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Method for integrated analysis of polycyclic aromatic hydrocarbons and organochlorine compounds in fish liver

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Abstract

An analytical method for integrated analysis of organochlorine compounds and polycyclic aromatic hydrocarbons (PAH) in large numbers of fish liver samples has been developed using one single clean-up step. Tissues are homogenized with anhydrous sodium sulphate and Soxhlet extracted with *n*-hexane–dichloromethane (4:1, v/v) for 24 h. The extracts are cleaned-up and fractionated with an alumina chromatographic column allowing the separation of the extracts in two fractions. One containing most organochlorine compounds, including hexachlorobenzene, DDTs and polychlorobiphenyls, and the other the hexachlorocyclohexane isomers and PAH. These two fractions are subsequently analysed by GC–MS. Tests of repeatability result in relative standard deviations mainly under 20%. Evaluation by the standard addition method shows good linearities and recoveries.

Keywords: Polycyclic aromatic hydrocarbons; Organochlorine compounds

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and organochlorine pesticides are ubiquitous contaminants in the environment [1,2]. These compounds have been widely studied because of their adverse effects on organisms, including humans [3–5]. Although their environmental concentrations are low they tend to accumulate in organic tissues due to their lipophilic character and persistence to degradation [6] which may eventually result in toxic concentration levels for organisms such as fish [7].

These compounds are persistent organic pollutants (POPs). Once released into the environment they may be transported over long distances and pass through many biogeochemical cycles, e.g. food web, without undergoing sensible degradation. Thus, despite the discontinued use of some of them, e.g. PCBs and most organochlorine pesticides, they are currently found in all sorts of environmental samples, including those collected in both polluted and remote sites [2]. In other cases their occurrence also reflects present production or increased delivery rates in recent decades. This is the case, for instance, of most PAHs whose concentration increased considerably in the 19th century as a consequence of the rise in fossil fuel combustion [8].

In these conditions, research is prompted to elucidate whether these compounds may have toxic

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effects on remote ecosystems, to ascertain how these potential effects are reflected in the species inhabiting these ecosystems and, most importantly, to identify the specific relationships between individual POPs and toxicity. In this respect, fish are ideal species for the study of remote aquatic systems since they are on top of the food web and therefore accumulate the highest POP concentrations.

However, studies of fish in remote sites such as high mountain lakes have to overcome major analytical difficulties such as low sample amounts and large numbers of POP to be determined. In addition to the sampling difficulties in these environments, the large number of measurements needed for the assessment of their health status limits the amount of material available for study. This is specifically the case for liver which, in turn, is the type of material from which more determinations may need to be performed, e.g. POPs, metals, histology, glycogen, enzymes and others [9]. In addition, high numbers of samples have to be analyzed in order to have representative measurements in each lake.

Thus, analytical methods allowing the analysis of large amounts of small size samples (ca. 1 g) have to be developed. Most of the presently available methods are devoted to the analysis of specific POP groups such as PCB and organochlorine pesticides (e.g. Refs. [7,10–13]) and PAHs (e.g. Refs. [14–16]). However, the stringent restrictions on sample amount require methods for the simultaneous analysis of organochlorine compounds and PAHs in fish liver. Development of these integrated methods in marine organisms has only been undertaken on a few occasions [17,18]. The present manuscript reports one of these methods based on a simple column chromatography clean-up procedure.

2. Materials and methods

2.1. Materials

Residue analysis *n*-hexane, dichloromethane, isooctane, methanol, acetone, NaOH pellets and anhydrous sodium sulphate for analysis were from Merck (Darmstadt, Germany). Aluminium foil was rinsed with acetone and let dry at ambient temperature prior to use. Neutral aluminium oxide type 507C was from Fluka AG (Switzerland). Cellulose extraction cartridges of 20 mm I.D. and 80 mm long were from Whatman (UK). The purity of the solvents was checked by gas chromatography–electron capture detection and no peaks were detected. Aluminium oxide, sodium sulphate, cartridges and NaOH pellets were cleaned by Soxhlet extraction with dichloromethane–methanol (2:1, v/v) for 24 h before use. Sodium sulphate and aluminium oxide were activated overnight at 400 °C and 120 °C, respectively.

γ-Hexachlorocyclohexane (γ-HCH) and tetrabromobenzene (TBB) were from Aldrich-Chemie (Steinheim, Germany). α-, β- and δ-HCH and PCBs (#28, #52, #101, #118, #138, #153, #180, #209) were from Promochem (Wesel, Germany), and pp′-DDE, pp′-DDT, PAHs mix9 and perdeuterated PAHs were from Dr. Ehrenstorfer (Augsburg, Germany). A standard mixture was prepared with the above mentioned HCH isomers, pp′-DDT, pp′-DDE, HCB, PCBs and PAHs (acenaphthene, acenaphthylene, naphthalene, phenanthrene, fluorene, fluoranthene, pyrene benz(a)anthracene, chrysene, benzo(b)-fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(ah)anthracene, indeno(1,2,3-cd)pyrene and benzo(ghi)perylene) at 50 ppb in isooctane.

