UNIVERSITY OF MILAN,
FACULTY OF VETERINARY MEDICINE

Customer Application Note 122

Determination of Persistent Organic Pollutants in Fish Tissues by Accelerated Solvent Extraction and GC-MS/MS

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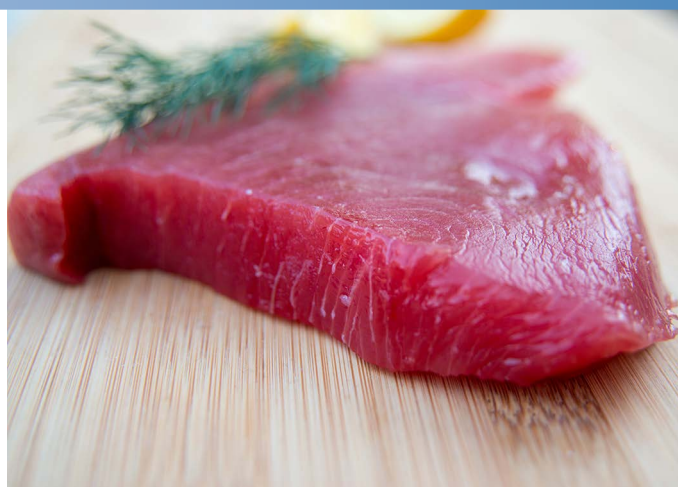
Key Words
Pressurized Fluid Extraction, Polychlorinated Biphenyls, Organochlorine Pesticides, Polybrominated Diphenyl Ethers, In-Line Clean Up, Rocket Evaporator, TSQ 8000 Triple Quadrupole GC-MS, Xcalibur, TraceFinder

Goal
To demonstrate an accelerated solvent extraction and GC-MS/MS procedure for persistent organic pollutants in fish tissues



Introduction
Polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs) belong to a broad family of synthetic organic compounds known as halogenated hydrocarbons. PCBs were domestically manufactured from 1929 until their manufacture was banned in 1979. They have a range of toxicity and vary in consistency from thin, light-colored liquids to yellow or black waxy solids. Due to their non-flammability, chemical stability, high boiling point, and electrical insulating properties, PCBs were used in hundreds of industrial and commercial applications including electrical, heat transfer, and hydraulic equipment; as plasticizers in paints, plastics, and rubber products; in pigments, dyes, and carbonless copy paper; and many other industrial applications. OCPs were widely used as insecticides throughout the 1950s and 1960s until their use was banned in Western countries in the 1970s. Polybrominated diphenyl ethers (PBDEs) are a class of compounds that have recently emerged as a major environmental pollutant. PBDEs are used as a flame-retardant and are found in consumer goods such as electrical equipment, construction materials, coatings, textiles, and polyurethane foam (furniture padding). Several nations have recently banned PBDEs and also introduced legislation that bans the sale of certain products containing PBDEs. Furthermore, based on the recommendation of the European Food Safety Authority (EFSA), the European Commission has asked member states to monitor the presence of PBDEs in food over the next two years. The capacity of the halogenated hydrocarbons to bioaccumulate in fatty tissues and biomagnify up the food chain, in combination with their resistance to degradation and their toxicity, make this class of chemicals a serious threat to environmental and human health.

Techniques such as Soxhlet and sonication are used for the extraction of halogenated hydrocarbons from food and environmental samples prior to their analytical determination. These techniques are, however, very labor intensive and suffer from high solvent consumption. Accelerated solvent extraction was developed to meet the new requirements of increased throughput and reduced solvent usage in sample preparation. With accelerated solvent extraction, extractions can be completed in very short periods of time and with minimal solvent as compared to conventional sample extraction techniques such as Soxhlet and sonication. Furthermore, interferences may be extracted along with desired analytes during those conventional extraction processes. These unwanted co-extractables can cause buildup of nonvolatile materials on the GC injection port and the analytical column resulting in poor analytical results and high instrument maintenance costs. For example, gel permeation chromatography (GPC) is often used as a post extraction clean-up for fish and meat tissues prior to analysis for halogenated compounds such as PCBs, OCPs, and PBDEs. However, one main disadvantage of the GPC systems is that it is difficult to remove all lipids. The remaining traces of lipids

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have to be removed in a second clean-up procedure, e.g., on an additional silica column or by a second GPC step. Additionally, challenges remain with high lipid content in which lipophilic pesticides may remain in the fatty layer even after the extraction. Recent advances using accelerated solvent extraction systems, as described in several publications,[1–19] include procedures for selective removal of interferences during sample extraction, thus combining extraction and purification into a single step.

