Chapter 1
Determination of Pesticide Residues in Fish

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Abstract Pesticide residues occurring in fish of marine and freshwater media is a continuous issue for food safety and environmental monitoring. In the last 40 years the analysis of organohalogen pesticides in marine fish has been conducted seeking to trace the bioaccumulation and biomagnification in food webs. The analysis of organochlorine, highly lipophilic compounds, was performed using sample preparation methods with a large solvent consumption followed by instrumental determination using gas chromatography with electron capture detectors. Fish as an analytical matrix presents particular challenges for residue analysis because of the large amount of different edible species, the variable proportion of fatty tissues and the they requirements for sample preservation. Moreover, different non-traditional tissues such as gills or the brain are of recent interest in environmental monitoring and pesticide exposure studies. In the last decades, new concepts for the sample preparation of trace contaminants in fish have been developed, based on sample

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miniaturization and minimal reagent consumption capable of analyzing a large palette of active principles, from non-polar to semi-polar ones.

The focus of this chapter is to present the complexity of the possible analytical matrices and the different strategies used for pesticide residues determination. The advent of liquid and gas chromatography coupled to tandem mass spectrometry, expanded the scope of target compounds monitored in fish, particularly for freshwater agroecosystems. The application of new mass spectrometry techniques for pesticide residue analysis in fish, along with the matrix effects observed with the applied instrumentation are deeply discussed. The evidence about different pesticides currently used worldwide and their occurrence in the tissues of freshwater fish at trace levels is presented. The bioaccumulation of banned persistent organic pollutants in edible fish is still a matter of interest for human exposure. Nevertheless, the regulation of maximum residue limits of currently used pesticides seeking a safe fish consumption is scarce.

Keywords Fish · Marine and freshwater · Currently used pesticides · Pesticide residues · Sample preparation · Gas chromatography · Liquid chromatography · Hyphenated mass spectrometry · Environmental monitoring · Food safety

1.1 Introduction

Different fish species, from marine and freshwater habitats are a key source of food for humans at global scale.

Synthetic organic contaminants have been monitored in edible fish from marine origin for more than 40 years. However, the compounds, named as legacy contaminants or persistent organic pollutants still play a role on health risks to aquatic ecosystems and human consumers (Boitsov et al. 2019; Pheiffer et al. 2018; Sun et al. 2018; Wu et al. 2018). The monitoring of organochlorine pesticides in fish muscle tissue initially started as a concern of bioaccumulation and biomagnification research. Highly persistent and non-polar compounds with log octanol-water coefficient higher than 5 such as hexachlorocyclohexane congeners, aldrin, and dichlorodiphenyltrichloroethane were found to occur in fish muscle tissues particularly associated to the high fat content (Pheiffer et al. 2018; Rios et al. 2019).

Widespread expansion of agricultural techniques based on application of pesticides led to an increasing use and disposal of pesticide residues in aquatic environments worldwide. Chemical diversity of pesticides has changed over the last decades and the currently used pesticides are, in general, more polar and less persistent. Nevertheless, significant exposure is evidenced in agricultural basins for freshwater fish species (Abalos et al. 2019; Ernst et al. 2018; Penland et al. 2018; Pheiffer et al. 2018; Picó et al. 2019; Vieira et al. 2019). The biological effect of the findings at sublethal levels is not well understood. The presence of pesticide residues in fish as multiple stressors in the aquatic environment can help to rationalize the effects of contaminants on non-target aquatic biota (Vieira et al. 2019). A multidisciplinary

approach would be helpful to integrate ecological, ecotoxicological and chemical observations that will give, through pesticide residues in fish, a holistic vision of the situation of a given environment.

Pesticide residues occurrence in fish is not only relevant from a toxicological point of view. Fish exposed to different environments are useful sources of information about the contamination level of the surrounding agroecosystems even at basin scale (Ernst et al. 2018; Ríos et al. 2019; Vieira et al. 2019). Pesticides can be incorporated onto fish tissues through the fish gills, either via water exposure or by the ingestion of contaminated feed. The route by which the pesticide integrates the fish body is relevant to assess contamination at muscle level. In the first case, no biotransformation occurs; the pesticide could be incorporated as such. In the latter case, the pesticide could be not only metabolized in the gastrointestinal tract but also stored in the viscera, normally, the non-edible portion of fish. Other situations, crucial to evaluate bioaccumulation or biomagnifications, are the habits of the animals, either migratory or not, and the existing trophic interactions (Ernst et al. 2018; Pérez-Parada et al. 2018; Picó et al. 2019). From a physicochemical point of view, when a compound shows a solubility in water lower than 0.0002 g/L, could be adsorbed to the suspended organic matter in freshwater environments and distributed within the fish tissues, no matter the route of exposure. Moreover, the amount of lipids plays a significant role in the storage capability of fish species. Lipid amount varies significantly seasonally, and has to be considered during pesticide residue analysis (Crane et al. 2007; Pérez-Parada et al. 2018; Ríos et al. 2019; Wickham et al. 1997).

Exposure to different currently used pesticide families led to recent reports showing pesticide occurrence ranging from pyrethroids and organophosphate insecticides to fungicides. Bioaccumulation of strobilurins, triazoles, triazines and chloroacetamide herbicides at μg/kg levels have been proved (Pérez-Parada et al. 2018). Additionally, some environmental organic contaminants like pharmaceuticals and semi-polar pesticides with log octanol-water coefficient higher than 2, has been detected in fish (Ernst et al. 2018; Picó et al. 2019; Rojo et al. 2019; Vieira et al. 2019). Considering the levels of pesticides residues found in fish it is important to pay attention to the safety of fish consumption. Agricultural pesticides in fish are assorted as extraneous chemicals. For that reason, extraneous maximum residue levels (E-MRLs) is matter of current interest at *Codex Alimentarius* (Pérez-Parada et al. 2018). Few pesticides are currently regulated and monitored in commercial trade from those existing based on specific national requirements for both persistent organic pollutants and currently used pesticides. Existing regulation deserves special attention on a few specific substances handling maximum tolerance levels and action levels (Food and Drug Administration 2011).

Summarizing, fish is one of the main aquatic organisms where an immediate impact of contamination because of agricultural activities can be observed. Furthermore, is considered a fundamental component of a balanced diet. Since fish contribution in nutrients is indisputable, is necessary to ensure the quality of fish to preserve human health. For this reason, the evaluation of the level of pesticide

residues in fish is important to ensure food safety (Molina Ruiz et al. 2015; Kalachova et al. 2013).

The interest in pesticide residues determination in seafood and particularly fish is actually not new. Interestingly, some well-established sample preparation methods for pesticide residues determination such as matrix solid phase dispersion were developed for organochlorine pesticides determination in fish (Long et al. 1991). New high throughput sample preparation methodologies, such as the variations of QuEChERS coupled to new and highly sensitive and selective instrumental techniques based on hyphenated mass spectrometry, has enabled a fast expansion of the scope to multi-class organic contaminants in fish at trace levels (Barbieri et al. 2019; Colazzo et al. 2019; Picó et al. 2019).

In this chapter, the determination of pesticides in fish, focused on contemporary sample preparation approaches will be discussed. The advantages and disadvantages as well as the coupling to modern tandem mass spectroscopic techniques will be addressed. Moreover, potential features of recent analytical tools aiming lower detectability of multi-class contaminants enabling an increase in knowledge of pesticide dynamics in the aquatic environment and food safety will be shown.

1.2 Fish Matrix Considerations

Fish and fish body parts are considered complex and variable matrices. As fish does not have a fixed chemical composition an approximate global composition, is generally accepted (Table 1.1). Furthermore, other minor components such as sugars, nucleotides and vitamins are also present. A thumb rule states that water and fat are inversely correlated, adding 80% of fish composition.

From an environmental point of view, the presence of compounds with high n-octanol-water partition coefficient such as persistent organic contaminants, pyrethroids and strobilurins in liver and viscera of fish give relevant information. Pesticide residues in the digestive apparatus of fish are indicative that the way of entry, is through food and water consumption. Therefore, the contaminants can be incorporated either from smaller fish, in a typical example of biomagnification or from sediments, catch tissue, as well as suspended organic matter. A higher frequency of dichlorodiphenyldichloroethylene findings in *Cnesterodon decemmaculatus*, a sediment feeding fish, caught in lagoons surrounded by farms where agricultural activities have been performed in the last hundred years was reported (Pareja et al. 2013).

Table 1.1 General proximate chemical composition of fish tissues

Chemical composition	Amount (%)
Water	65–90
Protein	10–22
Fat	1–20
Mineral	0.5–5

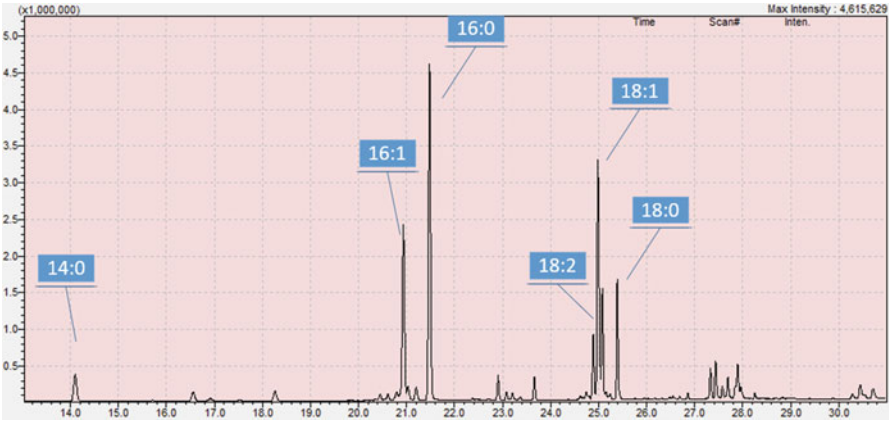

Fig. 1.1 Shows a typical fatty acid composition in fish fillet of *Prochilodus lineatus* captured in Uruguay River. The chromatogram was obtained by gas chromatography mass spectrometry in full scan mode. The main fatty acids are highlighted in blue; C14:0 myristic acid, C16:0 palmitic acid; C-18:0 stearic acid, C-18:1 oleic acid, C-18:2 linoleic acid, C-18:3 linolenic acid

Pesticide findings in fish fillets are a relevant issue from the food safety point of view. The possibility for the occurrence of residues in fish matrix is a combination of physicochemical properties and body compartments that could allow the partition between blood and tissues. Fat deposits are frequent in fish muscle, being the depositories for the most lipophilic compounds. As described above, the characteristics of the matrix varies not only with the fish species but also with the part of the fish to be studied. Moreover, matrix composition changes dramatically within species seasonally. The lipid amount increases when food is available but drops down when fish are in the spawning season. In addition, different amounts and types of lipophilic compounds such as vitamins, triglycerides, phospholipids, glycolipids, sterols as well as long-chain polyunsaturated fatty acids, among others could be present depending on the age and the fish species (Fig. 1.1). The composition of fish matrix should be taken into account when designing the sample preparation and instrumental determination technique because the described compounds can affect the determination of the target residues in different ways.

The size of the animal is also of paramount importance. Little fish, smaller than 5 cm like *Pomoxis spp, crappies,* or *Cnesterodon decemmaculatus,* madrecitas, are analyzed completes (Fig. 1.2). The aforementioned species are useful specimens for caged experiments in water contamination research studies where the animal exposure to the aquatic environment can be monitored in a given period of time. The information gathered has relevance when evaluating the environmental status of a given stream or catchments (Pareja et al. 2013).