Fish liver tissues were used to validate the methodology. They were cut, wrapped in pre-cleaned aluminium foil and kept frozen until extraction.

2.2. Soxhlet extraction

Liver tissue was ground with activated anhydrous sodium sulfate (7–9 g) and was introduced into a previously cleaned cellulose cartridge. TBB, PCB#209 [19] and perdeuterated anthracene, pyrene and benzo(ghi)perylene were used as internal standards. These five compounds used as internal standard mixtures were added for the study of the recoveries for the organochlorine compounds and polycyclic aromatic hydrocarbons. The cartridge was then Soxhlet extracted with *n*-hexane–dichloromethane (4:1, v/v) for 20 h. Then, the extract was vacuum evaporated until 1 ml and further concentrated to 50 μl in isooctane under a gentle nitrogen flow.

2.3. Alkaline digestion

The same internal standard mixture used for Soxhlet extraction was added to fish liver. This was

then introduced in an alkaline solution of 6 *M* NaOH for 14 h at 40 °C. A mixture of *n*-hexane–dichloromethane (4:1, v/v) was used to extract the compounds from the alkaline media. Then, the extract was vacuum evaporated until 1 ml and further concentrated to 50 μl in isooctane as described above.

2.4. Chromatographic fractionation and clean-up

The extracts obtained by Soxhlet extraction were fractionated by adsorption chromatography with glass columns (30 cm length and 0.8 cm I.D.) containing 5 g of aluminium oxide. Two fractions were collected: F1, 16.5 ml of *n*-hexane–dichloromethane (19:1, v/v) and 3 ml of *n*-hexane–dichloromethane (1:2, v/v); F2, 13 ml of *n*-hexane–dichloromethane (1:2, v/v). Both fractions were concentrated by vacuum rotary evaporation to 1 ml, transferred to vials, and further concentrated until 50 μl in isooctane as described above.

2.5. Recovery and standard addition test

Recoveries were calculated using 0.4-g liver fractions which were spiked with solutions containing 50 ng/ml of the standard mixture PAH and organochlorine compounds, 25 μl and 50 μl, respectively (Section 2.1).

Overall linearity of the method was calculated from concentrations of 0, 12, 25 and 62 ng/g for PAHs and 0, 0.4–3.1, 1.8–16 and 4–31 ng/g for organochlorine compounds which were prepared from aliquots of the standard mixture added to 0.4-g liver portions of the same freshwater trout. These subsamples were Soxhlet extracted and chromatography fractionated as previously described.

2.6. Instrumental analysis

Before chromatographic analysis, an internal standard of tetrachloronaphthalene and octachloronaphthalene was added (25 μl) to the vial to correct for instrument variability. Samples were analyzed by GC (Carlo Erba GC 8000) coupled to a quadrupole mass spectrometer (MS, Fisons MD800) with a 50-m HP-5MS column (0.25 mm I.D. and 0.25 μm film thickness). The machine operated in splitless mode (isooctane; hot needle technique) and electron impact mode (EI, 70 eV). The oven temperature program started at 90 °C (held for 1 min) to 120 °C at 10 °C/min, and then to 310 °C at 4 °C/min (holding time 15 min). Injector, transfer line, and ion source temperatures were 280 °C, 280 °C and 200 °C, respectively. Stringent precautions were observed for maintenance of the injector under clean conditions avoiding adsorptions that could deviate the system from linearity and increase the limits of detection and quantification. Under these conditions, repeated analysis of pp′-DDT standards at 250 °C, 270 °C and 280 °C showed no formation of pp′-DDE derivatives. Injection at 280 °C was preferred for the high molecular mass of some PCB congeners present in the samples. Helium was used as carrier gas (flow-rate 1.1 ml/min). Data acquisition was in selected ion recording mode at 40 ms of dwell time. The ion mass programs used for quantification are described elsewhere [1,20]. Quantification was performed by reference to calibration curves of the compounds included in Table 1 and subsequent correction using the internal standards for extraction and injection. The curves were generated by progressive dilution of standard mixtures and subsequent instrumental analysis. The regression lines of the curves closer to the lowest detectable concentration ranges were used to evaluate injector performance for linearity and sensitivity.

3. Results and discussion

3.1. Comparison of extraction methodologies

The method for the simultaneous analysis of organochlorine compounds and PAHs in fish liver developed in the present study is shown in Fig. 1. The recoveries of both organochlorine compounds and polycyclic aromatic hydrocarbons are better for Soxhlet extraction than alkaline digestion (Fig. 2). For Soxhlet extraction PAH and organochlorine compound recoveries were between 78.5–99% and 79–99%, respectively. In alkaline digestion these recoveries were between 67–95% and 0–81%, respectively. HCHs and pp′-DDT are destroyed as a consequence of the alkaline digestion which prevents their analysis [21,22]. These results show that Soxhlet extraction should be the method of choice.