The method reported here is applicable for the determination of 29 halogenated hydrocarbons (6 PCBs, 16 OCPs, and 7 PBDEs) in fish tissues. The concentration ranges are 1–100 ng/g for PCBs, 0.5–10 ng/g for PBDEs, and 5–1000 ng/g for OCs.

Experimental

Sample Collection

A total of 79 bluefin tuna (*Thunnus thynnus*) originating from different Food and Agriculture Organization (FAO) catch areas were selected for this study (Table 1). All tuna samples were provided by the main Italian tuna industry and by the fish market of Milan. Representative samples from each fish were obtained by sampling tissue from three different anatomic zones (proximal, ventral, and caudal); each sample was then stored at -22 °C until its analysis.

Table 1. Number of bluefin tuna samples from each FAO catch area.

Number of Samples	FAO Catch Area	Geographical Origin
20	51	Indian Ocean, Western
20	71	Pacific Ocean, Western Central
20	34	Atlantic Ocean, Eastern Central
19	37	Mediterranean Sea

Equipment

A Radwag analytical balance was used for weighing the fish tissues. The extractions were carried out using a Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extractor (P/N 083114 (120 V) or 083146 (240 V), shown in Figure 1A, equipped with 34 mL stainless steel extraction cells. The extracts were collected in 60 mL vials (Thermo Scientific, P/N 048784), treated with sodium sulfate and directly concentrated in a 2 mL autosampler glass vial (Thermo Scientific Chromacol VAGK ISP: GC 2-SVW + 9-SCK(B)-ST1) with the Genevac Rocket Evaporator (P/N 075904 (120 V) or 082766 (240 V) (Figure 1B). The samples were analyzed using a Thermo Scientific™ TRACE™ 1310 Gas Chromatograph equipped Split/Splitless injector, a fused-silica capillary column (Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane, 35 m × 0.25 mm × 0.25 µm, from Restek) and a Thermo Scientific™ TSQ™ 8000 Triple Quadrupole GC-MS/MS.

Chemicals and Reagents

A solution of PCBs congeners (PCB 28; PCB 52; PCB 101; PCB 138; PCB 153 and PCB 180), PCB 209 (internal standard for PCBs), solution of PBDEs (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 154) and FBDE, and an internal standard (IS) for flame retardants, were purchased from AccuStandard (New Haven, USA). Standard solution of 16 OCs (α-HCH; Hexachlorobenzene; β-BHC; Lindane; Heptachlor; Aldrin; Heptachlor epoxide; Trans Chlordane; 4,4'-DDE; Endosulfan I; 2,4'-DDT; Endrin; 4,4'-DDD; Endosulfan II; 4,4'-DDT and Endosulfan sulfate) was purchased from Restek (Bellefonte, PA, USA). Silica gel 60 (0.063–0.200mm) was purchased from Merck (Darmstadt, Germany). Hexane, isooctane, acetone (special grade for pesticide residue analysis (Pestanal)) and 4-nonylphenol (IS for OCs) were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Working solutions were prepared by diluting the stock solution in hexane and then storing at –40 °C. The mixed compound calibration solution, in hexane, was prepared from the stock solutions and also used as a spiking solution (10 µg/mL).

Figure 1. Dionex ASE 350 accelerated solvent extractor (A) and Genevac Rocket Evaporator (B).

Extraction, Concentration, and Measurement

A cellulose filter (Thermo Scientific, P/N 056780) was placed in the bottom of a 34 mL extraction cell (Figure 2), followed by 10 g of activated silica gel and another cellulose filter. A representative portion of tuna (300 g) was obtained from each fish and minced. A 3 g sample was homogenized with an equal weight of Thermo Scientific Dionex ASE Prep DE (Thermo Scientific, P/N 062819) and sodium sulfate and transferred into the cell. Into this mixture, 1.0 mL isooctane solution containing the three internal standards was added. The remaining empty volume was filled with Thermo Scientific™ Dionex™ ASE™ Prep DE. The Accelerated Solvent Extractor was programmed according to the method conditions listed below.