In order to select a "fit for purpose" sample preparation method; the lipid content in the tissues should be calculated before pesticide residues determination, (Choi et al. 2016; Ernst et al. 2018; Molina Ruiz et al. 2015; Morrison et al. 2016; Yao et al. 2016). Different procedures are used for lipid determination: ISO 734-1:2008, Blight

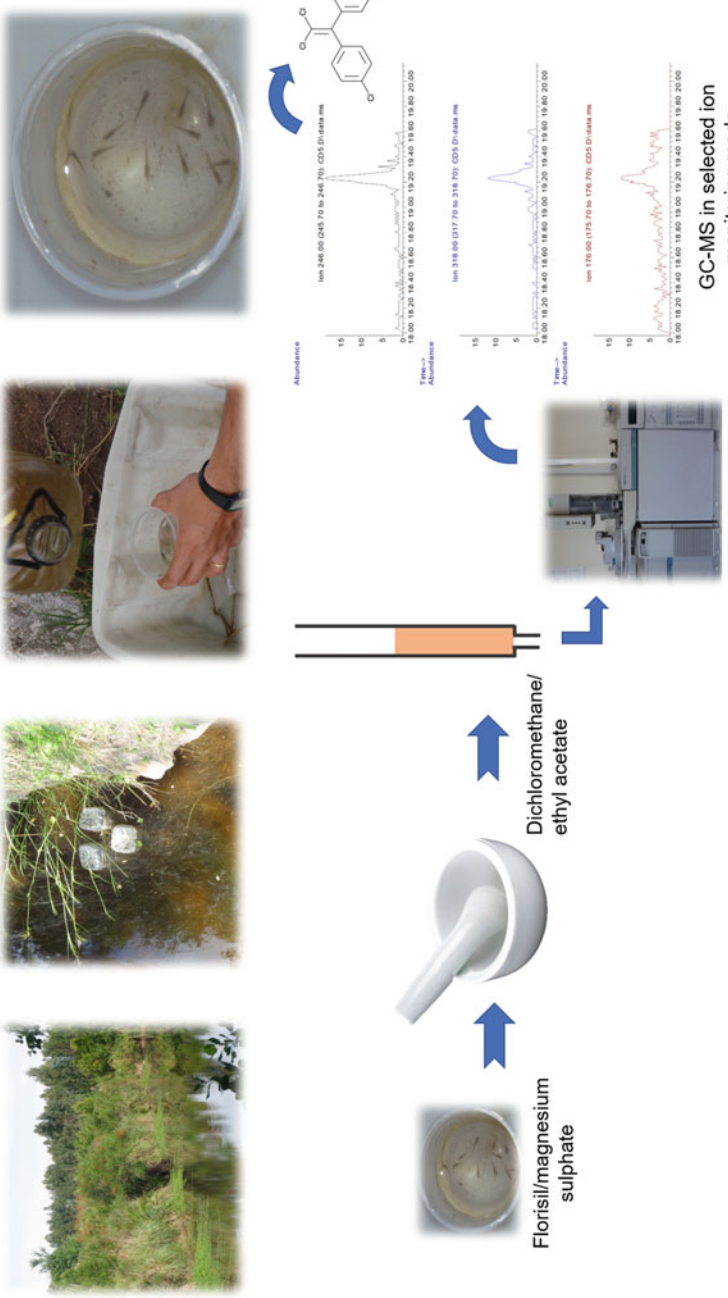

Fig. 1.2 Experimental design of a study performed at Cañada del Dragón, in Montevideo, Uruguay, for the analysis of pesticides in *Cnesterodon decemmaculatus*. The sample is extracted by matrix solid phase dispersion with gas chromatography mass spectrometry analysis in selected ion monitoring mode

& Dyer and Folch methods, among others (Ramalhosa et al. 2012). Moreover, in some cases moisture content is also evaluated (Sapozhnikova et al. 2015).

1.3 Importance of Fish Analysis

Pesticides have been considered as one of the most relevant environmental pollutants due to the persistence, mobility and long-term health effects on living organisms (Hernández et al. 2019). In the last decades, the interest was concentrated on persistent organic substances such as organochlorine and organophosphate pesticides because of the wide use, persistence and ability to bioaccumulate through trophic chains (Greco et al. 2010; Robinson et al. 2016). However, in recent years new types of widespread use organic contaminants were developed, and should be included in the analytical scope.

1.4 Analytical Methodologies for Pesticide Residues Determination in Fish Samples

Plenty of different analytical methods are reported in the literature to determine the presence of pesticide residues in fish. The targeted methodologies employed for these determinations must ensure true and precise results at low concentration levels for all the selected analytes, in accordance to the international guidelines. Moreover, straightforward, cheap and environmentally friendly methodologies should be preferred to carry out the task (Wilkowska and Briziuk 2011).

Generally, pesticide determination involves several steps; sampling, sample preparation including homogenization, extraction, clean-up, separation, detection, identification, quantification and data analysis (Samsidar et al. 2018).

1.4.1 Sampling, Transport, Traceability and Storage of Laboratory Samples

Fish tissue suffers very rapid spoilage, in terms of a few hours after harvest or catching, with a consequent potential change in matrix conditions over a reduced time. Therefore, fast storage under refrigerated or freezer conditions of the samples or performing the analysis immediately after sampling is recommended. All the reports stated that, once the sampling is performed, the samples should be transported refrigerated upon arrival at the laboratory and stored at −20 °C until analysis.

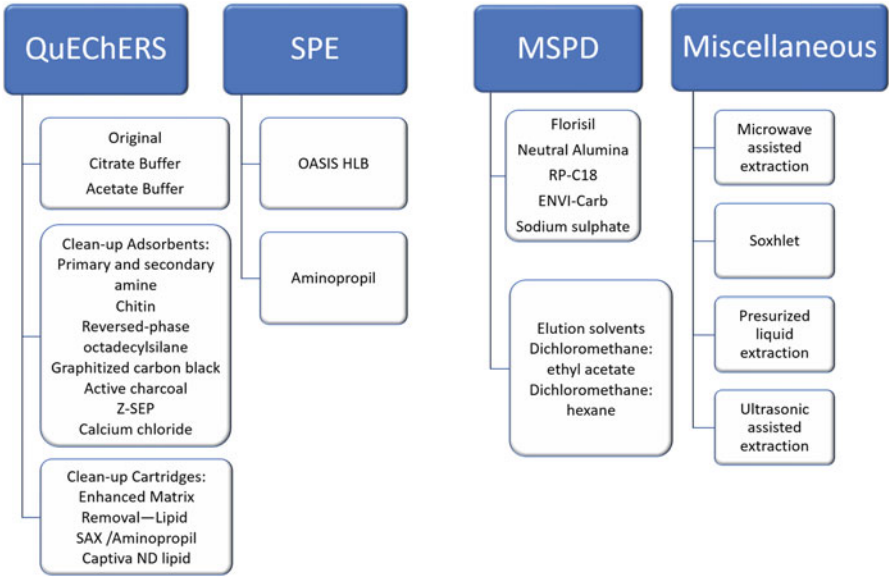

Fig. 1.3 Main extraction and clean up methodologies reported for the sample treatment of fish during pesticide residue analysis. *ASE* accelerated solvent extraction, *MSPD* matrix solid phase dispersion, *SPE* solid phase extraction, *UAE* ultrasonic assisted extraction, *MAE* microwave assisted extraction, *PLE* pressurized liquid extraction

Several authors reported the use of a lyophilized fish sample (Choi et al. 2016; He et al. 2017; Masiá et al. 2013; Mijangos et al. 2019; Picó et al. 2019; Zhao et al. 2019), while others prefer to use fresh or refrigerated samples. Regarding the homogenization of the samples, different approaches are described. Baduel et al. (2015) and Zhou et al. (2008), processed the samples with a kitchen blender, whereas Choi et al. (2016), removed the skin and used an ultra-dispenser to homogenize the fish muscle. Other protocols grinded the entire fish, including muscles, skin and bones (Picó et al. 2019). The main workflows reported for fish matrix are summarized in Fig. 1.3.

The most common tissues to be sampled are muscle fillet with or without the skin and liver tissue. According to the working document on the nature of pesticide residues in fish by SANCO, tissue samples should be dissected and the weight of each tissue recorded in the raw data. If the samples cannot be analyzed immediately, the sample should be stored frozen at or below −18 °C (European Commission 2013). Several reports advised to separate fish meat from bones and skin before homogenization (Chatterjee et al. 2016). Lipid peroxidation is one of the most problematic points when preparing the sample for analysis. Sample comminuting at room temperature, even of frozen fish causes heating of the sample, air absorption and lipid deterioration. The present peroxides can degrade the pesticides under study. Because of the possible degradation, assisted milling with dry ice or liquid nitrogen is advised. The sample obtained using the described methodologies is a

homogenized fine powder that allows an easy and representative extraction of the studied compounds (Sapozhnikova et al. 2015).

1.4.2 Extraction and Clean-Up Methods for Pesticide Residues Analysis in Fish

The selection of a suitable solvent for the extraction step is one of the most important aspects to obtain acceptable results when developing pesticide residue analysis in complex matrices. Pesticide extraction from tissues depends not only on the physicochemical properties but also in the matrix composition.

Generally, the sample treatment comprises two main steps: the extraction and the clean-up step. The first one consists of the homogenization of the sample with an organic solvent or solution water: organic solvent in different proportions with or without pH adjustment. The extraction could be assisted using different shaking devices; namely an ultrasonic bath, a blender or a homogenizer. The clean-up step seeks to separate the target compounds from any other matrix components that may interfere during the instrumental analysis, either causing detection or quantitation problems (Bennet et al. 1997). The clean-up is performed using particular adsorbents and salts in a suitable combination for the removing of the specific compounds from each matrix, sometimes the procedure could be also done in two steps, for example the typical dispersive one and a freeze out procedure in order to better precipitate the fatty compounds (Anastassiades et al. 2003).

According to Lazartigueset al. (2011a, b) the speed of use of an Ultra-turrax during homogenization is of crucial importance; while a low speed might not be useful. Nevertheless, a high speed could potentially induce pesticide losses. A middle speed was the best option when maintaining the sample in a water bath. Ideally, the exchange area between the comminuted sample and the extraction solvent should be maximized (Lazartigues et al. 2011a, b).

Several protocols have been reported for pesticide determination in fish (Fig. 1.4). However, the most used for fish matrix are the QuECHERS template, matrix solid phase dispersion and solid phase extraction. However, other approaches, like Soxhlet extraction, pressurized liquid extraction or ultrasonic assisted extraction are reported especially for organochlorine residues determination in fish are described in Sect. 1.4.2.4.

1.4.2.1 Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) Extraction

In recent years, the scope of the analytical methodologies has been broadened from a single-family class of pesticides to several different chemical classes as well as other anthropogenic contaminants. QuEChERS provides a versatile template of several

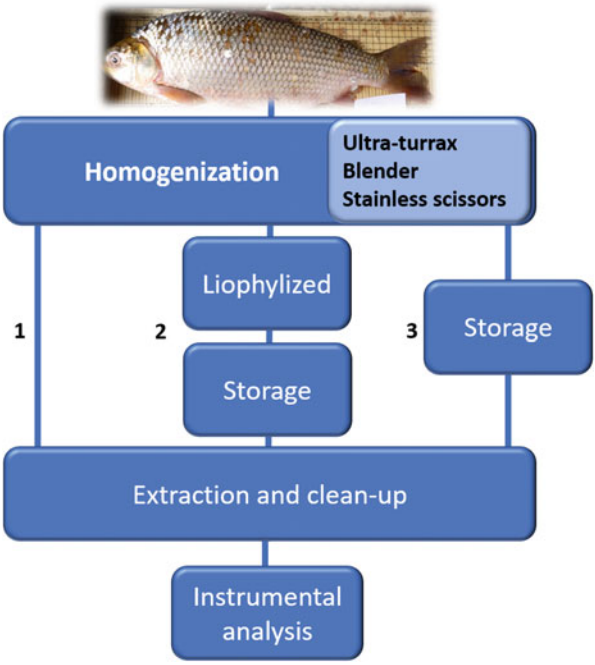

Fig. 1.4 Summary of three main workflows used for the sample treatment during pesticide residues analysis in fish. The general process consists of the homogenization of the sample followed in some cases by sample storage. Then, an extraction and clean-up step and finally the Instrumental analysis

protocols depending on the type of pesticide, matrix, instrument used or aim of the analysis. QuEChERS is a nice alternative with few steps, which provides in general, good analytical results.

The extraction is carried out with acetonitrile or acidified acetonitrile with citrate or acetate salts to buffer the extraction media. Phase separation is induced through a salting–out with sodium chloride followed by the addition of anhydrous magnesium sulfate as drying agent (Anastassiades et al. 2003; Anastassiades 2007; Lehotay et al. 2005). The formic acid as well as acetic acid is recommended to improve the stability of pesticides aforementioned methiocarb, chlorothalonil, or alachlor (Kaczyński et al. 2017).

The typical clean-up step of QuEChERS consists in a dispersive solid phase extraction, d-SPE, based on different sorbents and salts. The ideal sorbent is one capable of retaining co-extractives without interfering with the selected pesticides turning into a procedure as simple and fast as possible. The degree of purification using the QuEChERS template ranges from none to significant removing of the metabolites from the sample.

