RESIDUES AND TRACE ELEMENTS

Rapid Method for the Determination of Organochlorine Pesticides and PCBs in Fish Muscle Samples by Microwave-Assisted Extraction and Analysis of Extracts by GC-ECD

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A procedure for the multiresidue determination of organochlorine pesticides and polychlorinated biphenyls in fish muscle samples has been developed. The method is based on the microwave-assisted extraction (MAE) of food samples from an acetonitrile–water (95 + 5, v/v) mixture followed by SPE cleanup of the extracts and analysis by GC with an electron capture detector. MAE operational parameters, such as the extraction solvent, temperature, and time, were optimized with respect to the extraction efficiency of the target compounds from food samples with 10–13% fat content. The chosen extraction technique allows reduction of the solvent consumption and extraction time when compared with methods already used. Acetonitrile is a good extraction solvent for low-fat matrixes (2–20% fat content), such as fish samples, because it does not significantly dissolve the highly polar proteins, salts, and sugars commonly found in food and gives high recoveries of a wide polarity range of analytes. For purification, SPE using LC-Florisil was shown to be sufficient for the removal of coextracted substances. Recoveries >78% with RSD values <15% were obtained for all compounds under the selected conditions. Method quantification limits were in the 5–10 μg/kg range. The method was applied to the analysis of samples of herring (*Clupea harengus*) purchased at the local fish market. The method is rapid and reliable for the determination of organochlorine analytes in fish muscle.

Although the use of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) has been banned because of their adverse effect on the environment, some are still in use, e.g., OCPs in developing countries remain the most economic and efficient way of controlling pests (1). Thus, it is hardly surprising that residues of highly chlorinated organic compounds have been detected in water and various commodities, including food samples (2–4). Such halogenated organic compounds enter the food chain from environmental media, mainly through the intake of animal or fish fats (meat, fish, and milk), thereby reaching wildlife and humans. Hence, it is of paramount importance for chlorinated residues in food (especially in fatty food) to be closely monitored.

The U.S. Food and Drug Administration (FDA) defines fatty foods as having ≥2% fat content, and nonfat foods as having <2 % fat (5). However, there is a big difference in the analysis of milk containing 3% fat and very fatty foods such as lard. To take this into account, Lehotay et al. (6) proposed that foods should be divided into nonfat (<2% fat), low-fat (2–20%), and high-fat (>20%), the fat content being calculated on a wet weight basis. Here we follow this terminology and classify fish samples as low-fat.

The conventional approach to analyzing organochlorine compounds in low- and high-fat foods involves solvents such as hexane, acetone, ethyl acetate, and dichloromethane for extraction in order to dissolve the lipids (7–9). However, a time-consuming and labor-intensive cleanup is usually needed to remove the coextracted fat from the extracts prior to the final analysis. For high-fat matrixes such as vegetable oils or animal fat (>20% lipids), there is no option but to use a nonpolar solvent to dissolve the fat to extract the organochlorine compounds. But in the case of low-fat matrixes, including nuts, milk, fish, shellfish, other seafood, poultry, pork, beef, eggs, nuts, wheat, corn, soybeans, and other grains, the postextraction cleanup will be less intensive as long as lipid extraction can be avoided while still achieving adequate coverage and detection limits for lipophilic analytes. From this point of view, acetonitrile is a good extraction solvent for low-fat matrixes because it does not significantly dissolve the highly polar proteins, salts, and sugars commonly found in food and gives high recoveries for a wide polarity range of analytes.