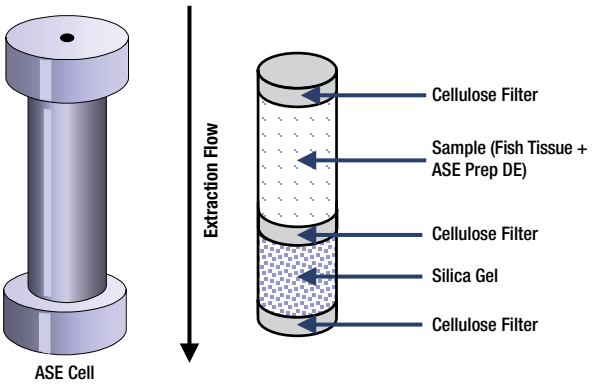

Figure 2. Extraction cell schematic.

Conditions for Accelerated Solvent Extraction	
Solvent	n-Hexane/Acetone (4:1, v/v)
Temperature	80 °C
Pressure	1500 psi
Static Cycles	3
Static Cycle Time	10 min
Rinse Volume	90%
Purge Time	90 s
Total Extraction Time per Sample	~ 40 min
Total Solvent Volume per Sample	~ 40 mL

The extracts were collected in 60 mL vials and treated with sodium sulfate to remove any possible humidity. After filtration, the organic phase was concentrated to dryness in the Rocket Evaporator, dissolved in 200 µL of isooctane, and submitted to analysis by GC-MS/MS. The GC conditions are summarized below.

GC and Injector Conditions	
Split/Splitless Injector	
Injector Temperature	250 °C
Liner	2 x 2.75 x 120mm
Injected Volume	1 µL
Splitless time	0.5 min
Splitflow	10 mL/min
Surge Pressure	5 kPa
GC Program	
GC Column	Rt-5MS (35 m x 0.25 mm x 0.25 µm)
Carrier Gas	Helium, 99.999% purity
Flow Rate	1.0 mL/min, constant
Initial Temperature	80 °C (3 min)
	10 °C/min to 170 °C
	3 °C/min to 195 °C
	2 °C/min to 240 °C
	3 °C/min to 280 °C
	10 °C/min to 310 °C
Final Temperature	310 °C (5 min)

Mass Spectrometer Parameters	
Source Temperature	250 °C
Ionization	EI
Electron Energy	70 eV
Emission Current	50 µA
Q2 Gas Pressure (Argon)	1.5 mTorr
Collision Energy	10 to 30 eV
Q1 Peak Width FWHM	0.7 Da
Q3 Peak Width FWHM	0.7 Da

The monitored selected reaction monitoring (SRM) transitions for PCBs, PBDEs and OCs are given in Tables 2–4. The star indicates the quantifier ion.

Table 2. SRM transitions for PCBs.

PCB #	Compound Name	Molecular Formula	Retention Time	Nominal Mass	Exact Mass	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
28	2,4,4'-Trichlorobiphenyl	$C_{12}H_7Cl_3$	18.76	258	255.9613	256	**186***	20
						258	186	25
52	2,2',5,5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$	20.25	292	291.9194	292	**222***	25
						292	257	10
101	2,2',4,5,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$	24.46	326	325.8804	324	254	25
						326	**256***	25
						328	256	25
138	2,2',3,4,4',5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$	28.99	361	359.8415	360	**290***	25
						360	325	10
153	2,2',4,4',5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$	30.25	361	359.8415	360	**290***	20
						360	325	30
180	2,2',3,4,4',5,5'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$	34.06	395	393.8025	394	**324***	25
						394	359	10
						396	324	25

Table 3. SRM transitions for PBDEs.

PBDE #	Compound Name	Molecular Formula	Retention Time	Nominal Mass	Exact Mass	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
28	2,4,4'-Tribromodiphenyl Ether	$C_{12}H_7Br_3O$	27.95	407	405.80266	246	139	10
						248	**139***	10
						408	248	10
33	2',3,4-Tribromodiphenyl Ether	$C_{12}H_7Br_3O$	28.05	407	405.80266	246	139	30
						248	**139***	30
						406	246	10
47	2,2',4,4'-Tetrabromodiphenyl Ether	$C_{12}H_6Br_4O$	34.34	486	485.7111	326	217	30
						328	219	30
						484	**326***	30
99	2,2',4,4',5-Pentabromodiphenyl Ether	$C_{12}H_5Br_5O$	38.17	565	563.6216	410	297	30
						406	294	30
						564	**404***	20
100	2,2',4,4',6-Pentabromodiphenyl Ether	$C_{12}H_5Br_5O$	39.05	565	563.6216	410	297	30
						406	297	30
						564	**404***	10
153	2,2',4,4',5,5'-Hexabromodiphenyl Ether	$C_{12}H_4Br_6O$	40.88	644	643.5301	484	377	25
						642	**482***	10
154	2,2',4,4',5,6'-Hexabromodiphenyl Ether	$C_{12}H_4Br_6O$	41.76	644	643.5301	484	324	30
						486	326	30
						644	**484***	20