Several authors report the implementation of QuEChERS for fish samples. Table 1.2 summarizes the different sample preparation methods developed for the analysis of pesticide residues based on the QuEChERS template in fish. Most of the reports used the traditional clean-up with; primary and secondary amine, reversed-phase octadecylsilane, graphitized carbon black and magnesium sulfate (Barbieri

Table 1.2 Summary of the QuEChERS based methods reported for the determination of pesticides in fish matrix

Number of pesticides	Matrix	Extraction conditions	Clean-up conditions	% Rec (% RSD)	References
24	*C. carpio, A. sturio*	2.5 g sample + 5 mL H_2O + 10 mL MeCN. Shaken by hand 1 min + 0.5 g $Na_2HCit.1.5H_2O$ + 1 g $Na_3Cit.2H_2O$ + 4 g $MgSO_4$ + 1 g NaCl. Shaken by hand 1 min. 1 mL $CHCl_3$ was added and shaken for 1 min. Centrifugation for 15 min at 8700 rcf	6 mL aliquot + 1 g $MgSO_4$ + 150 mg PSA + 50 mg SAX + 50 mg NH_2. Shaken by hand for 1 min + centrifugation for 5 min at 5000 rcf. 4 mL aliquot + 1 mL $CHCl_3$ and 100 mg RP-C18. Shaken by hand for 1 min + Centrifugation for 5 min at 5000 rcf. 4 mL extracts + 40 μL 5% formic acid in MeCN, and evaporated under N_2 at 40 °C to dryness. Re-dissolved in 1 mL hexane. Freezed-out at −26 °C overnight. Separation of the precipitates by decantation.	61–122 (1–13)	Molina-Ruiz et al. (2015)
15	*Specie: not specified*	5 g sample + 5 mL H_2O vortexed for 1 min + 15 mL 1% acetic acid in MeCN + 2 mL hexane. Shaken by hand 1 min. 6 g $MgSO_4$ + 1.5 NaAc + vortex for 2 min. Centrifugation at 5000 r.p.m. for 5 min.	A portion of the middle layer freezed-out for 20 min at −20 °C + 100 mg $CaCl_2$ + 150 mg $MgSO_4$. 1 mL extract purified with 50 mg PSA + 50 mg Florisil + 150 mg RP-C18 + 150 mg $MgSO_4$, vortexed for 1 min and centrifuged for 5 min at 10,000 r.p.m.	60–127 (1–11)	Chatterjee et al. (2016)
5	*Specie: not specified* (Liver and muscle)	2 g muscle/0.5 g liver + 5 mL H_2O vortexed for 1 min + 10 mL 1% acetic acid in MeCN + 6 g	7 mL aliquot + 900 mg $MgSO_4$ + 150 mg RP-C18 + 150 mg PSA, shaken and centrifugation for	Muscle: 66–195 (0.09–13)	Han et al. (2016)

(continued)

Table 1.2 (continued)

Number of pesticides	Matrix	Extraction conditions	Clean-up conditions	% Rec (% RSD)	References
		$MgSO_4$ + 1.5 g NaAc. Shaken by hand 1 min. Centrifugation for 2 min at 2364 *x g*.	2 min at 2364 x*g*. 5 mL extract evaporated to dryness. Re-dissolved in 200 µL MeOH, separate into two aliquots which were dried again. One reconstituted with 100 µL MeOH: H_2O. The other in dichloromethane for analysis	Liver: 56–286 (0.02–9)	
52	*T. thymallus, L. cephalus, C. gobio, S. trutta marmoratus, S. trutta fario*	10 g sample + 5 mL H_2O + 10 mL MeCN. Shaken by vortex for 1 min at 2500 r.p.m. Centrifugation for 5 min at 4000 r.p.m.	7 mL aliquot +400 mg PSA + 400 mg RP-C18 + 1200 mg $MgSO_4$. Vortex 1 min at 2500 r.p.m. Centrifugation 5 min at 4000 r.p.m.	21–106 (3.3–20.6)	Barbieri et al. (2019)
5	*O. niloticus*	0.5 g + 9.2 mL acetone: hexane (1:1) shaken by hand 1 min + 2 g Na_2SO_4. Shaken manually 1 min + 4 $MgSO_4$ + 1 g NaCl, vortexed 1 min + sonication for 10 min. Centrifugation at 4 °C, for 10 min at 4000 r.p.m.	Supernatant evaporated to dryness, resuspended in 2 mL acetone: hexane (1:1, v/v) + 100 mg PSA + 23 mg RP-C18 mg + 400 mg $MgSO_4$, vortexed 1 min + Centrifugation at 4 °C, for 10 min at 5000 r.p.m. Filtration through 0.2 µm nylon filter for analysis	77.3–110.8 (3.4–12.6)	Stremel et al. (2018)
340	*E. Lucius, B. bjoerkna, S. cephalus, P. fluviatilis, L. aspius, T. tinca, S. lucioperca, A. brama, C. carassius, R. rutilus, L. lota* (Muscle and liver)	5 g liver or 10 g muscle + 10 mL 1% formic acid in MeCN. Shaken manually 1 min + 0.5 g $Na_2HCit.1.5H_2O$ + 1 g $Na_3Cit.2H_2O$ + 4 g $MgSO_4$ + 1 g	Extract + 1 g chitin (muscle) + 1 g chitin + 50 mg GCB (liver). Shaken by hand 1 min. Centrifugation for 10 min at 4000 r.p.m. Filter 1 mL supernatant through a 0.2 µm PTFE filter for analysis	70–120 (≤20)	Kaczynski et al. (2017)

		NaCl. Shaken 1 min. Centrifugation for 10 min at 4000 r.p.m.			
67	*M. obtusidens* (Muscle)	10 grams frozen sample + 10 mL MeCN. Shake vigorously by hand during 1 min + 1.5 g NaCl + 4 g $MgSO_4$. Shake vigorously by hand during 4 min. Centrifugation at 2260 *xg* during 5 min.	7 mL aliquot + 350 mg PSA + 180 mg RP-C18 + 1 g $MgSO_4$. Vortex for 30 s. centrifugation at 2260 x g for 5 min. Filter 1 mL supernatant through a 0.22 μm PTFE filter. Collect into a 2 mL screw-cap vial for analysis. Evaporate to dryness 4 mL solution under nitrogen stream. Re-dissolve in EtOAc for analysi-s.	36–123 (3- ≥ 20)	Colazzo et al. (2019)
42	*L. barbussclater, C. carpus*	1 g lyophilized sample + 10 mL MeCN. Shake vigorously by hand during 1 min + 1.5 g NaCl + 6 g $MgSO_4$. Shake vigorously by hand	50 mg PSA, 15 mg active coal, 50 mg RP-C18 and 150 mg anhydrous $MgSO_4$. Vortexed for 30 s. Centrifugation 5 min at 5000 r.p.m.	68–140 (5–22)	Masiá et al. (2013)
50	*B. guiraonis, M. salmoides, C. carpus*	2 g sample + 8 mL H_2O + 15 mL MeCN. Shake vigorously by hand during 1 min + 1.5 g NaCl + 6 g $MgSO_4$ + vortex 30 s. Centrifugation at 3000 r.p.m. at 20 °C for 5 min.	50 mg PSA, 15 mg active coal, 50 mg RP-C18 and 150 mg anhydrous $MgSO_4$. Vortexed for 30 s. centrifugation at 3000 r.p.m. at 20 °C for 5 min. Filter 1 mL supernatant through a 0.22 μm PTFE filter.	58–140 (5–22)	Masiá et al. (2015)
72	*Hoplias malabaricus Rhamdia quelen Pimelodus maculatus Paraloricaria vetula Hypostomus commersonni Salminus brasiliensis Megaleporinus obtusidens Prochilodus lineatus* (muscle)	10 g frozen sample + 10 mL MeCN. Shake vigorously by hand during 1 min + 1.5 g NaCl + 4 g $MgSO_4$. Shake vigorously by hand 4 min. Centrifugation at 2260 *x g*, 5 min.	7 mL aliquot + 350 mg PSA, 180 mg RP-C18 + 1 g $MgSO_4$. Vortexed for 30 s. centrifugation at 2260 x g, 5 min. Filter 1 mL supernatant through a 0.22 μm PTFE filter for analysis. 4 mL extract to dryness under a	36–123 (3– ≥ 20)	Ernst et al. (2018)

(continued)

Table 1.2 (continued)

Number of pesticides	Matrix	Extraction conditions	Clean-up conditions	% Rec (% RSD)	References
			nitrogen stream. Re-dissolve in EtOAc analysis.		
37	*Catfish, tilapia*	10 g lyophilized sample + 10 mL 1% acetic acid in MeCN. Shake vigorously by hand during 1 min + 1 g NaCl. Shake vigorously by hand during 1 min + 1.7 g NaAc + 0.3 g $MgSO_4$. Shake vigorously by hand during 1 min. Centrifugation 8 min at 3400 r.p.m.	3 mL aliquot + 75 mg PSA + 375 mg RP-C18 + 450 mg $MgSO_4$. Shake 1 min. Centrifugation 8 min at 3400 r.p.m. Filtration through 0.2 µm nylon filter for analysis	34.3–120.0 (2.1–20.0)	Munaretto et al. (2013)
27	*Specie: not specified*	10 g sample + 10 mL MeCN + Ultra-turrax for 1 min + 1 g NaCl + 4 g $MgSO_4$ + ceramic homogenizer, vortex 30 s, shake 1 min. Centrifugation 10 min x 3500 r.p.m.	Extract + freeze out −20 °C min 4 h, Extract 1 Non polar analytes: Extract 1 + 50 mg Z-sep, 150 mg $MgSO_4$, Vortex 1 min, shake 1 min. Centrifugation 10 min × 10.000 r.p.m., Vortex + Filtration through 0.2 µm PTFE filter for analysis Polar compounds: 500 µL xtract 1 + 1% Formic acid in MeCN, filtration through Captiva ND lipid cartridge. Evaporation to dryness ad reconstitute MeOH:H_2O (2:8, *v/v*) for analysis.	76–128 (2.2–27.5)	Baduel et al. (2015)
3	*Prawns, salmon, shark, tilapia, trout, tuna, marlin, arctic char*	5 g sample + 5 mL 1% Acetic acid in MeCN, sonication for 10 min + 0.5 g NaAc + 2 g $MgSO_4$.	1 mL extract +150 mg $MgSO_4$ + 50 mg PSA + 50 mg RP-C18, Vortex 1 min, shake	70–115 (2.9–14)	Rawn et al. (2010)

		Shake 2 min. Centrifugation 5 min at 3000 r.p.m.	1 min, Centrifugation 5 min at 3000 r.p.m. 500 µL aliquot concentrated to dryness + internal standard. The extract diluted with isooctane + Na_2SO_4, vortexed for analysis.		
50	*Specie: not specified*	2 g lyophilized fish + 8 mL H_2O + 15 mL MeCN + 6 g $MgSO_4$ + 1.5 g NaCl	2 mL extract + 300 mg $MgSO_4$, 100 mg PSA, 100 mg RP-C18 + 15 mg GCB.	58–140 (5–22)	Ccanccapa et al. (2016)
153	*R. quelen*	10 g sample + 10 mL MeCN shaken 1 min + 4 g Na_2SO_4 + 1 g NaCl. Manual shaking + centrifugation.	1 mL extract + 150 mg Na_2SO_4, 25 mg PSA + 25 mg RP-C18.	20–120 (1–22)	Munaretto et al. (2016)
40	*Iberian gudgeon, brown trout, European eel, bleak, pumpkinseed, Eastern iberian barbel, largemouth bass*	2 g lyophilized sample + 8 mL H_2O + 15 mL MeCN, shaken 30 s + 6 g $MgSO_4$ + 1.5 g NaCl, shaken for 1 min. Centrifugation 4 min at 4000 r.p.m.	2 mL extract + 300 mg $MgSO_4$, 100 mg PSA, 100 mg RP-C18 + 15 mg activated charcoal. Vortex for 30 s. Centrifugation 4 min at 4000 r.p.m. Filtration through 0.22 µm PTFE filter for analysis	<20 – >120 (< 20)	Belenguer et al. (2014)
113	*P. hypophthalmus*	5 g sample + 5 mL H_2O + vortex 1 min + 10 mL 1% Acetic acid in MeCN, vortexed 1 min + 6 g $MgSO_4$ + 1.5 g NaAc, vortex 2 min. Centrifugation 5 min at 1699 x *g*.	2 mL extract 20 min at −20 °C. 1.5 mL extract + 150 mg $MgSO_4$ + 100 mg $CaCl_2$. The supernatant + 50 mg PSA + 50 mg RP-C18 + 50 mg Florisil + 150 mg $MgSO_4$. Centrifugation 5 min at 10621 x *g*. Filtration through PTFE filter for analysis	62–119 (1–17)	Chatterjee et al. (2015)
6	*White sturgeon* (liver and gonad)	5 g sample + 5 mL MeCN + Ultra-turrax.	1.5 mL extract + 0.5 mL MeCN + 150 mg $MgSO_4$ + 50 mg		Morrison et al. (2016)

(continued)

Table 1.2 (continued)