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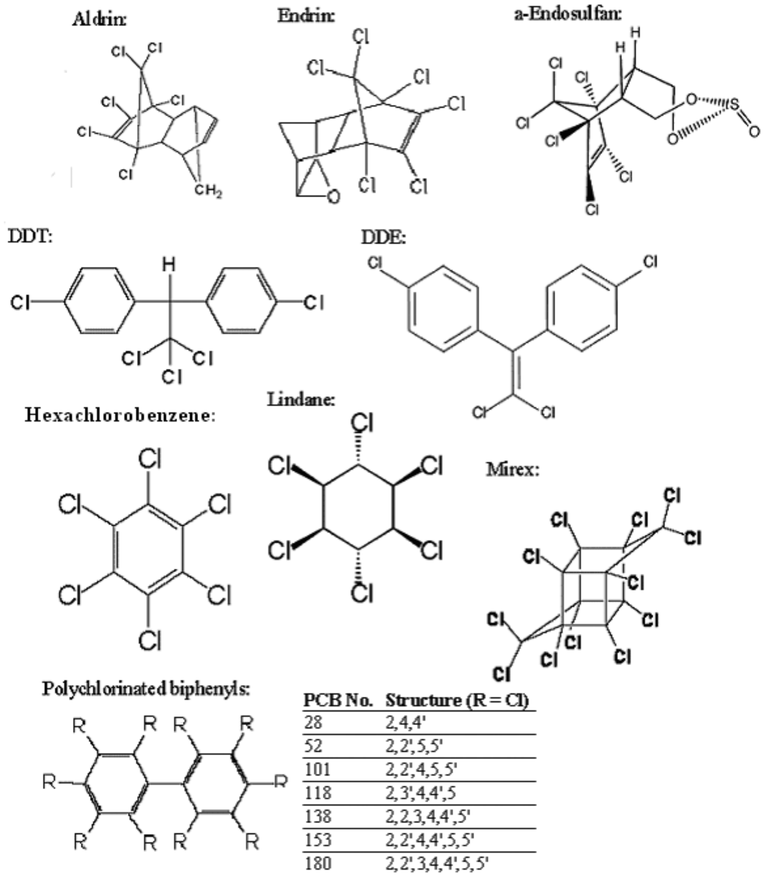

PCB No.	Structure (R = Cl)
28	2,4,4'
52	2,2',5,5'
101	2,2',4,5,5'
118	2,3',4,4',5
138	2,2,3,4,4',5'
153	2,2',4,4',5,5'
180	2,2',3,4,4',5,5'

Figure 1. Chemical structures of the OCPs and PCBs investigated.

When compared with already used procedures, the developed method avoids a multistep purification of extracts. For example, Pan et al. (10) proposed a two-step procedure with preliminary cleanup and Florisil column fractionation for purification of fish muscle extract after hexane extraction. For preliminary cleanup, sulfonation by concentrated sulfuric acid was applied. The results showed that proposed procedure to be time-, work-, and solvent-consuming and cannot be used for some pesticides (dieldrin, endrin, and endrin aldehyde) due to complete loss of the analytes after sulfonation.

Microwave ovens, initially used for sample digestion, have also been used for extraction; they offer such benefits as improved efficiency, reduced extraction time, low solvent consumption, and a high level of automation compared to conventional extraction techniques (11, 12). The principle of the method lies in the fact that microwave energy is absorbed by the extractant, which, in turn, transfers it to the sample in the form of heat. The partitioning of the analytes from the sample matrix to the extractant depends mainly on the temperature and the nature of the extractant. Unlike classical heating, microwaves heat the entire sample simultaneously (13). Microwave-assisted extraction (MAE) has been used mainly for extracting persistent organic pollutants from a variety of matrixes (mostly soils and sediments), whereas other methods have been reported for the analysis of PCBs and pesticide residues in plant and animal tissues, e.g., fish (14–16), cod livers (17), sesame seeds (18), mussels (19), and vegetables (20).

The aim of the present study was to develop a method for the rapid and efficient extraction of OCPs and PCBs (Figure 1) from food samples using MAE followed by LC-Florisil cleanup and final analysis by GC with an electron capture detector (GC-ECD). Several of the parameters known to affect extraction were optimized, and the method was applied to the analysis of samples of herring (*Clupea harengus*) purchased at the local fish market.

Experimental

Chemicals and Reagents

Organic solvents (*n*-hexane, acetone, isooctane, and acetonitrile) for GC were obtained from E. Merck (Darmstadt, Germany).

The analytical pesticide standards γ-HCH (lindane), aldrin, endrin, α-endosulfan, *p*,*p*'-DDT, *p*,*p*'-DDE, mirex, hexachlorobenzene (HCB), and PCBs (International Union of Pure and Applied Chemistry Nos. 28, 52, 101,