Table 4. SRM transitions for OCPs.

Compound Name	Molecular Formula	Retention Time	Nominal Mass	Exact Mass	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
α-HCH	$C_6H_6Cl_6$	15.27	297	295.8772	181	**145***	10
					181	146	10
					219	183	10
Hexachlorobenzene	C_6Cl_6	15.45	285	283.8102	284	**249***	20
					286	214	30
					286	251	20
β-HCH	$C_6H_6Cl_6$	16.69	297	295.8772	181	**145***	10
					183	148	10
					219	183	10
Lindane (γ-HCH)	$C_6H_6Cl_6$	16.44	297	295.8772	181	**145***	10
					183	145	10
					219	183	10
Heptachlor	$C_{10}H_5Cl_7$	19.27	373	371.8181	272	**237***	10
					274	237	10
					274	239	10
Aldrin	$C_{12}H_8Cl_6$	20.84	365	363.8728	261	**191***	30
					263	193	30
					265	193	30
Heptachlor epoxide	$C_{10}H_5Cl_7O$	22.77	389	387.8130	353	**263***	10
					353	282	10
					355	265	10
trans-Chlordane	$C_{10}H_6Cl_8$	23.96	410	409.7919	373	264	20
					373	**266***	20
					375	266	20
Endosulfan I	$C_9H_6Cl_6O_3S$	24.64	407	405.8139	373	**266***	20
					375	266	20
					377	268	20
pp'-DDE	$C_{14}H_8Cl_4$	25.96	318	317.9351	246	**176***	30
					248	176	30
					328	248	20
Endrin	$C_{12}H_8Cl_6O$	27.06	381	379.8677	245	173	30
					263	**193***	30
					281	245	10
Endosulfan II	$C_9H_6Cl_6O_3S$	27.65	407	405.8139	195	**159***	10
					241	206	10
pp'-DDD	$C_{14}H_{10}Cl_4$	28.18	320	319.9507	235	**165***	20
					237	165	20
op-DDT	$C_{14}H_9Cl_5$	28.27	354	353.9117	235	**165***	20
					237	165	20
Endosulfan sulfate	$C_9H_6Cl_6O_4S$	29.88	423	421.8088	272	**237***	10
					274	237	10
					274	239	10
pp'-DDT	$C_{14}H_9Cl_5$	30.36	354	353.9117	235	**165***	20
					237	165	20

Results and Discussion

The proposed method was optimized for the multi-residue analysis of 29 persistent organic pollutants (POPs). Total ion current chromatograms (GC-MS/MS) of tuna spiked with POPs and a naturally contaminated fish sample are shown in Figures 3 and 4. The optimization of the MS/MS method consisted of:

(1) Acquisition of respective MS spectra in full-scan mode (*m/z* 100–1000 mass range)

(2) Selection of precursor ions

(3) Product ion scans at different collision energies (10, 20, and 30 eV)

(4) Final tuning of the collision energy in selected reaction monitoring mode.

For each compound, two MS/MS transitions were chosen to fulfill the generally applied identification criteria: according to SANTE 2015 (guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed), one precursor ion with two product ions or two precursor ions with one product ion should be available for unbiased identification of the target analyte. An overview of the quantitative and confirmation MS/MS transitions