Number of pesticides	Matrix	Extraction conditions	Clean-up conditions	% Rec (% RSD)	References
		The tubes were allowed to sit at room temperature for 30 min + sonication 15 min + 2 g $MgSO_4$ + 0.5 g NaCl, vortex 2 min. Centrifugation 5 min at 3000 *x g*.	PSA + 50 mg RP-C18. Centrifugation 5 min at 10621 x *g*. Repeat twice. Vortex 1 min + Centrifugation 5 min at 7200 *x g*.	60.8–108 (5.4–28.2)	
103	*Specie: not specified*	5 g sample + 5 mL H_2O + 10 mL MeCN. Shaken manually 1 min + 0.5 g $Na_2HCit.1.5H_2O$ + 1 g $Na_3Cit.2H_2O$ + 4 g $MgSO_4$ + 1 g NaCl. Shaken by hand 1 min + shaker 20 min. Centrifugation for 5 min at 8000 r.p.m. (10,560 *x g*).	6 mL extract + 900 mg $MgSO_4$ + 150 mg GCB + 150 mg RP-C18 + vortex 1 min. Centrifugation 5 min at 8000 r.p.m. (10,560 x *g*). 200 µL extract + 200 µL H_2O for analysis.	42–116 (1–11)	Zhang et al. (2017)
13	*Carp, catfish, Saury*	5 g sample + internal standards + room temperature for 30 min + 10 mL MeCN + vortex 30 s + 4 g $MgSO_4$ + 1 g NaCl + vortex 30 s + ultrasonic bath (40 kHz frequency and 200 W, 10 min). Centrifugation for 5 min at 5000 r.p.m.	1 mL extract + 50 mg PSA + 6 mL H_2O + 40 µL 1-undecanol + vortex 30 s. Centrifugation 5 min at 9000 r.p.m. The tube maintained in ice bath to solidify the organic extraction phase. The solidified portion transferred into a vial and melted at room temperature. Centrifugation 1 min at 5000 r.p.m. Separate the organic phase for analysis.	71.4–109.8 (≤ 15)	Wang et al. (2017)
18	Catfish	10 g sample + 10 mL MeCN, shaken by hand 30 s + 4 g	1 mL aliquot + 150 g $MgSO_4$ + 50 mg Z-SEP Plus.	71–119 (3–19)	

		$MgSO_4$ + 1 g NaCl. Shaken by hand 1 min. Centrifugation for 2 min at 3250 rcf.	Shaken + centrifugation for 2 min at 3250 rcf. 0.5 mL extract transferred to a vial an injection + standard addition for analysis.		Sapozhnikova and Lehotay (2013)
2	Croaker, salmon	4 g sample + 4 mL MeCN. Shaken for 10 min on a pulsed-vortexer at 80% in maximum pulsing. 2 g ammonium formate were added. Shaken for 1 min + centrifugation for 2 min at 3700 rcf.	Filtration of 0.5 mL extract through 0.2 μm PVDF filter + 75 mg $MgSO_4$ + 25 mg PSA + 25 mg Z-Sep + 25 mg RP-C18. Shaken by hand and transferred to an autosampler vial.	–	Sapozhnikova et al. (2015)

$CaCl_2$ calcium chloride, *H_2O* water, *$CHCl_3$* Chloroform, *EtOAc* ethyl acetate, *GCB* graphitized carbon black, *MeCN* acetonitrile, *MeOH* methanol, *$MgSO_4$* magnesium sulphate, *NaAc* sodium acetate, *$Na_2HCit.1.5H_2O$* disodium hydrogencitrate sesquihydrate, *$Na_3Cit.2H_2O$* trisodium citrate dihydrate, *Na_2SO_4* sodium sulphate, *N_2* nitrogen, *NH_2* aminopropyl, *NaCl* sodium chloride, *PSA* primary and secondary amine, *PTFE* polytetrafluoroethylene, *PVDF* polyvinylidene fluoride, *RP-C18* reversed-phase octadecylsilane, *SAX* strong anion exchange adsorbent

et al. 2019; Colazzo et al. 2019; Ernst et al. 2018; Munaretto et al. 2013; Stremel et al. 2018).

Different combinations of other adsorbents have also been assayed. Kaczysnki et al. (2017), reports a novel step extraction-clean-up strategy using chitin for the determination of 340 pesticides in fatty fish and liver matrix. Some others, highlight the use of a purification step with primary and secondary amine, reversed-phase octadecylsilane, magnesium sulphate and Z-sep® sorbent (Sapozhnikova and Lehotay 2015; Sapozhnikova et al. 2015). The authors also studied the efficiency of the afford mention dispersive solid phase extraction method and observed that Z-sep sorbent combination removed 83–95% of co-extracted matrix in incurred salmon and cracker (Sapozhnikova et al. 2015).

Each one of the mentioned sorbents interacts with different types of compounds from the matrix. The primary and secondary amine, removes polar pigments, acids and sugars, the graphitized carbon black interacts with color pigments, reversed phase octadecyl, is used for the removal of fats and non-polar components, whereas Z-sep® or Zep+® sorbent are for pigments and lipids.

Molina-Ruiz et al. (2015) evaluated a dual dispersive solid phase extraction method clean-up, consisting of the addition of chloroform with an octadecyl reversed phase, after the dispersive solid phase extraction combination of primary and secondary amine, strong anion exchange and aminopropyl resins. The strong anion exchange sorbent is ideally suited for the removal of carboxylic acids whereas; the amino group is a weak anion exchanger capable to extract fatty acids and sugars, among other carboxylates. The authors reported that strong anion exchange sorbent for the clean-up removes a high amount of fat and waxes with good recoveries and high sensitivity in samples with high lipid content.

Chitin, calcium chloride are also salts used in some cases; the first one for the removal of lipophilic compounds and other biomolecules while calcium chloride precipitate fatty acids and removes water providing better clean-up efficiency in some cases. Thus, ionic interactions with the sorbent or H-binding ionic become stronger providing better clean-up results. For example, Chatterjee and co-workers reported an exhaustive purification step based on cooling the samples at −20 °C for 20 min, followed by a dispersive solid phase extraction step with calcium chloride and magnesium sulfate, finally the obtained extract is again purified using primary and secondary amine, Florisil, reversed phase octadecyl, with magnesium sulfate as dissecant. Apparently, the simultaneous use of Florisil and primary and secondary amine removes co-extractives more efficiently than each one individually (Chatterjee et al. 2016).

More specific approaches to remove lipids are also depicted in the literature. For example, Lazartigues et al. (2011a, b), report the addition of 3 mL of hexane after the extraction and homogenization step but before the addition of the citrate salts. This approach generates less viscous extract that improves the liquid chromatography tandem mass spectrometry analysis. Baduel et al. (2015), optimized a new clean-up protocol for the determination of polar pesticides with Captiva ND lipid cartridges that removes proteins, lipids, phospholipids and other impurities providing lower matrix effects and avoiding low recoveries for some compounds.

Regarding the effect of the pH on the extraction efficiency, Baduel et al. (2015), evaluated the use of formic acid to acetonitrile with the citrate buffer during the salting out step. As known, buffered procedures provide satisfactory recoveries for acid-sensitive compounds. Different concentrations of formic acid at different amounts: 0%, 0.1%, 1% and 2% were tested. Two compositions of salts were also evaluated; the citrate buffer salts and the original salts of QuEChERS. No significant differences on the most lipophilic compounds with the citrate buffering or the addition of formic acid were found. However, for polar compounds the extraction is more pH dependent, mostly with those compounds, which present a carboxylic group in the molecule. Moreover, Rawn et al. (2010) used the acetate buffered version for the analysis of pyrethroids in several types of fish species with very good recoveries and coefficient of variation (Table 1.2).

The use of new absorbent for QuEChERS was also evaluated for pesticide residues analysis of fish. The Enhanced Matrix Removal of Lipids sorbent, was tested in salmon, a fish with a high lipid content. The lipid removal was around 80%, for 65 studied pesticides and the recoveries were between 70% and 120%. Nevertheless, recoveries of more lipophilic contaminants such polybrominated diphenyl ethers, polycyclic aromatic hydrocarbons and polychlorinated biphenyls were below 70% and adjustments using internal standards were needed for a proper determination of the above-mentioned compounds (Han et al. 2016).

1.4.2.2 Matrix Solid Phase Dispersion

Matrix solid phase dispersion methodology involves the extraction and clean-up in one single step. Is a rapid and easy procedure with few sample and solvent requirements in which the sample is blended with proper sorbents for example: Florisil, reversed-phase octadecylsilane, alumina, among others, until the homogenization is completed.

The main drawbacks are the great number of parameters to optimize like amounts of sample and sorbent, sorbent materials, clean-up characteristics and elution steps, as well as the automation (Long et al. 1991; Samsidar et al. 2018). Furthermore, the use of co-columns, consisting of other solid phase or chromatographic supports, incorporated at the bottom of the solid phase extraction column assists in analyte isolation or further clean-up, thus improving the matrix effects. Another way to eliminate co-extractives is to apply a sequential series of eluting solvents trying to remove the contents of the entire sample, including macromolecular components of the matrix. Besides the fact that the use of a solvent gradient is quite easy and cheap, the sequential elution had been applied to a limited number of compounds.

Barriada-Pereira et al. (2010), developed a protocol for the analysis of 20 organochlorine pesticides in muscle and liver fish. The adsorbent used was ENVI-Carb™ and sodium sulphate, after the matrix solid phase dispersion, the obtained extract is purified in a solid phase extraction cartridge containing ENVI-Florisil. A simple matrix solid phase dispersion procedure, mentioned above, was developed by Pareja et al. (2013). Small fishes are comminuted and blended with Florisil and magnesium

sulfate. Then the analytes are eluted with a mixture of ethyl acetate and dichloromethane. The small lipid content of the selected class of fish allowed the direct injection by gas chromatography coupled to electron capture detection, for the determination of dichlorodiphenyldichloroethylene at trace level.

Another approach was reported by Li et al. (2017), for the determination of 21 organochlorine pesticides in several fish species. One gram of sample is blended with 6 g neutral alumina. The mixture is placed in an empty solid phase extraction column containing a polypropylene frit and 1 g of neutral alumina as a co-column adsorbent. The selected pesticides were eluted with hexane:dichloromethane (50:50 *v/v*) and 10 mL eluate were collected. The extract was concentrated to dryness and dissolved in isooctane for analysis.

Hela and Papadopoulos (2013), estimated the uncertainty associated for the analysis of chlorinated compounds in 4 different fish species. The method was based on the extraction of 1 g of sample which was blended with reversed-phase octadecylsilane:Florisil (80:20) at a ratio dispersant to sample equal to 2:1. The clean-up mixture is then placed into a 20 mL-syringe barrel-column containing a portion of glass wool and 2 g of a mixture reversed-phase octadecylsilane:Florisil (40:60). The elution solvent was dichloromethane:hexane (50:50, *v/v*). The eluate was concentrated and dissolved in hexane for analysis. The methodology allowed the determination of 19 compounds with recovery percentages between 68% and 115% and relative standard deviations below 10%.

1.4.2.3 Solid Phase Extraction

Solid phase extraction technique is combined in general with liquid-liquid extraction or solid-liquid extraction for better enrichment and purification. Solid phase extraction has been reported to be fast, and efficient for pesticide monitoring in various matrices. The main advantages of the method are low solvent consumption, short analysis time, and high efficiency in removing co-extractives (Samsidar et al. 2018).

Regarding fish matrix, Gan et al. (2016), performed a solid-liquid extraction combined with solid phase extraction for the analysis of 6 organofluorine pesticides. The methodology consists of the homogenization of 5 g of fish sample with 10 mL acetonitrile, using a vortex for 60 s and centrifugation at 5000 r.p.m. for 10 min. The previous step is repeated twice and then 10 mL hexane are added to the extract, the mixture is vortexed 30 s and centrifuged with the previous conditions. The supernatant is discarded and the addition of hexane is repeated. Finally, the remaining extract is subjected to SPE clean-up. The solid phase extraction was optimized by the comparison of the efficiency of different types of cartridges: reversed-phase octadecylsilane, Florisil, aminopropyl, OASIS®-HLB, and neutral alumina. The recoveries of the analytes using reversed-phase octadecylsilane, Florisil and neutral alumina were in the range from 30% to 86% whereas the use of aminopropyl and OASIS®-HLB resulted in better recoveries. Therefore, aminopropyl cartridges were used due to the high cost of the other ones.

1.4.2.4 Miscellaneous Techniques

Soxhlet method has been applied for the analysis of organochlorine pesticides in fish. However, is time-consuming, expensive regarding amounts of solvent, analyst time and energy (Le Doux 2011). Moreover, high-moisture samples need a desiccation step to ensure the penetration of the solvent into the samples (Muralidharan et al. 2009; Suchan et al. 2004).

Muralidharan et al. (2009), determined 10 organochlorine pesticides in 10 fish species using a Soxhlet extraction with hexane for 6 h, from a 10 g portion of fish muscle followed by an acid treatment with sulfuric acid, neutralization with saturated sodium hydroxide solution after a clean-up with silica packed in a glass column was performed eluting the analytes with 250 mL hexane.