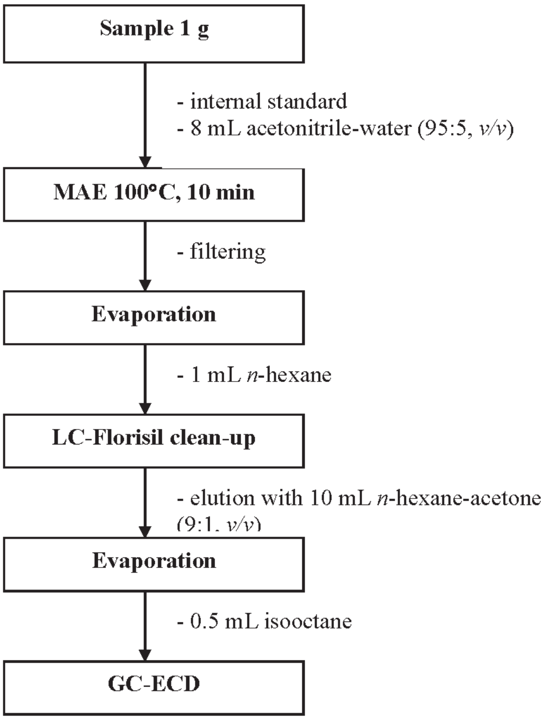

Figure 2. Analytical procedure for chlorinated compounds in fish muscle samples.

118, 138, 153, 180, and 209, as internal standards) were from LGC Standards (Teddington, UK). Stock solutions of the above analytes at 100 µg/mL were made in isooctane; working standard solutions containing each analyte at 0.025, 0.1, 0.25, 0.5, 1, and 2.5 µg/mL were also prepared in isooctane. These solutions were used for plotting the calibration curves and the preparation of fortified fish muscle samples. Stock solutions of individual compounds were stored in amber-colored bottles (to prevent photolytic degradation) at –23°C; working standard solutions were also stored at –23°C.

Distilled water was obtained with a Milli-Q system (Millipore Corp., Billerica, MA).

Apparatus

(**a**) *Syringes*.—5, 10, 50, and 100 µL (Hamilton, Bonaduz, Switzerland).

(**b**) *MAE*.—A MARS V laboratory microwave system (CEM, Matthews, NC) equipped with a 12-vessel carousel operated in the closed-vessel mode was used for the MAE step. The extraction vessels were Teflon-lined, and during operation both temperature and pressure were monitored in a single vessel.

(**c**) *SPE*.—A Baker SPE 12G system with pump (No. N022.AN18; J.T. Baker, Deventer, The Netherlands) was used. LC-Florisil columns (2 g/12 mL, No. 57115) were purchased from Supelco (Bellefonte, PA).

(**d**) *Evaporation/concentration*.—A Caliper LifeSciences Turbovap LV (Hopkinton, MA) workstation (40°C, 5 psi) was used for the concentration of organic solvents.

(**e**) *GC*.—Samples were analyzed with a GC 6000 Vega Series 2 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a 63Ni ECD (Finnigan, Waltham, MA). Chromatographic analysis was carried out on a ZB-5MS capillary column (30 m × 0.25 mm id, 0.25 µm film thickness; Phenomenex, Torrance, CA) with a 5 m precolumn (fused silica).

Procedures

(**a**) *Preparation of fish muscle samples*.—Fish muscle samples with an average moisture content of 70% and a fat content of 10–13% were mashed in a food processor. The fat content of fish muscle samples was determined using the method described by Lee et al. (21). The samples were then fortified by the addition of 0.1 mL portions appropriate stock solution to 1 g portions of mashed fish. After thorough mixing, the samples were left to stand overnight.

(**b**) *MAE*.—Portions (1 g) of the mashed fish muscle samples were transferred to microwave extraction vessels and suspended in 8 mL acetonitrile–water (95 + 5, v/v). Extraction was carried out at 100°C for 10 min. Following extraction, the vessels were allowed to cool to 30°C, after which the extracts were withdrawn from the clear supernatants, allowed to percolate through anhydrous sodium sulfate, and collected in 12 mL tubes. The solvent was removed by means of a Caliper LifeSciences Turbovap LV workstation; the residue of each sample was redissolved in 1 mL *n*-hexane and subjected to LC-Florisil cleanup.

(**c**) *Sample cleanup*.—Even though lipids are not very soluble in acetonitrile, a certain amount of fat will be coextracted and should be removed prior to chromatographic analysis. Sample cleanup to remove the coextracted substances was carried out on columns packed with 2 g LC-Florisil. Before use, each column was conditioned by passing 12 mL *n*-hexane through it. After sample application, the target compounds were eluted with 2 × 5 mL *n*-hexane–acetone (9 + 1, v/v). The eluates were collected, the solvent was evaporated, and the residue was redissolved in 500 µL isooctane.