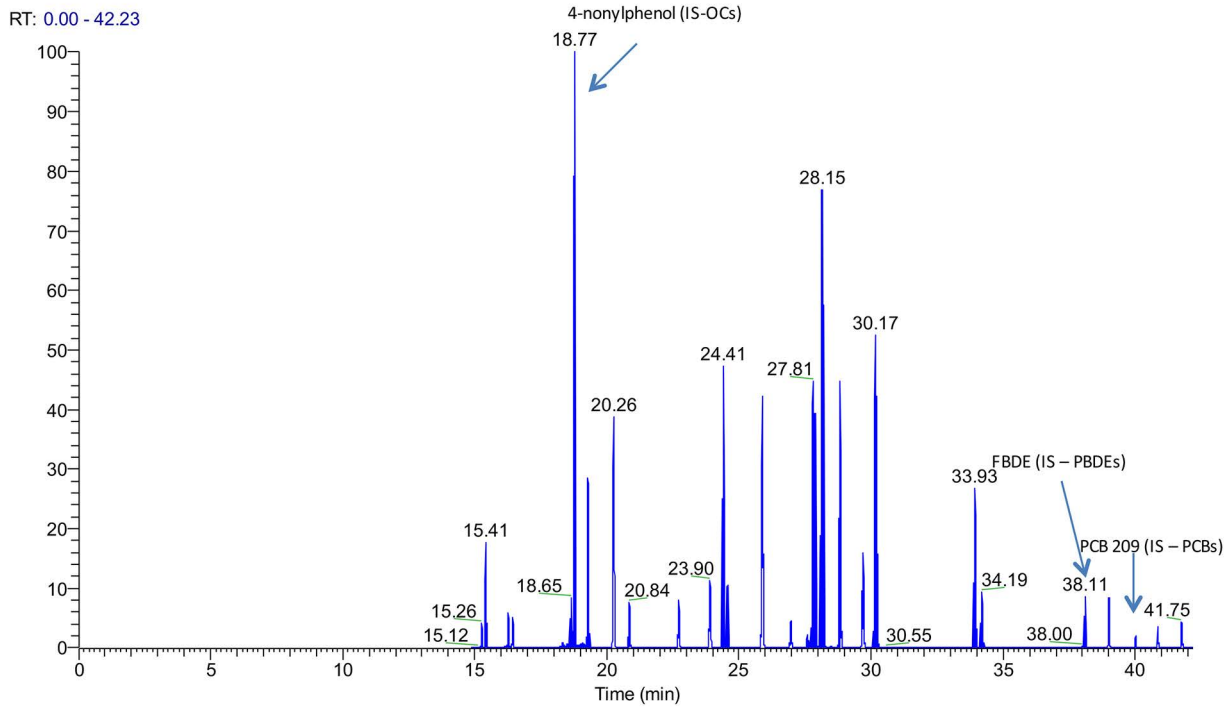

Figure 3. Total ion current (GC-MS/MS) chromatogram of tuna spiked sample.