Zhou et al. (2008) optimized a Soxhlet method based on the extraction of 10 g of sample grounded with 30 mg sodium sulfate and 200 mL of hexane:acetone (3:1, *v:v*) for 8 h. After the concentration of the extract, the lipid percentage was determined gravimetrically in an aliquot of the sample. The clean-up depended on the type of compound; for acid stable pesticides 3 g of activated ash, sulfuric acid impregnated and hexane washed silica was placed in an empty cartridge and the fat residue was loaded. The elution was performed with 2 × 5 mL hexane:dichloromethane (3:1, *v/v*). The eluates were concentrated and dissolved in hexane for analysis. For the non-acid stable pesticides, the fat extract was purified in a cartridge containing 2 g of alumina, silica gel and Florisil, impregnated with a 15% potassium hydroxide methanolic solution (50%, *v/v*). The elution was done with 2 × 5 mL hexane: dichloromethane (3:1, *v/v*). The eluates were concentrated and dissolved in hexane for analysis. The recovery percentages varied from 78% to 89% and the relative standard deviations were in the range 5–10%.

Matrix solid phase dispersion was used coupled to a Soxhlet procedure avoiding the use of sulfuric acid in sample dehydration step (Miglioranza et al. 2013). The protocol has been applied to the systematic study of persistent organic pollutants occurrence in different aquatic environments. The samples were homogenized with sodium or magnesium sulfate, Soxhlet extracted with hexane: dichloromethane (50:50, *v/v*), purified through silica gel and microgel permeation using Biobeads X300. The extract thus obtained was clean enough to perform large monitoring programs of persistent organic pollutants including all the legacy organochlorine pesticides with gas chromatography electron capture detection.

Pressurized liquid extraction or accelerated solvent extraction has been used for the extraction of organochlorine pesticides from fish liver and muscle. The method involves little solvent consumption and short times of extraction step. One of the disadvantages is that samples with high water content needs a desiccation step before the extraction. The methodology uses conventional solvents at elevated pressures, 10–15 MPa, and temperatures in the range 50–200 °C for short periods of time, from 5 to 10 min.

Suchan et al. (2004), optimized the extraction conditions of pressurized liquid extraction and compared the performance in terms of repeatability of the developed

method with Soxhlet extraction. The relative standard deviations obtained were in the range from 3% to 14%, which were comparable to conventional Soxhlet. Usually, the worst repeatability was obtained for hexachlorohexane isomers for both methods. Regarding the comparison of the time needed for both methods, the authors concluded that pressurized liquid extraction requires less time per analysis. In addition, the cost per sample was calculated being also higher using Soxhlet extraction.

Choi et al. (2016) developed an extraction and clean-up method using pressurized liquid extraction for the analysis of 14 organochlorine pesticides in fish. The extraction solvent was hexane:dichloromethane (7:3, *v/v*) and the samples were extracted using a modification of the method 3545A of the US Environmental Protection Agency. The selection between alumina, Florisil, acid-silica, and different combinations, for lipid removal was performed using a type of fish with around 23% fat, being alumina and acid silica the adsorbents which removed the highest amount of lipids. Method validation was performed with certified reference materials and spiked fish. The recoveries were from 79% to 108% with relative standard deviations below 10%.

The pressurized liquid extraction method was also reported for the analysis of 18 organochlorine pesticides in a lyophilized fish sample. Two grams of sample were mixed with 10 g sodium sulphate and transferred to 33 mL sample cell and kept in an equilibrium state for at least 12 h before extraction. The sample was extracted with dichloromethane:hexane (1:1, *v/v*) at 10.3 MPa and 100 °C, 3 × 5 min cycle. The extracts were evaporated until constant weight and then reconstituted in hexane for the clean-up. Lipids removal was achieved with an acidic silica gel column of 300 mm length and 15 mm of internal diameter, packed with 1 g activated silica gel, 8 g 44% acidic silica gel, 8 g 22% acidic silica gel, 1 g activated silica gel and 4 g sodium sulphate. After conditioning with hexane, the extract was loaded and eluted with 100 mL dichloromethane:hexane (1:1, *v/v*). The eluate was evaporated to 1 mL and purified on a multi-layer silica gel basic alumina oxide and Florisil column. The recoveries of the method ranged from 54% to 115% with relative standard deviations between 7% and 20% (Zhao et al. 2019).

Microwave-assisted extraction has also been tested for both organochlorine and organophosphates pesticides extraction from fatty fish tissue.

He et al. (2017) reported the use of microwave-assisted extraction methodology for the determination of 25 organochlorine pesticides in fish tissues. The method was based on the extraction of 2 g of sample with 25 mL solvent with the following instrument conditions: working power 1200 W, and gradient temperature program increased to 100 °C within 10 min constant for 10 min and then to ambient temperature within 30 min. The filtered extract was concentrated to 1 mL and 10 mL ethyl acetate:cyclohexane (1:1, *v/v*) were added. The solvent was again concentrated up to 2 mL and centrifuged at 4 °C for 15 min at 12,000 r.p.m. The supernatant was filtered and purified by gel permeation chromatography, graphitized carbon black, using a mobile phase of ethylacetate:cyclohexane (1:1, *v/v*) with a flow rate of 5 mL/min and a Biobeads SX-3 (300 × 200 mm) column. The fraction was collected from minute 10 to 39 and purified in a multi-layer chromatography column

with a 10 mm internal diameter containing; 12 cm wet of neutral alumina, 12 cm wet of neutral silica gel and 1 cm sodium sulfate. The elution was made with 10 mL hexane which were discarded and then 50 mL of dichloromethane:hexane (1:1, *v/v*). The extract was concentrated to dryness and dissolved in hexane for analysis.

In 2011, an extraction of 8 g of sample with 5 g of sodium sulphate and 15 mL acetone by ultrasonication for organophosphate pesticides analysis was reported. Two times extracts were combined and concentrated under reduced pressure vacuum and the residue was dissolved in 8 mL methanol:water (5:95, *v/v*). The extract was cleaned-up using a hollow fiber liquid-phase microextraction device. Several parameters influence the efficiency of hollow fiber liquid-phase microextraction technique, including an organic solvent, speed of agitation, pore size, porosity internal diameter and wall thickness. The recovery percentage differed between the fish species evaluated, but in general were in the range of 71.8–95.5% with relative standard deviations below 18.9% (Sun et al. 2011).

Another multi-class methodology based on the use of ultrasonic extraction of fish matrix was described by Kong et al. (2018) using 2 g of sample that were extracted with 10 mL acetonitrile and 200 μL 0.1 M ethylene diamine tetraacetic acid disodium solution with a grinder for 30 s. The mixture is agitated by vortex for 5 min, placed in an ultrasonic bath at 40 °C for 10 min and again agitated for another 5 min. The extract was centrifuged at 10000 × *g* for 10 min and the supernatant transferred to a glass tube. Afterwards 10 mL ethyl acetate were added to the sample and the extraction procedure is repeated. The extracts were combined, dried under a nitrogen flow at 40 °C and the residue was dissolved in 1 mL of 0.1% formic acid in acetonitrile:water (2:3, *v/v*).

Liu et al. (2017), reported a straightforward methodology for the analysis of 10 pesticides in fish. In this method, 2 g of sample were treated in a sonicator for 10 min with 10 mL ethyl acetate. After 5 min centrifugation at 10,000 r.p.m. at 4 °C the supernatant was passed through an OASIS PRIME HLB cartridge and collected. The eluate was concentrated to dryness, dissolved in 70% methanol and filtered through 0.22 μm filter for analysis.

Mijangos et al. (2019), applied a pulsed time of 0.8 s, 10% amplitude, at 0 °C in an ice-water bath to sonicate 0.5 g freeze dried liver or muscle sample or 0.1 g gills and brain with 7 mL methanol: water (95:5, *v/v*) for 30 s. The extract was filtered through 0.45 μm, evaporated to 1 mL approx. and diluted with 6 mL water adjusted at pH 2 with formic acid and an appropriate volume of ethylene diamine tetraacetic acid disodium solution. The extract was loaded in a 200 mg-OASIS HLB previously conditioned cartridge and eluted with 6 mL water for cleaning purposes and finally eluted with 6 mL methanol. For liver matrix the extract was concentrated to approx. 1 mL and poured into a 30 mL vessel where 6 mL water with 30% sodium chloride were incorporated twice; first adjusted to pH 2 with formic acid and then adjusted with ammonium hydroxide to pH 10. The final concentration was achieved by adding ethylene diamine tetraacetic acid disodium. Afterwards, pre-cleaned 50 mg-portions of polyethersulfone were added. The vessels were closed and extracted at room temperature overnight at 800 r.p.m. The polymer was removed, washed with milli-Q water, dried and desorbed in 1 mL methanol by soaking for

32 min in an ultrasonic bath. The method was validated for 41 multi-class compounds in which four of them were pesticides. The recoveries ranged from 69% to 145% with relative standard deviation in the range 2–33%.

Shin (2006), developed a method for the determination of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid in frog and fish tissues. The developed method was based on the ultrasonic extraction of 5 g sample with 20 mL 1 M sodium hydroxide:acetone (1:9, *v/v*) for 5 min. Then the extract was concentrated to 0.2 mL, dissolved in 9 mL methanol-1 N sodium hydroxide (1:2, *v/v*) and heated for 15 min at 70 °C. The solution was extracted with 6 mL methyl, ter-butyl ether and the organic phase discarded. The aqueous solution was adjusted to pH 2 with 20% Hydrochloric acid and extracted with 8 mL of petroleum ether. Afterwards, 2 mL 10% sulfuric acid in methanol were incorporated, the mixture was heated for 30 min at 80 °C, concentrated to 0.2 mL for analysis. The 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid presented recovery percentages of 96.8% and 93.8% with relative standard deviations below 6% at 2 μg/kg.

A single solid-liquid extraction method was developed by Lazartigues et al. (2011a, b). An Ultra-turrax was used for the extraction of 3 g of sample maintained at 20 °C in a water bath with 10 mL acetonitrile:water (50:50 *v/v*), and 4 g of magnesium sulphate. The tube was shaken 10 min and centrifuged at 12,000 g for 10 min at 25 °C. Then another solid-liquid extraction was performed with ethyl acetate:cyclohexane (75:25, *v/v*) in the same conditions. After evaporation of the extract the residue was reconstituted with 0.9 mL acetonitrile:water (10:90, *v/v*) and stored in the dark at −20 °C for analysis. Recovery rates were from 36% to 115%, with relative standard deviations below 22% for all the evaluated compounds.

1.5 Separation and Determination

As previously stated, the analysis of fish samples is a challenging task which involves working with a complex matrix, low concentration of target compounds and analytes with a broad spectrum of physico-chemical properties. In this review, we focused on the most important and used chromatographic methods, which represent the vast majority of the studies in the literature, related to fish analysis. Gas chromatography was the most widespread method for the separation and determination of pesticides. However, the use of liquid chromatography has gained grown since new pesticides with more polar characteristics were released.

1.5.1 Gas Chromatography

Gas chromatography is the most common technique and the preferable instrumentation when dealing with less polar, highly volatile or easily vaporized pesticides.

Different detectors are available, such as electron capture detector, flame photometric detector, nitrogen phosphorus detector and flame ionization detector. Nowadays for pesticide residues analysis involving food safety and environmental monitoring the most frequent coupling implies the use of a mass spectrometer in single mode or in tandem configuration.

Conventional detectors such as electron capture detector or flame photometric detector have been largely applied for the determination of pesticide residues in fish. These detectors are intended for the analysis of halogenated compounds like organochlorine and organophosphate residues can be combined with the determination of other contaminants like polychlorinated biphenyl compounds (Zhou et al. 2008; Sun and Chen 2008; Malhat and Nasr 2011; Stremel et al. 2018).

The coupling of a mass detector is widely reported and several configurations are used. In gas chromatography coupled to mass spectrometry different options can be used, from low-resolution single quadrupole to a high-resolution magnetic sector, either working with electron impact ionization or negative chemical ionization (Colazzo et al. 2019; Zhao et al. 2019; Li et al. 2017; Ernst et al. 2018; Molina-Ruiz et al. 2015).

In gas chromatography tandem mass spectrometry better sensitivity and selectivity can be achieved with a triple quad or hybrid quadrupole-time of flight. Therefore, is implemented in the analysis of multi-class pesticides, such as organochlorine. organophosphate, pyrethroids, diverse fungicides and herbicides. To fulfill the current requirements from the guidelines for pesticides residues analysis about identification and confirmation of the studied analytes, the tandem mass spectrometer, in particular the triple quad is the default detector of choice (SANTE 2017a). As can be seen in Table 1.3, several examples of pesticide residue analysis in fish by gas chromatography coupled to different detectors have been reported.