In preliminary experiments to test the efficiency of *n*-hexane, two cleanup steps—freezing-lipid filtration and SPE—were necessary to remove the lipid materials. The *n*-hexane extract was dried and redissolved in acetonitrile. The acetonitrile extract was placed in the freezer at –23°C for 30 min to freeze the lipids, after which the cold extract was immediately filtered (Whatman filter paper, 8 µm, Grade No. 2) to remove the frozen lipids. The filtration was repeated using the same procedure. Acetonitrile was removed and the sample residue, redissolved in 1 mL *n*-hexane, was subjected to LC-Florisil cleanup

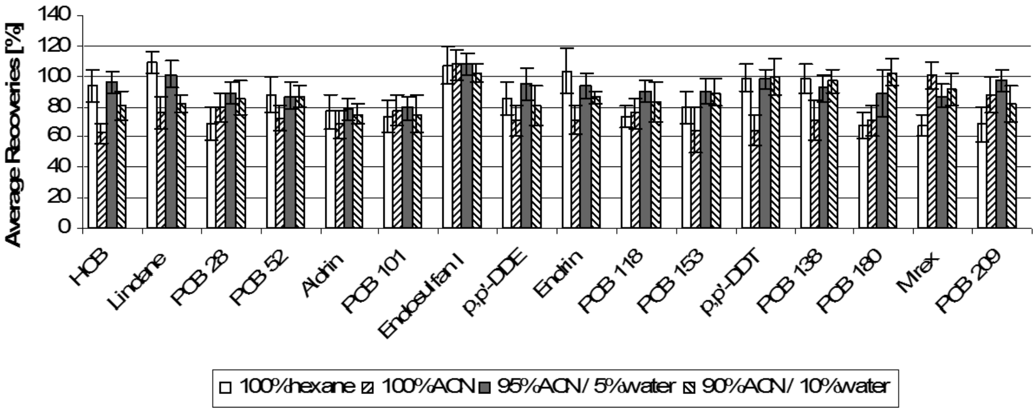

Figure 3. Effect of extraction medium on target analyte recoveries (%). Error bars represent the RSD (n = 4).

(as described above). Figure 2 outlines the sample preparation scheme.

(**d**) *GC-ECD analysis*.—Hydrogen (from a hydrogen generator, purity 99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min. The initial oven temperature was set at 80°C. Subsequently, the temperature was raised to 180°C at a rate of 15°C/min. A ramp to 300°C was performed at a rate of 10°C/min with a final hold time of 3 min. The on-column injector was set at 80°C, and 2 µL portions were injected. The detector temperature was 350°C.

(**e**) *Statistical analysis*.—The data were analyzed using the Graphpad PRISM data analysis program (Version 5.0). One-way analysis of variance followed by Tukey's test was used. These parametric statistical tests could be used as the data were normally distributed. P <0.05 was considered statistically significant.

Results and Discussion

Extraction Optimization

Several parameters affecting the performance of the MAE method were evaluated. The microwave system was programmed to increase the temperature to the predetermined setting by performing a linear temperature ramp of 10 min. This was done to avoid the abrupt heating of the extraction vessels that would occur if the maximum available microwave energy was delivered at a constant rate. According to the manufacturer's recommendation, a 1 g sample was selected for all experiments; this is perfectly adequate for trace residue analysis and minimizes variations caused by sampling.

Traditionally, tissues with high oil and fat contents were extracted with nonpolar solvents (hexane and chloroform); along with the target compounds, large amounts of lipid matter were coextracted (8, 9) that subsequently had to be removed prior to chromatographic analysis. When the efficiency of n-hexane was tested in preliminary experiments (Figure 3), recoveries ranged from 68% (mirex and PCB 180) to 109% (lindane) with an average of 85%, but a further cleanup step—freezing-lipid filtration—was necessary. Because large amounts of lipids were extracted when n-hexane was used as the solvent and two cleanup steps (freezing-lipid filtration and SPE) were required to remove the lipid materials, we tested other solvents.