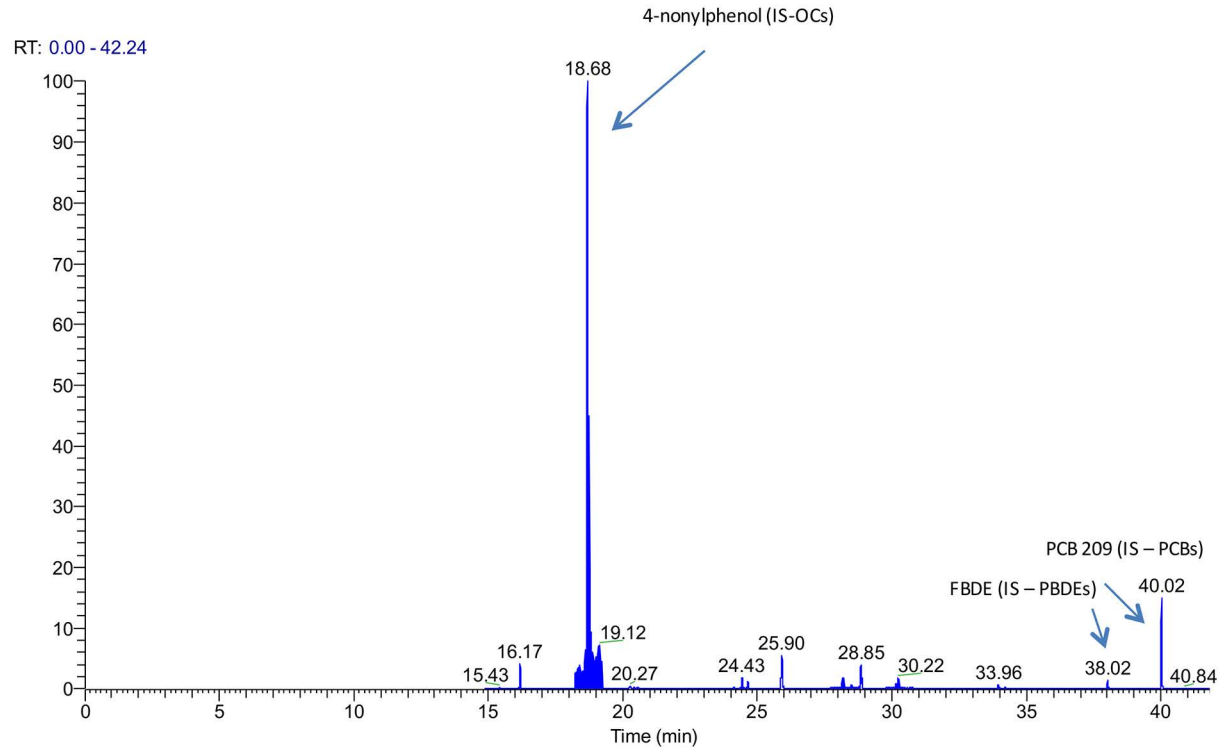

Figure 4. Total ion current (GC-MS/MS) chromatogram of raw fish sample.

and the collision energies selected for each compound in EI mode is given in Tables 2–4. In general, MS/MS allows for minimal matrix component interferences, and at the same time, due to the possibility of selecting suitable precursor and product ions, makes possible identification and quantification of the above-mentioned contaminants even at (ultra)trace concentrations. Notwithstanding that a highly selective triple quadrupole mass spectrometer is used, because GC-MS instruments are generally rather intolerant of non-volatile matrix impurities, the choice of an appropriate sample preparation strategy is also important to avoid poor ionization, background noise, and contamination of the whole GC-MS system. All results obtained confirm the efficacy of the present method for the determination of multiresidue pollutants in fish tissue.

The method showed a good linearity with coefficients of determination equal to or higher than 0.99 for all the compounds investigated, as well as good repeatability, confirming the present method as useful to monitor compounds belonging to different chemical classes (Table 5). The recoveries ranged from 108–119% for PCBs, from 91–102% for PBDEs, and from 75–96% for OCs. The CVs ranged from 4–14%. The one-step accelerated solvent extraction method using silica as fat retainer is both rapid and cost-effective and minimizes waste generation compared to the classic methods. The time required in the laboratory is reduced 50% by combining the extraction and the two clean-up steps (i.e., GPC and SPE) in one single accelerated solvent extraction step, thus doubling the number of samples that can be analyzed per day.

Table 5. Recoveries (%, RSD), LOD, LOQ, and coefficient of determination (r^2).

Contaminants	LOD (ng g^{-1})	LOQ (ng g^{-1})	Recovery % (RSD)	Coefficient of Determination (r^2)
Polychlorobiphenyls (PCBs)				
PCB 28	0.08	0.24	102 (7)	0.9994
PCB 52	0.07	0.21	103 (7)	0.9999
PCB 101	0.04	0.12	97 (4)	0.9999
PCB 138	0.05	0.15	105 (4)	0.9999
PCB 153	0.02	0.06	102 (4)	0.9999
PCB 180	0.06	0.18	98 (9)	0.9999
Polybrominated Diphenyl Ethers (PBDEs)				
PBDE 28	0.01	0.03	100 (9)	0.9991
PBDE 33	0.02	0.06	98 (9)	0.9999
PBDE 47	0.02	0.06	97 (8)	0.9996
PBDE 99	0.03	0.09	102 (7)	0.9998
PBDE 100	0.01	0.03	103 (7)	0.9998
PBDE 153	0.03	0.09	97 (10)	0.9992
PBDE 154	0.02	0.06	100 (12)	0.9999
Organochlorines (OCs)				
α-HCH	0.99	2.97	78 (10)	0.9959
Hexachlorobenzene	1.26	3.78	80 (12)	0.9945
β-HCH	1.17	3.51	85 (12)	0.9995
Lindane (γ-HCH)	0.79	2.39	96 (10)	0.9985
Heptachlor	0.95	2.84	93 (12)	0.9996
Aldrin	0.85	2.55	75 (14)	0.9991
Heptachlor epoxide	0.91	2.73	77 (14)	0.9994
trans-Chlordane	1.48	4.44	92 (10)	0.9993
Endosulfan I	1.13	3.38	80 (13)	0.9992
pp'-DDE	0.85	2.55	97 (12)	0.9994
Endrin	0.99	2.98	88 (11)	0.9998
Endosulfan II	1.14	3.42	90 (10)	0.9993
pp'-DDD	0.91	2.74	87 (14)	0.9986