Not only pesticides are included but also others contaminants of relevance like polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers and polychlorinated biphenyl compounds (Chatterjee et al. 2015, 2016; Sapozhnikova and Lehotay 2015; Baduel et al. 2015; Nácher-Mestre et al. 2014).

1.5.2 Liquid Chromatography

Liquid chromatography is frequently used for high polarity, low volatility and thermally labile compounds. UV/vis detector can be used for trace analysis in fish, but a lot of work must be done in relation to the sample treatment as it is not a specific detector. The application of liquid chromatography for pesticide residue analysis has been reported worldwide. Jabeen et al. (2015) worked with liquid chromatography for the determination of nine pesticides including pyrethroids, carbamates and neonicotinoids.

Liquid chromatography hyphenated with triple quad, quadrupole time of flight coupled and linear trap quadrupole-Orbitrap mass spectrometry has been adopted for multi-residue pesticide analysis in fish. This alternative enhances sensitivity and

Table 1.3 Instrumental conditions for determination of contaminants in fish samples

Number of pesticides	Extraction method	LOQ (μg/kg)	Instrumentation			References
			Chromatography column	Mobile phase	Detector	
52	QuEChERS	0.04–87.1	Purospher STAR RP-18e column (150 × 2.1 mm, 2 μm)	MeCN/Water	LC-MS/MS (ESI-QqQ)	Barbieri et al. (2019)
51	Ultrasound and SPE; QuEChERS	0.0015–15	N/R	N/R	LC-MS/MS	Pico et al. (2019)
44	QuEChERS	1–10	Zorbax Eclipse XDB C-18 (150 × 24.6 mm, 5 μm)	MeCN/0.1% formic acid in water	LC-MS/MS (ESI-QqQ)	Colazzo et al. (2019)
23		15–50	TRACET TR-5MS (30 m × 0.25 mm, 0.25 μm)	Helium	GC-MS (EI-Q)	
18	ASE	0.5	DB-5-MS (60 m × 0.25 mm, 0.25 μm)	N/R	GC-MS (EI/NCI-Magnetic Sector)	Zhao et al. (2019)
44	QuEChERS	0.6–9.6	Zorbax Eclipse XDB C-18 (150 × 24.6 mm, 5 μm)	MeCN/0.1% formic acid in water	LC-MS/MS (ESI-QqQ)	Ernst et al. (2018)
28		11–85	TRACET TR-5MS (30 m × 0.25 mm, 0.25 μm)	Helium	GC-MS (EI-Q)	
7	MeCN/AcOEt extraction		Accucore RP-MS C-18 (100 × 2.1 mm, 2.6 μm)	MeCN/Water (both containing 0.1% formic acid)	LC-MS/MS (ESI-Q-Orbitrap)	Kong et al. (2018)
4	FUSLE	0.2–0.5	Kinetex F5 100 Å core-shell (100 × 2.1 mm, 2.6 μm)	MeOH:water (95:5)/water:MeOH (95:5)	LC-MS/MS (ESI-QqQ)	Mijangos et al. (2019)
340	QuEChERS	5–50	KINETEX C-18 (100 × 2.1 mm, 2.6 μm)	MeOH/Water (both containing 0.5% of formic acid and 5 mM of ammonium formate)	LC-MS/MS (ESI-QqQ)	Kaczyński et al. (2017)

103	QuEChERS	1–10	Poroshell 120 EC-C-18 (100 × 3.0 mm, 2.7 μm)	MeOH/0.1% formic acid 5 mM ammonium acetate in water	LC-MS/MS (ESI-QqQ)	Zhang et al. (2017)
21	MSPD	0.037–0.100	HP-5MS (30 m × 0.25 mm, 0.25 μm)	Helium	GC-MS (NCI-Q)	Li et al. (2017)
10	AcOEt extraction followed by SPE	0.03–2.50	ZORBAX SB-Aq column (100 × 3.0 mm, 1.8 μm)	MeCN/0.1% formic acid in water	LC-MS/MS (ESI-QqQ)	Liu et al. (2017)
5	QuEChERS + ultrasound	0.8–9.6	DB-5-MS (30 m × 0.32 mm, 0.25 μm)	Nitrogen	GC-ECD	Stremel et al. (2018)
82	MeCN extraction	2–13	VF-5MS (30 m × 0.25 mm, 0.25 μm)	Helium	GC-MS/MS (EI-QToF)	Chatterjee et al. (2016)
153	QuEChERS	5–25	Zorbax Eclipse Plus C-18 (50 × 2.1 mm, 1.8 μm)	MeOH/Water:MeOH (92:2) (both containing 0.1% (v/v) of formic acid and 5 mmoL/L of ammonium formate)	LC-MS/MS (ESI-QToF)	Munaretto et al. (2016)
6	MeCN extraction	0.3–0.5	Atlantis T3 C-18 (150 × 2.1 mm, 3 μm)	MeOH/0.1% formic acid, 5 mM ammonium acetate buffer (pH = 5)	LC-MS/MS (ESI-QqQ)	Gan et al. (2016)
150	QuEChERS with Pulsed-vortexing shaker	N/R	Rti-5MS (15 m × 0.53 mm, 1 μm)	Helium	LPGC-MS/MS (QqQ)	Sapozhnikova et al. (2015)
105	MeCN (1% acetic acid) extraction	1–11	HP-5MS (30 m × 0.25 mm, 0.25 μm)	Helium	GC-MS/MS (EI-QToF)	Chatterjee et al. (2015)
50	QuEChERS	0.23–11.25	Luna 18 (150 × 2.0 mm, 3 μm)	MeOH/Water (both containing 10 mM ammonium formate)	LC-MS/MS (ESI-QqQ)	Masiá et al. (2015)
24	QuEChERS	4–9	HP-5MS (30 m × 0.25 mm, 0.25 μm)	Helium	GC-MS (EI-Q)	Molina-Ruiz et al. (2015)
18	QuEChERS			N/R		

(continued)

Table 1.3 (continued)

Number of pesticides	Extraction method	LOQ (μg/kg)	Instrumentation			References
			Chromatography column	Mobile phase	Detector	
		0.08–2.0	DB-5-MS (30 m × 0.25 mm, 0.25 μm)		GC-MS/MS (EI-QqQ)	Baduel et al. (2015)
9		0.4–3.0	XDB-C-18 (100 × 2.1 mm, 1.8 μm)	MeOH/Water (both containing 0.1% formic acid)	LC-MS/MS (ESI-QToF)	
9	AcOEt extraction	N/R	Supelcosil TM LC-18 (250 × 4.5 mm, 5 μm)	MeCN/Water	HPLC-UV/Vis	Jabeen et al. (2015)
6	QuEChERS	N/R	Rti-5MS (15 m × 0.53 mm, 1 μm)	Helium	LPGC-MS/MS (QqQ)	Sapozhnikova et al. (2015)
54	QuEChERS	N/R	Kinetex XB C-18 (50 × 2.1 mm, 1.7 μm)	MeOH/Water (both containing 0.1% formic acid)	LC-MS/MS (ESI-LTQ-Orbitrap)	Farré et al. (2014)
133	QuEChERS	N/R	DB-5MS (30 m × 0.25 mm, 0.25 μm)	Helium	GC-MS (APCI-QToF)	Nácher-Mestre et al. (2014)
40	QuEChERS	0.03–11.25	Luna 18 (150 × 2.0 mm, 3 μm)	MeOH/Water (both containing 10 mM formic acid)	LC-MS/MS (ESI-QqQ)	Belenguer et al. (2014)
50	QuEChERS	0.9–11.25	Luna 18 (150 × 2.0 mm, 3 μm)	MeOH/Water (both containing 10 mM ammonium formate)	LC-MS/MS (ESI-QqQ)	Masiá et al. (2013)
37	QuEChERS	1–10	VF-5-MS (30 m × 0.25 mm, 0.25 μm)	Helium	GC-MS/MS (EI-QqQ)	Munaretto et al. (2013)
18	QuEChERS	N/R	Rti-5-MS (15 m × 0.53 mm, 1 μm)	Helium	LPGC-MS/MS (QqQ)	Sapozhnikova and Lehotay (2013)
14		N/R		Nitrogen	GC-FPD	

	Chloroform/acetone extraction		PAS-1701 (30 m × 0.32 mm, 0.25 μm)			Malhat and Nasr (2011)
13	MeCN/water extraction	0.1–49	Zorbax XDB-C-18 (50 × 2.1 mm, 1.8 μm)	MeCN/0.0125% (v/v, pH = 4.04) acetic acid	LC-MS/MS	Lazartigues et al. (2011a, b)
8	QuEChERS	N/R	HP-5 (30 m × 0.32 mm, 0.25 μm)	Helium	GC-MS (NCI-Q)	Rawn et al. (2010)
1	MeCN extraction	N/R	DB-608 (30 m × 0.53 mm, 0.83 μm)	Nitrogen	GC-FPD	Sun and Chen (2008)
13	Acetone/hexane extraction	N/R	DB-5 (30 m × 0.32 mm, 0.25 μm)	Nitrogen	GC-ECD	Zhou et al. (2008)

APCI atmospheric-pressure chemical ionization, *ASE* accelerated solvent extraction, *AcOEt* ethyl acetate, *EI* electron impact ionization, *ESI* electrospray ionization, *FUSLE* focused ultrasonic solid-liquid extraction, *GC-ECD* gas chromatography electron capture detection, *GC-FPD* gas chromatography flame photometric detector, *GC-MS* gas chromatography mass spectrometry, *GC-MS/MS* gas chromatography tandem mass spectrometry, *HPLC-UV/Vis* High Performance Liquid Chromatography ultraviolet-visible detector, *LC-MS/MS* liquid chromatography tandem mass spectrometry, *LPGC-MS/MS* low pressure gas chromatography tandem mass spectrometry, *LTQ* linear trap quadrupole, *MeCN* acetonitrile, *MeOH* methanol, *MSPD* matrix solid phase dispersion, *NCI* negative chemical ionization, *Q* quadrupole, *QqQ* triple quadrupole, *QToF* quadrupole time-of-flight, *SPE* solid phase extraction

selectivity, improving the quantification limit of the method at sub part per billion levels and, depending on the configuration, allowing the identification of non-target compounds.

One of the most frequently used mass spectrometric detectors is the triple quad, which has the multiple reaction monitoring mode of data acquisition, that allows the determination of a larger number of targeted compounds in a single run with the highest sensibility. Several authors reported the use of triple quadrupole tandem mass spectrometry for the analysis of fish samples.

Barbieri et al. (2019) validated a methodology for the determination of 52 medium to highly polar pesticides in fish muscle with low quantification limits. Ernst et al. (2018) and Colazzo et al. (2019) reported the analysis of 44 multi-class pesticides residues in fish muscle with electrospray ionization in multiple reaction monitoring mode. Kaczyński et al. (2017) determined 340 pesticides residues in fatty fish with detection limits between 0.05 and 1.2 μg/kg.

High resolution mass spectrometry was also introduced to pesticide residue analysis in fish. time-of-flight detector is also available to be coupled with liquid chromatography and has the advantage of allowing high resolution and high sensitivity when analyzing non targeted compounds in the full scan mode.

Munaretto et al. (2016) evaluated two scan modes: full scan and all ions mass spectrometry, to assess the best option for screening analysis in spiked fish fillet samples. The study included the analysis of 153 pesticides residues among other organic contaminants from different chemical classes such as veterinary drugs and personal care products. The authors found that full scan acquisition was more reliable as allowed to detect around 84% of the compounds in the automatic identification and quantification mode whereas "all ions" mass spectrometry detected 72%. Most of the compounds presented a screening detection limit value of 10 μg/kg in both full scan acquisition modes.

In 2015, Baduel et al. (2015) presented a straightforward multi-residue method to analyze polar and non-polar compounds in biological matrices such as fish. Baduel and co-workers combined targeted multi-residue analysis using gas chromatography–triple quadrupole mass spectrometry and multi-targeted analysis complemented with non-target screening using liquid chromatography quadrupole time-of-flight mass spectrometry with limits of quantification between 0.2 and 9 μg/kg.

The Orbitrap™ mass analyzer, implements the principles of Fourier transform through an electrostatic axially harmonic orbital trapping technique to yield high-resolution mass spectra. It provides high mass resolution, higher than 15,000 full widths at half maximum, and high mass accuracy, below 2 ppm, but without mass selection (Farré et al. 2014). This detector in combination with a single quadrupole or a linear ion trap quadrupole has been used in the analysis of pesticides and pharmaceutical residues in fish and aquatic food (Kong et al. 2018; Farré et al. 2014).