Lipids are not very soluble in acetonitrile or water, or their mixtures; the fats typically form an oily film on the surface of these solvents or an emulsion during extraction. The lipophilic compounds remain or partition into the undissolved fats, which leads to their low recovery in the acetonitrile extract. However, if the fat composition of a sample is small, all or a high percentage of the fat dissolves in the acetonitrile, and recoveries are high. This is why acetonitrile can be used for low-fat foods (22, 23). In experiments where the efficiency of acetonitrile was tested, recoveries ranged from 62% (HCB) to 108% (α-endosulfan) with an average 76%; these were considered satisfactory since only a small volume (8 mL) of solvent was used (Figure 3). However, the amount of coextracted lipid matter was still significant and had to be minimized. Subsequent extractions were carried out with mixtures of 5 and 10% water in acetonitrile. Figure 3 presents the results. Although there was no significant difference in the recovery values (Tukey test, α = 0.05), most extracts were cleaner and contained less lipid matter. Moreover, there were no adverse effects on column performance after the repeated injection of those extracts into the chromatographic system. The larger amount of extracted pigments, as demonstrated by the yellow color of the acetonitrile–water extracts, did not pose a problem since they were efficiently removed prior to the GC-ECD determination by the LC-Florisil columns used for sample cleanup. The 95 + 5 (v/v) acetonitrile–water mixture was, therefore, selected as it generally yielded higher recoveries than the 90 + 10 (v/v) mixture.

Temperature is one of the most important experimental parameters in MAE; therefore, a series of extractions was performed at 60, 80, 100, and 110°C. Recoveries were greatly affected by the increase in extraction temperature

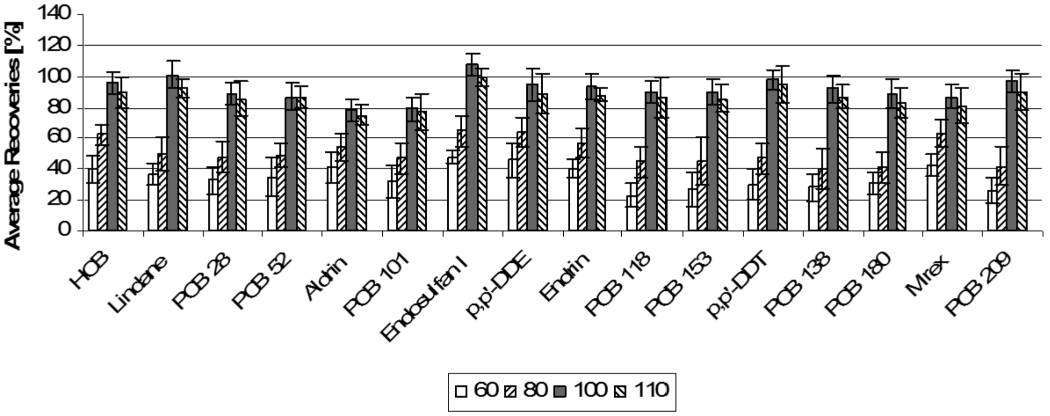

Figure 4. Effect of extraction temperature on target analyte recoveries (%). Error bars represent the RSD ($n = 4$).

(Figure 4). At 60°C they were in the 23–48% range, increasing to 40–65% at 80°C and 78–108% at 100°C; at 110°C recoveries were lower than at 100°C (range 75–100%). Elevated temperatures usually improve extraction efficiency as long as the analytes are stable at those temperatures (11). Hence, all subsequent extractions were performed at 100°C.

Because extraction time is usually a very important experimental parameter, a series of extractions was carried out for periods of 5, 10, and 20 min. Figure 5 shows that the extraction of most of the analytes was not affected by its duration (whether 10 or 20 min); in any case, 5 min is too short a time to extract analytes.

In accordance with the manufacturer's recommendation, an extractant volume of 8 mL was selected to ensure sufficient extraction of all the analytes.

Sample Cleanup and GC-ECD Analysis

Sample cleanup was necessary to remove coextracted polar substances. LC-Florisil columns were used for this purpose because these adsorbents had proved to be very efficient for the cleanup of food samples (18, 24). The columns were chosen arbitrarily from among several commonly used columns. Most of the compounds were quantitatively recovered using LC-Florisil, LC-Alumina, and LC-NH2 columns (data not shown); the LC-Florisil column was selected for use in these experiments. The efficiency of the cleanup procedure was manifested by the lack of detrimental effects on column performance after the analysis of a large number of fish muscle samples. Readers who are interested in more information on this subject can compare the efficiency of different sorbents used during cleanup of extracts for determination of PCB and pesticide residues in low-fat food reported in an earlier article (25).