All the PCBs investigated were detected in all tuna samples (Figure 5), with the exception of PCB 153, which was always present in FAO area 37, but detected in only five samples from the other three areas (two in FAO area 34 and three in FAO area 51). The concentrations of PCBs in the samples from FAO area 37 were much higher than those from the other three areas; in fact, they ranged from 0.535 to 69.7 ng/g, while in the other areas they ranged from 0.0331–1.52 ng/g. As it is a semi-closed basin, the Mediterranean Sea has limited exchange with the open ocean, facilitating the accumulation of these pollutants.

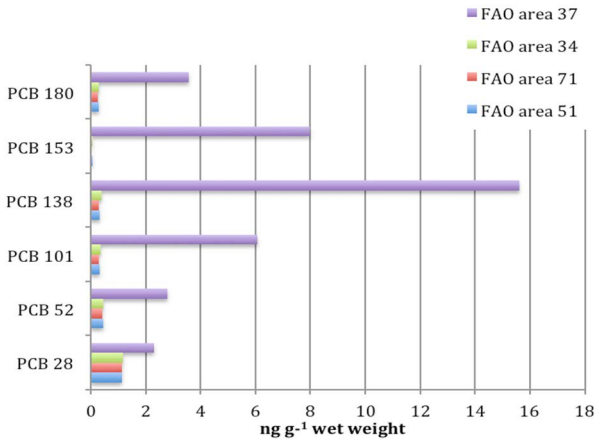

Figure 5. Spatial distribution of PCBs among FAO catch areas.

Moreover, in FAO area 37, PCBs 101, 138, 153, and 180 were at higher concentrations compared to PCB 28 and PCB 52; the abundance of these congeners is consistent with their molecular structure, their high prevalence in technical mixtures, and their high lipophilicity, stability, and persistence, which facilitate adsorption to sediments and accumulation in the aquatic ecosystem.

In examining PBDEs, the 47, 100, and 154 congeners were detected in all samples with concentrations between 0.415 pg/g and 6.29 ng/g; PBDE 99 and PBDE 153 were found in FAO area 51 and FAO area 37, while the remaining congeners (28 and 33) were detected only in FAO area 37 (Figure 6). These data show that, as with PCBs, all the PBDEs investigated were detected in the Mediterranean Sea, probably for the aforementioned reasons.

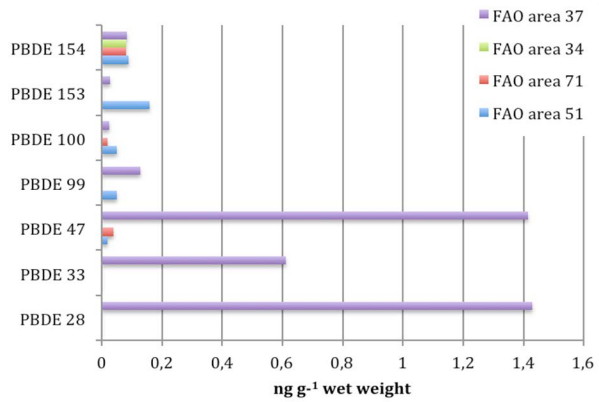

Figure 6. Spatial distribution of PBDEs among FAO catch areas.

Results of the OCP analysis showed that only five compounds were detected in tuna samples. Endosulfan sulfate was detected in all FAO areas, with a concentration of about 7 ng/g in each area, and a prevalence between 65–89%. Endrin was present in FAO areas 51, 71, and 34, with concentrations ranging from 1.71–39.0 ng/g and frequency from 5–30%.

Summary

An analytical method was developed and applied to evaluate POP residues in tuna samples from different FAO areas. The method proved to be simple and rapid, requiring small sample sizes and minimizing solvent consumption, due to use of accelerated solvent extraction with an in-line clean up step. Detection via MS/MS provides both quantitative information and confirmation of POP residues in tuna, confirming that the one-step accelerated solvent extraction method is a valid faster alternative to classic extraction methods because the analytical quality is comparable.

Acknowledgments

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