1.6 Matrix Effect

Matrix effect is defined as the influence on the changes in the intensity of the detector response due to compounds present in the matrix, which generally co-elute with the analyte. Matrix effect depends therefore on the matrix compounds present in the extracts to be analyzed, the chromatographic behavior and the differential sensibility towards the detectors employed for the determination. The effect could be noticed as an increase of the signal named: signal enhancement or decrease, signal suppression. The comparison of these effects with the neat signal yielded by the compound of interest in the conditions of the analysis are classified as positive in the former case and negative in the latter. The evaluation can be performed by comparing the slopes values of the matrix match calibration curve and the solvent using the formula

$$\text{Matrix effect}\% = [(\text{Slope matrix}/\text{Slope solvent}) - 1] \times 100$$

The signal enhancement gives positive matrix effects whereas negative values correspond to signal suppression. Matrix effects are supposed to be compensated using matrix matched calibration curves. European Commission SANTE guidelines establish that the potential for matrix effects to occur should be assessed during method validation. In general, matrix effect is notoriously variable in occurrence and intensity, but some techniques are particularly prone to them. When the techniques used are not inherently free from such effects, quantification should be performed by matrix-matched calibration. Extracts or samples of blank matrix preferably of the same type as sample may be used for calibration purposes (SANTE 2017a).

The matrix effect can be observed in any type of detector. In specific detectors, such as flame photometric detector in gas chromatography or fluorescence in high performance liquid chromatography which are related to a relatively narrow specific chemical property or composition of the molecule, these effects can be minimal, but matrix effect should be evaluated in any case. Very dirty samples can cause signal enhancement or mask completely the analyte signal, for example in flame photometric detector when looking for trace organophosphate compounds as the emission due to the C-H bond of organic matter can be noticeable in the detector.

Matrix effects are classified as negligible when the value is less than 20%, medium between 20% and 50% and high when the value is above 50% (Kmellar et al. 2008). Sometimes matrix effect could be higher than 100%. The 20% of matrix effect can be assigned to the inherent uncertainty of pesticide residue analysis, and therefore, have no biased influence in the determination. As the matrix effects grows, the uncertainty of the identity of the detected analyte could also increase.

Residue identification in trace analysis using chromatography coupled to mass spectrometry is performed by the similarity ($\pm$ 0.1 s) of the retention time with respect to a given standard. Matrix matched calibrations can compensate for these effects, as for high load of co-extractives pesticide solutions, changes in retention times of the compounds are commonly observed.

The other identification criteria in trace analysis are that the ratio between qualifier and quantifier ions should not differ more than 30% when compared with the same relation determined in solvent. Matrix components can alter the ratio and therefore identification can be hampered. In some cases, matrix effect can be overcome by using exact mass measurements. The probability of wrongly assign an ion using an error mass of 5 ppm is below 5%.

Exact mass measurements also prevent in a high extent misleading identification due to co-elution of matrix components. The SANTE document, proposed that mass resolutions higher than 50,000 identify unambiguously any compound with a molecular weight below 600 Da, the common situation for pesticide molecules (SANTE 2015).

1.6.1 Matrix Effect in Gas Chromatography Analysis

Matrix effects can have distinctly sources in pesticide residue analysis. The most accepted one is the signal enhancement observed when an extract containing pesticide residues is injected in the gas chromatography. Signal enhancement is due to the minimization of analyte degradation when going through the inlet of the chromatograph by the extract components. The liner is usually not completely deactivated, although the glass surface has been silanized. The Si-OH groups that remained free react with the analytes at the hot temperature of the inlet, degrading them.

The extracts for pesticide residue analysis usually contain co-extractives of different chemical origin that reacts with silanol groups hampering pesticide degradation, acting as analyte protectants. Analyte protectants having a polyol structure have been proposed to be added to the working solutions, allowing to overcome the "liner degradation effect". Nevertheless, the compounds proposed do not achieve a proper protection of the late eluting compounds, such as the temperature sensitive pyrethroids. Thus, the protective effect of extracts can be rapidly lost, as the liner accumulates carbonized organic matter. When enough amount of "dirty" extracts has been injected, the degraded organic matter, react with the analytes giving poor defective peaks e.g. tailing, bad shape or variable area.

Fish extracts from QuEChERS procedures are very dirty. Although, in gas chromatography analysis, most of the analysts do not inject the acetonitrile extract as such and prefer to change the solvent prior injection to a less expansible in the vapor phase and polar ones, like ethyl acetate or isooctane, the co-extracted poly unsaturated fish lipids deposit and degrade quickly in the injector and the liner. The highly reactive peroxides formed, degrade more quickly the analytes.

The most commonly employed conventional detector in the determination of organochlorine and halogenated contaminants in fish is the electron capture detector due to the high sensibility and specificity of the electron capture detector (Ballesteros et al. 2014). One of the main drawbacks of electron capture detector is that can be easily inactivated by lipidic compounds in the matrix. Lipophilic compounds not necessarily co elute with the matrix co-extractives but are of paramount importance

as tend to deposit and passivate the detector. To keep the detector functioning at adequate levels of sensibility an exhaustive clean-up of the sample is required.

The traditional sample treatments include gel permeation chromatography procedures that have been actually miniaturized. Nowadays, combinations of absorbents such as reversed-phase octadecylsilane, Z-sep or the enhanced matrix removal of lipid adsorbent contribute to eliminate the triglycerides of the sample extract, allowing a better and longer detection performance.

As pointed out above, actually the routinely applied detectors in pesticide trace analysis, are the mass spectrometer, used either alone or in tandem. The most employed configurations are low resolution quadrupoles. High resolution configurations such as time of flight and Orbitrap instruments have up to now been scarcely applied to the analysis of pesticide residues in fish.

In gas chromatography mass spectrometry, the most common ionization mode is electronic impact at 70 eV. When using electronic impact ionization technique, isobaric matrix interferences could hamper the determination of some pesticides. Matrix effects in gas chromatography mass spectrometry have been described as low to medium ones in a recent publication (Colazzo et al. 2019). In this report 25 gas chromatography-amenable analytes showed matrix effect below 20%, in agreement with a previous study of pesticides residues in fish, performed using low pressure gas chromatography tandem mass spectrometry that showed negligible matrix effects for most of the analyzed compounds (Colazzo et al. 2019; Sapozhnikova and Lehotay 2013).

1.6.2 Matrix Effect in Liquid Chromatography Analysis

On the other hand, the matrix effects in pesticide residue analysis during liquid chromatography electrospray ionization tandem mass spectrometric determination arise in the ionization step. The matrix co-extractives compete with the analytes to reach the droplet surface. As a consequence, less analyte molecules are capable of ionize and the signal is lowered. The overall effect is called “signal suppression”. When lipophilic compounds are present tend to occupy the droplet surface lowering even more the possibility to the analytes of reaching the surface.

According to the literature, although the complexity of the fish matrix, and despite the difficulties at the injection port in gas chromatography that fish extracts showed, the matrix effects in liquid chromatography tandem mass spectrometry were negligible for more than 90% of the analytes when modification of the unbuffered QuEChERS version was employed to determine 65 currently used pesticides in freshwater fish (Colazzo et al. 2019).

Matrix effects in fish depend on the type of extract and the fish species. Extracts with high lipid content cause the most common problems during the determination. In order to avoid interference during the analysis, the use of specific absorbents such as reversed-phase octadecylsilane, zirconium dioxide and Z-sep, or freeze out techniques is desirable. From that point of view, the use acetonitrile as a solvent is

useful. On the other hand, acetonitrile has a relatively high polarity and cannot compete effectively with triglycerides, composed by highly unsaturated fatty acids, where the less polar compounds such as the organochlorine pesticides are trapped.

Although time consuming, the freeze out is particularly useful to eliminate the triglycerides. In order to be effective, lower temperatures than the usual −20 °C have to be reached. Dry ice or cryo-cooler systems that reach up to −70 °C, are advisable to be employed. Freeze out time is dramatically lowered when very low temperatures are reached, from 10 h or overnight to 15–30 min.

The type of extract and the whole sample preparation procedure are the clue to perform a proper determination of pesticide residues in fish. Some of the inherent limitations of the instrumental systems are not easy to overcome once the extract is injected into the chromatograph. Matrix effects are complex and needs a deeply evaluation on a case by case basis.

1.7 Occurrence of Pesticides in Fish

Monitoring studies on the occurrence of pesticides and other contaminants in fish have two distinctive goals. On one side, the general food safety concern on the widespread occurrence of toxic pollutants in the environment and the well-known capability of fish to bioaccumulate or biomagnified non-polar compounds. On the other hand, the use of aquatic wildlife for biomonitoring strategies at both, temporal and spatial scales (Pérez-Parada et al. 2018).

As stated above, major focus on the occurrence of persistent organic pollutants has been evidenced in seawater and freshwater species (Penland et al. 2018; Rose et al. 2015). Recent findings report pesticides in fish usually at μg/kg levels.

Environmental quality standards in biota have been defined for a bunch of persistent organic pollutant compounds. The standards were defined as the concentration below which no harmful effects are expected to wildlife or humans and applied as an approach for persistent organic pollutants monitoring (European Commission 2013).

Biota monitoring is particularly important in the case of hydrophobic substances that tend to accumulate in sediments or in the fat tissues of living organisms. Outcomes in large-scale monitoring of pollutants in fish evidenced the co-occurrence of organochlorine compounds, polychlorinated dibenzo p-dioxins and polychlorinated dibenzofurans, polychlorinated biphenyl compounds, polybrominated diphenyl ethers as well as perfluorinated compounds in European freshwater basins (Abalos et al. 2019; Picó et al. 2019).

Growing evidence in Spanish river basins showed a plethora of chemicals being accumulated in fish. However, an increasing relevance for perfluorinated compounds and ultraviolet filters occurring in fish state the significance of emerging freshwater pollution (Picó et al. 2019). Several persistent organic pollutants including dichlorodiphenyltrichloroethane and hexachlorocyclohexane congeners were found to occur in remote areas such as in Antarctic or Arctic areas. Organochlorine pesticides were

found in Antarctic notothenioid fish species (Lana et al. 2014). Distribution of these persistent organic pollutants among different tissues such as muscle, liver, gonads, and gills, has evidenced the role of lipid content on the pattern of persistent organic pollutants distribution in tissues. Authors showed that while gonads showed higher levels of dichlorodiphenyltrichloroethane and hexachlorohexane, the most significant polybrominated diphenyl ethers flame retardant concentrations were recorded in gills (Lana et al. 2014).

Similarly, recent reports showed levels of dichlorodiphenyltrichloroethane, hexachlorohexane and hexachlorobenzene in Arctic fish with economic importance such as *Gadus morhua* and *Melanogrammus aeglefinus* in Barens Sea, Norway. The authors showed an apparent decrease from 1992 to 2015 period over the persistent organic pollutant levels found in these fishes where the concentrations decrease from higher than 100 μg/kg in 90's to 20 μg/kg in 2015. However, the authors questioned the possibility of removing the pollutants from the marine environment in the foreseeable future, considering the permanent leakage from continents and the fact that are still being produced in some parts of the world (Boitsov et al. 2019).

Concerning freshwater resources, organochlorine levels were recently studied in muscle and liver tissues from fish at central Andes streams in Argentina. *Oncorhynchus mykiss* and *Odontesthes bonariensis* fish species were found having different persistent organic pollutant but mainly dichloro-diphenyl-trichloroethane congeners rounding 500 μg/kg levels at lipid weight. Hexachlorohexane, chlordane and hexachlorobenzene were also found but in a lower extent (Ríos et al. 2019). The authors positively correlated the lipid content of target tissues to the uptake levels of organochlorine pesticides highlighting the relevance of fat content in tissue (Ríos et al. 2019).

As seen, most knowledge is currently evidenced for compounds with proven bioaccumulation ability exposed by larger n-octanol-water partition coefficient values for most organochlorine compounds. Recent findings underline the relevance of new families of chemicals n-octanol-water partition coefficient lower around 3; such as S-metolachlor an herbicide, the insecticide chlorpyrifos or trifloxystrobin fungicide (Ernst et al. 2018; Picó et al. 2019). However, other pesticides such as carbendazim having a n-octanol-water partition coefficient close to 1 were found in freshwater fish.

Table 1.4 shows relevant reports of currently used pesticides and persistent organic pollutant in freshwater fish. As stated, the advent of liquid chromatography tandem mass spectrometry monitoring enabled an increase in the scope of analytes among different pesticides occurring in different fish tissues. Many reports state the occurrence of organic pollutants at trace levels with very low n-octanol-water partition coefficient (Ernst et al. 2018; Picó et al. 2019; Rojo et al. 2019). In addition, currently used pesticides have been reported in freshwater fish with different feeding and migratory habits over a wide range of concentrations worldwide (see Table 1.4). The Olmstead-Tukey diagram indicates an easy way for contaminant classification among levels found and frequency of detection for a set of samples. The diagram enables to categorize among dominant, frequent, occasional and rare compounds between the reported analytes, Fig. 1.5 (Ernst et al. 2018).