In the preliminary experiments to test the efficiency of *n*-hexane two cleanup steps, freezing-lipid filtration and SPE, were necessary to remove the lipid materials. However, this procedure was very labor-intensive and time-consuming.

QA/QC

QA/QC protocols included the analysis of fortified samples, nonfortified samples, and procedural blanks. Peaks were identified by retention times compared to standards. The reported data were not corrected for

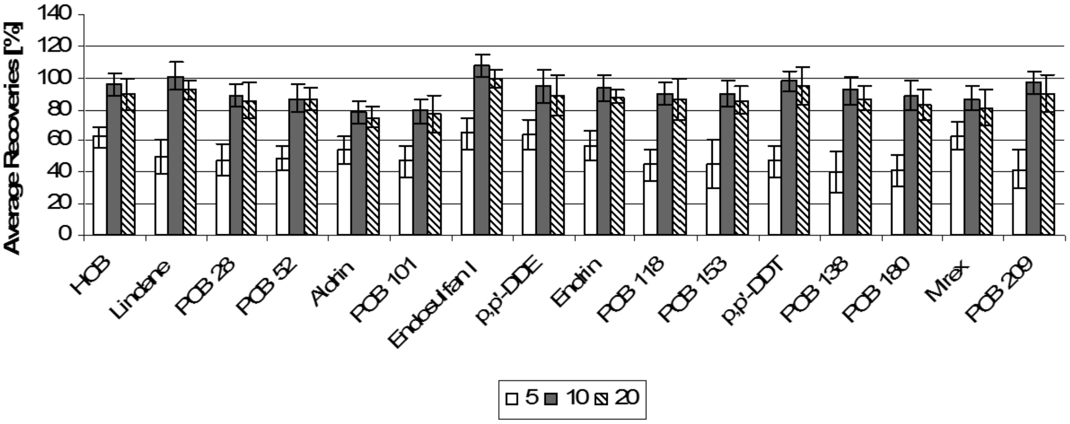

Figure 5. Effect of extraction time on target analyte recoveries (%). Error bars represent the RSD ($n = 4$).

Table 1. Recovery, RSD, MQL, and MRL values obtained from the analysis of fortified fish muscle samples

No.	Compound	MQL, μg/kg	MRL, μg/kg[a]	Average recovery, % ± RSD, %[b]
1	Hexachlorobenzene	7		96 ± 7
2	Lindane	7	20[c]	101 ± 9
3	PCB 28	6		89 ± 7
4	PCB 52	6		87 ± 8
5	Aldrin	7	10	78 ± 7
6	PCB 101	6		79 ± 8
7	α-Endosulfan	7	100[d]	108 ± 7
8	*p,p*'-DDE	10	50[e]	95 ± 11
9	Endrin	10	10	94 ± 8
10	PCB 118	6		90 ± 7
11	PCB 153	6		90 ± 8
12	*p,p*'-DDT	10	50[e]	98 ± 7
13	PCB 138	6		92 ± 9
14	PCB 180	6		89 ± 15
15	Mirex	5		87 ± 8
Internal standard	PCB 209	10		97 ± 7

[a] MRL given for pesticides only.
[b] $n = 4$.
[c] ∑-HCB = sum of HCB.
[d] ∑-Endosulfan = sum of endosulfan.
[e] ∑-DDT = sum of DDT.

recoveries. Samples below the LOD were assigned a value of 0 in the data analysis.

Fish muscle samples fortified with chlorinated compounds were repeatedly analyzed to determine the recovery, reproducibility, and LOD of the method. Table 1 lists the mean and RSD of the recoveries, calculated as the measured amount divided by the fortified amount. Recoveries of pesticides and PCBs are between 78 and 108%, and RSDs are 7–15% (average 92%). Recoveries >80% (78% only for aldrin) and RSDs <15% fulfill the requirements of the European Union (EU) guidelines (26).

