
Fish	3.5	6.5
Milk	2.0	4.0
Eggs	2.5	5.0

mated. For solid samples, pressurized liquid extraction (PLE) is employed. The method can be applied to a wide range of food matrices, including animal fat, meat, liver, milk, eggs, fishery products, and vegetable oils.

2 Materials

High-purity grade solvents and reagents must be used. Solvents can be purchased as suitable for PCDD/F and PCBs analysis, or tested at the laboratory for interfering peaks. All glassware must be previously rinsed with dichloromethane and hexane. Labeled standard solutions must have a minimum of 99% purity. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Reagents and Materials

– Capillary chromatographic column for GC-MS/MS, DB-5MS UI – 60 m; 0.25 mM internal diameter; 0.25 μm film thickness (Agilent Technologies, USA).
– Graphitized carbon C (Carboblack® C 80–100 mesh, Restek, USA).
– Diatomaceous earth, acid washed (Celite 545 AW, Supelco, USA).
– Dichloromethane.
– Florisil (60–100 mesh).
– Helium 5.0, for GC-MS/MS.
– *n*-hexane.
– Nitrogen 5.0 and 6.0 (for GC-MS/MS).
– Nonane.

- Set of columns for LC-TECH equipment: Universal Column; Aluminum oxide Column; Carbon Column (LC Tech GmbH, Obertaufkirchen, Germany).
- Silica gel 60–63-200 μm, 60 Å, 70–230 mesh.
- Sodium sulfate (anhydrous).
- Sulfuric acid.
- Toluene.

2.2 Standards

2.2.1 Commercial Standard Solutions

- Native standard solution containing 17 PCDD/F, EPA1613 Stock from Wellington Laboratories, or EDF7999-10× from Cambridge Isotope Laboratories, Inc.
- Native standard solution containing NO-PCBs, CIL EC-4986 from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).
- Native standard solution containing MO-PCBs, CIL EC-4987 from Cambridge Isotope Laboratories, Inc.
- PCDD/F isotopically labeled internal standard solution, EPA1613LCS from Wellington Laboratories or EDF8999 from Cambridge Isotope Laboratories, Inc.
- NO-PCB isotopically labeled internal standard solution, CIL EC-4187 from Cambridge Isotope Laboratories, Inc.
- MO-PCB isotopically labeled internal standard solution, CIL EC-4188 from Cambridge Isotope Laboratories, Inc.
- Syringe standard, containing labeled internal standard $^{13}C_{12}$–1,2,3,4-TCDD; $^{13}C_{12}$–1,2,3,7,8,9-HxCDD, EDF8999 from Cambridge Isotope Laboratories, Inc.

2.2.2 Working Standard Solutions

All standard solutions are prepared in nonane.

1. Native standard solution for PCDD/F and NO-PCBs are combined while being diluted (50 and 200×, respectively), in order to obtain a working solution containing 0.8 pg μL^{-1} of tetra-; 4.0 of μL^{-1} for penta-, hepta- and 8.0 pg μL^{-1} of octa-CDD/Fs. NO-PCBs are used at a concentration of 5.0 pg μL^{-1}.
2. The working solution for native MO-PCBs, at a concentration of 40 pg μL^{-1}, is obtained by diluting 25 times the MO-PCB native standard solution.
3. The internal standard solution containing labeled ^{13}C-PCDD/F and ^{13}C-NO-PCB congeners are also combined in order to obtain a working solution containing 1 pg μL^{-1} of tetra-, penta-, hexa-, and hepta-chlorinated and 2 pg μL^{-1} of octa-CDD/F. ^{13}C-NO-PCBs is used at a concentration of 5.0 pg μL^{-1}.
4. The working solution for labeled internal standard ^{13}C-MO-PCBs, at a concentration of 40 pg μL^{-1}, is obtained by diluting