Table 1.4 Summary of recent findings of pesticide residues in freshwater fish species

Water course	Fish specie	Detected pesticides	Concentration range in fish muscle (μg/kg) wet basis	References
Argentina. Mendoza River and Yaucha River at Andes region	*Oncorhynchus mykiss* *Odontesthes bonariensis*	OCs (mainly DDT, HCH, and HCB)	<LOQ to 614	Ríos et al. (2019)
Argentina: Pergamino River	*Jenynsia multidentata*	17 CUPs (mainly fenvalerate and bifenthrin)	<LOQ to 1500	Brodeur et al. (2017)
Argentina: La Peregrina Lake	*Odontesthes bonariensis* *Oligosarcus jenynsii* *Cyphocharax voga*	OCs (mainly endosulfan)	<LOQ to 26	Barni et al. (2016)
Australia: Rivers at Southeast Queensland	*Gambusia holbrooki*	5 CUPs (mainly chlorpyrifos, atrazine and linuron)	<LOQ to 54	Scott et al. (2018)
India: Rivers at Andhra Pradesh and Kerala regions	*Pangasianodon hypophthalmus*	6 CUPs (parathion-methyl, malathion, chlorpyrifos, fipronil, quinalphos, etofenprox) and 2 OCs (p,p′-DDE, p,p′-DDT)	<LOQ to 473	Chatterjee et al. (2015)
China: Pearl River and Dongjiang River	*Cirrhinus molitorella* *Tilapia nilotica* *Hypostomus plecostomus* *Channa argus* *Channa asiática* *Monopterus albus*	OCs (mainly β-HCH and p,p′ DDE)	14–5560	Sun et al. (2018)
Poland: Biebrza River and Narew River	*Perca fluviatilis* *Leuciscus aspius* *Tinca tinca* *Sander lucioperca* *Abramis brama* *Carassius carassius* *Rutilus rutilus* *Blicca bjoerkna* *Lota lota*	S-metolachlor and OCs (DDTs and HCHs)	<LOQ to 14	Kaczynski et al. (2017)
Spain: Guadalquivir, Júcar, Ebro and Llobregat Rivers	*Luciobarbus sclateri* *Cyprinus carpio* *Salmo trutta* *Gobio lozanoi* *Pseudochondrostoma polylepis* *Micropterus*	25 CUPs (mainly chlorpyrifos)	<LOQ to 840[a]	Picó et al. (2019)

(continued)

Table 1.4 (continued)

Water course	Fish specie	Detected pesticides	Concentration range in fish muscle (μg/kg) wet basis	References
	salmoides *Barbus guiraonis* *Lepomis gibbosus* *Alburnus alburnus* *Anguilla anguilla* *Esox lucius* *Barbus graellsii* *Silurus glanis*			
Uruguay: Uruguay River and Negro River	*Hoplias malabaricus* *Rhamdia quelen* *Pimelodus maculatus* *Paraloricaria vetula* *Hypostomus commersonni* *Salminus brasiliensis* *Megaleporinus obtusiden,* *Prochilodus lineatus*	30 CUPs (mainly metolachlor, pyraclostrobin, trifloxystrobin)	<LOQ to 194	Ernst et al. (2018)
United States: Yadkin-Pee Dee River	*Anguilla rostrata* *Ictalurus furcatus* *Lepomis macrochirus* *Ictalurus punctatus* *Cyprinus carpio* *Micropterus salmoides* *Moxostoma collapsum* *Moxostoma macrolepidotum* *Ictiobus bubalus* *Cyprinella nivea*	OCs (β-HCH and heptachlor epoxide)	<LOQ to 49	Penland et al. (2018)

LOQ limit of quantification, *CUPs* currently used pesticides, *DDT* dichloro-diphenyl-trichloroethane, *HCH* hexachlorocyclohexane, *HCB* hexachlorobenzene, *OCs* organochlorine pesticides, *p,p′-DDE*: dichlorodiphenyldichloroethylene

[a]Report on dry basis (dry weight, dw)

Active biomonitoring approaches using caged fish has been reported as strategy for freshwater monitoring in agricultural areas. Pesticides identified in fish tissues, particularly liver, has been reported and correlated with different biological markers of endosulfan exposure (Vieira et al. 2019). Perspectives in monitoring approaches are focusing the development of non-target screening in different fish tissues that will lead to an increase in evidence of detailed exposure and metabolism of these pollutants.

Some analytical issues should be considered in large scale analysis. Due to the high n-octanol-water partition coefficient of persistent organic pollutant, partition

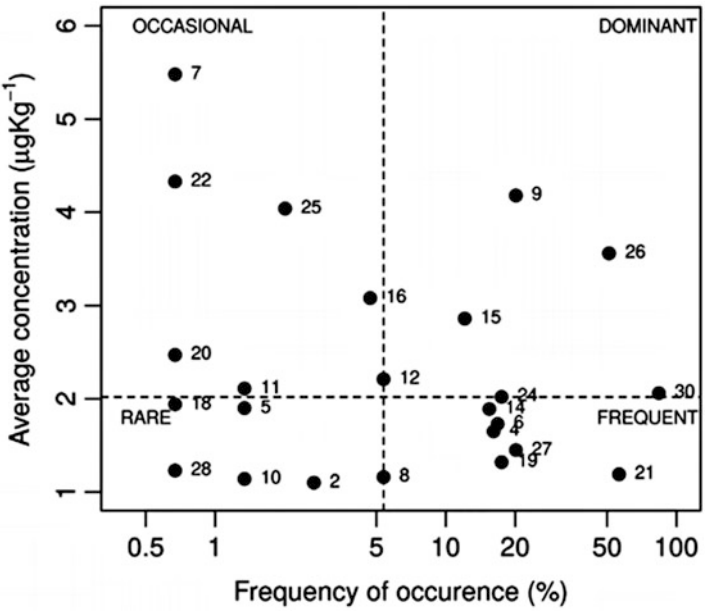

Fig. 1.5 Olmstead-Tukey diagram for the pesticides found at sampling sites. Frequency of occurrence in percentage vs the average concentration in µg/kg of the quantified pesticides for the whole data set. (Reprinted with permission of (Science of the Total Environment, Elsevier B.V) from Ernst et al. 2018)

with acetonitrile is sometimes not enough to extract them properly from the fatty portions of the samples. Some approaches using acetone or toluene as extraction solvent followed by gel permeation chromatography to clean-up the lipidic fraction have been used for monitoring programs seeking the good maintenance of the chromatographic equipment (Ballesteros et al. 2014; Miglioranza et al. 2013).

An interesting study of contaminant occurrence in fish in a salty lake showed that after the toxicological evaluation of organochlorine pesticides levels found the acceptable daily intake for endosulfan and polychlorinated biphenyl compounds were largely overpassed in fish from "La Chiquita" Lake in Argentina (Ballesteros et al. 2014).

Other possible aim of monitoring studies regarding fish is to evaluate the aquatic environment and the effect of the surrounding land. Different approaches for monitoring studies of aquatic environments using fish as target organisms should be performed. If the interest lies on the effect of contaminants on biota, field studies provide a richer information on the effect of combined stressors that otherwise cannot be properly evaluated under laboratory conditions.

Water quality can be assessed performing cage trials with small fishes, like "madrecitas" *Cnesterodon decemmaculatus*, that act as samplers of different chemicals present in the water streams. The strategy has the advantage of performing a selective water "sampling" for weeks. Figure 1.2 shows the experimental design of

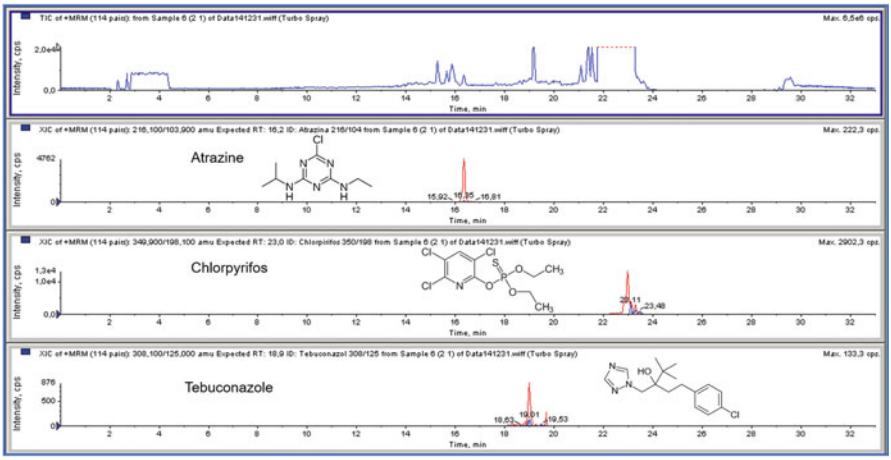

Fig. 1.6 Extracted ion chromatogram obtained by liquid chromatography tandem mass spectrometry in multi reaction monitoring mode of a *Luciopimelodus pati* sample from Uruguay River. The chromatogram indicates the presence of three pesticides, atrazine, chlorpyrifos and tebuconazole in a concentration range 0.1–30 μg/kg

such a study, performed at the Cañada del Dragón, in Montevideo, Uruguay, in a traditional farming region. After analyzing the whole *C. decemmaculatus* exposed to the muddy waters of the stream during 3 weeks of experiment for persistent organic pollutants determination, dichloro-diphenyl-dichloroethylene was detected (Pareja et al. 2013). The study of fish from different agroecosystems in Uruguay showed the presence of pesticide residues directly related to the agrochemicals used in different crops (Pareja et al. 2013) evidencing the suitability of fish as environmental biomonitors of aquatic systems surrounding farm activities. Moreover, a study of 50 fish samples from the Uruguay River reported that atrazine, chlorpyrifos and tebuconazole were the most frequently found pesticides, in a concentration range between 0.1 and 30 μg/kg. Figure 1.6 shows the extracted ion chromatograms of a positive sample in which atrazine, chlorpyrifos and tebuconazole were detected. Interestingly, some pesticides supposed having rather short half-life in water and soil accumulate in fish bodies. Despite trifloxystrobin is not one of the most employed fungicides in Uruguay and that the soil half-life is less than 2 days, the fungicide was found in 84% of the analyzed samples (Pérez-Parada et al. 2018).

1.8 Conclusions

Currently, monitoring of environmental pollutants such as pesticide residues in fish is of great concern for ensuring food safety and evidence environmental fate of semi-polar and non-polar pesticides in aquatic media.

Fish has shown a high potential as a biomonitor of the aquatic environment even at basin level. Compounds that are not supposed to be found in freshwater have been detected accumulated in fish. The relevance of the findings to fish health is still unknown.

Despite the fact that there are several multi-residue methodologies for pesticide residues, multi-class or multi-residue methods for highly polar compounds are needed for broadening exposure surveillance. The trend for the multi-class analysis is to develop environmentally friendly and cheap techniques capable to determine in one-sample preparation step different types of contaminants at the lowest possible concentrations.

Although state-of-the-art for pesticide residues determination in fish is based on target screening, metabolites are commonly present in the environmental compartments and therefore the determination of the transformed products in fish should also be considered. Therefore, non-target analysis particularly dedicated to organic multi-contaminants, metabolites and possible degradation products of pesticides with toxicological relevance using high-resolution mass spectrometry is expected to be incorporated soon in order to bring a better knowledge on the chemical pollution of the aquatic environment.

Further research is needed to spread analytical outcomes aiming potential hazards and ecotoxicological effects characterization.

The challenge is to face the analysis of pollutants whose relevance for the environment health is unknown. Pesticide watch lists elaborated by regulatory authorities are needed to preserve water bodies and implement regulations that enable environmental and public health protection as well as studies looking for the implementation of maximum residue levels for extraneous substances in fish.

Non-target analysis is a trend in environmental chemistry but is also of enormous importance in food security and nutritional capacity evaluation, as fish is one of the natural resources with relevance for the world population. So far, high resolution mass spectrometry techniques have been implemented in several environmental matrices such as wastewater and surface water. The use of similar protocols to analyze marine and freshwater fish will open new and exciting fields for future research.

Finally, the global maximum residue limits settlement for fish consumption to ensure food safety is missing. Maximum residue limits are associated with good agriculture practice accomplishment, but the findings of residues in fish from agricultural pesticides cannot be linked to any possible good agriculture practices. However, the risk for consumers health cannot be avoided and is desirable to expand the concept of extraneous-maximum residue limits suggested by the *Codex Alimentarius* to current used pesticides, within realistic risk assessment studies. The new regulatory frameworks will surely foster the development and expansion of pesticide residue analysis in fish, reaching new analytical advances, better understanding of the pesticide uptake and effect on fish and the discovering new horizons to broad the scope food safety science.

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