The method detection limit (MDL; 27) is a term that should be applied to the extraction of specific analytes within a matrix and to the methods developed for their analysis. MDL can be defined as the smallest amount of an analyte that can be reliably detected or differentiated from the background for a particular matrix by a specific method. All matrix interference must be taken into consideration when determining MDL. Similarly, the method

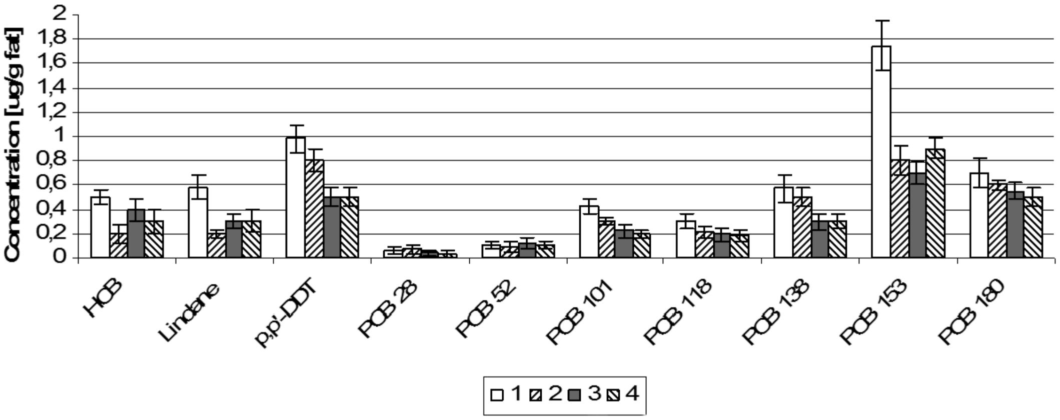

Figure 6. Concentrations of OCPs and PCBs in herring from the local fish market, 2008–2009.

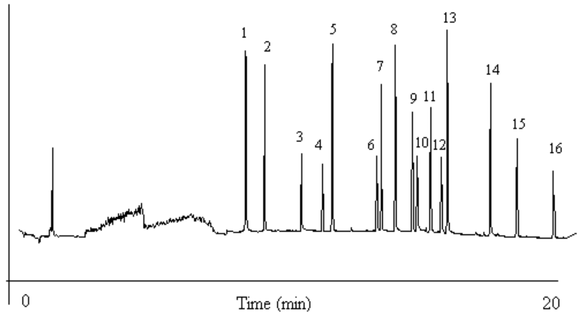

Figure 7. GC-ECD chromatogram of a spike fish sample. Peak identities are (1) HCB, (2) lindane, (3) PCB 28, (4) PCB 52, (5) aldrin, (6) PCB 101, (7) endosulfan I, (8) 4,4' DDT, (13) PCB 138, (14) PCB 180, (15) mirex, (16) PCB 209.

quantification limit (MQL) can be defined as the smallest amount of an analyte that can be reliably quantified with a certain degree of reliability within a particular matrix by a specific method. We followed this terminology.

MDLs were found to be <5 μg/kg for almost all compounds; moreover, MQLs for all pesticides were below the maximum residue limit (MRL) established by the EU (28; the MQL for endrin was equal to the EU MRL). The FDA requires that infant foods, eggs, milk, and other dairy products, fish and shellfish, poultry, and red meat contain no more than 0.2–3 ppm of PCBs (29). The MQLs also met these requirements. For all compounds, there was linearity in the range of 80–80 000 pg of the amount injected, with correlation coefficients >0.92.

The method was applied to the analysis of samples of herring (*C. harengus*) obtained from the local fish market in 2008 and 2009. Levels of chlorinated compounds in the samples were low (Figure 6); pesticide and PCB concentrations are expressed on a lipid-weight basis. Figure 7 illustrates a chromatogram of a spiked sample. Positive real samples were confirmed by GC/MS, but MS detection was used only for confirmation.

Conclusions

A method was developed to provide a rapid and efficient means of determining PCB and OCP residues in fish muscle samples. It was based on an MAE technique that allowed the simultaneous processing of 12 samples under strictly controlled conditions. The extraction was completed in just 25 min, yielding extracts containing small amounts of lipid materials that did not pose a serious problem. Solvent consumption/sample was low, thus reducing waste disposal and keeping evaporation time to a minimum. Recoveries were >78% with corresponding RSDs <15% for all analytes, and MQLs were ≤10 μg/kg, thus allowing for the accurate determination of trace levels of residues. For the determination of OCP analytes in fish muscle, the method is rapid and reliable. Further applications to analyze chlorinated pollutants in the presence of lipids in other types of food samples are to be investigated.

Acknowledgments

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