Table 1
Results of the repeatability (relative standard deviation) and standard addition (recovery and r^2) tests for the integrated method of organochlorine compound and PAH analysis in fish liver (spiking standard mixture indicated in Section 2.5)

Compound	Relative standard deviation (%)	Recovery (%)	r^2	Limits of detection (pg/g)	Limits of quantitation (pg/g)	Compound	Relative standard deviation (%)	Recovery (%)	r^2	Limits of detection (pg/g)	Limits of quantitation (pg/g)
Acenaphthene	6.7	71	0.9988	15	16	α-HCH	6.4	110	0.9916	7.8	8
Fluorene	11	86	0.9945	17	17	γ-HCH	4.7	90	0.9896	6	6.2
Phenanthrene	15	130	0.994	8	8	HCB	3.7	68	0.9906	6.6	5.8
Anthracene	14	130	0.9789	10	10	PCB≠28	5.5	110	0.9821	1.4	1.8
Fluoranthene	16	110	0.9988	11	13	PCB≠52	10	79	0.9984	11	11
Pyrene	1.3	110	0.9987	12	12	PCB≠101	11	83	0.9951	13	13
Benz(a)anthracene	16	75	0.9517	18	18	PCB≠118	7.3	100	0.9986	21	21
Benzo(b)fluoranthene	16	79	0.9015	20	20	pp′-DDE	5.9	89	0.9988	1	1.2
Benzo(a)pyrene	16	84	0.8138	21	21	PCB≠153	9.1	93	0.9996	23	25
Indeno(1,2,3-cd)pyrene	21	83	0.7803	26	26	PCB≠138	8.4	88	0.9936	13	14
Dibenzo(ah)anthracene	23	80	0.7307	28	30	pp′-DDT	8.2	100	0.9968	22	22
Benzo(ghi)perylene	23	74	0.8135	25	26	PCB≠180	3.0	92	0.9999	7.8	9.6

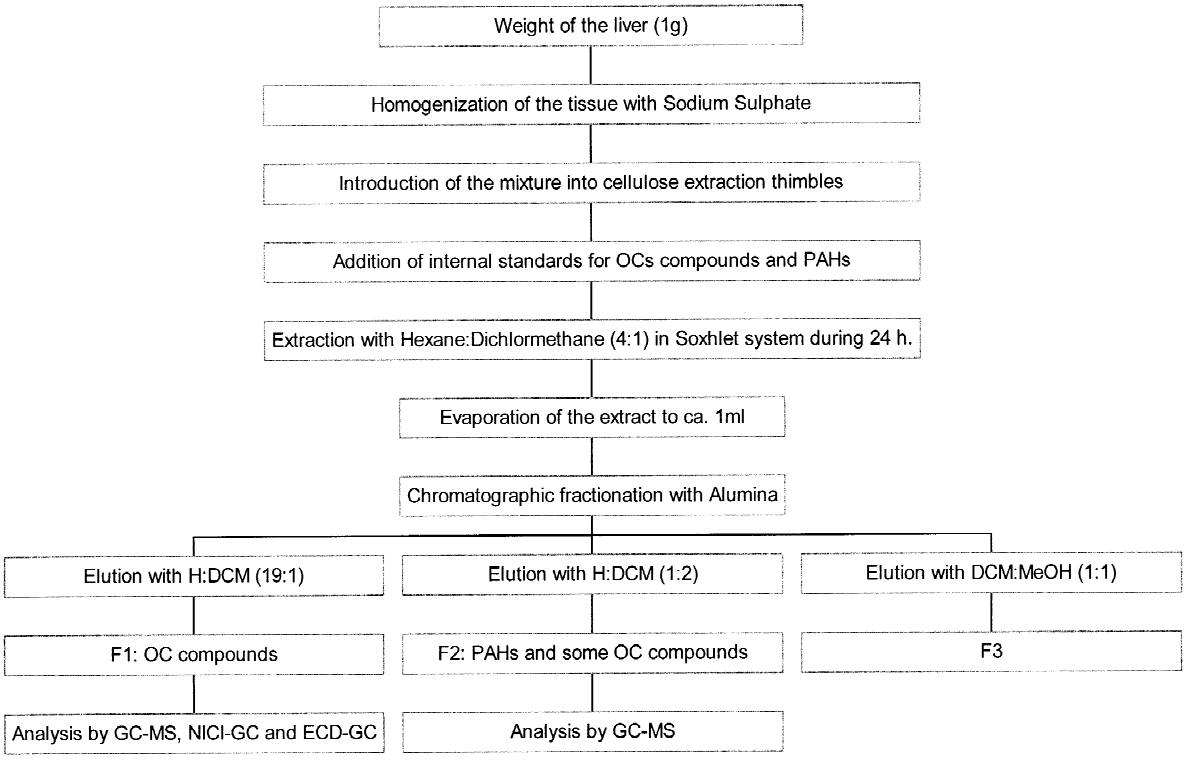

Fig. 1. Scheme of the analytical method for the integrated study of organochlorine compounds and PAH.

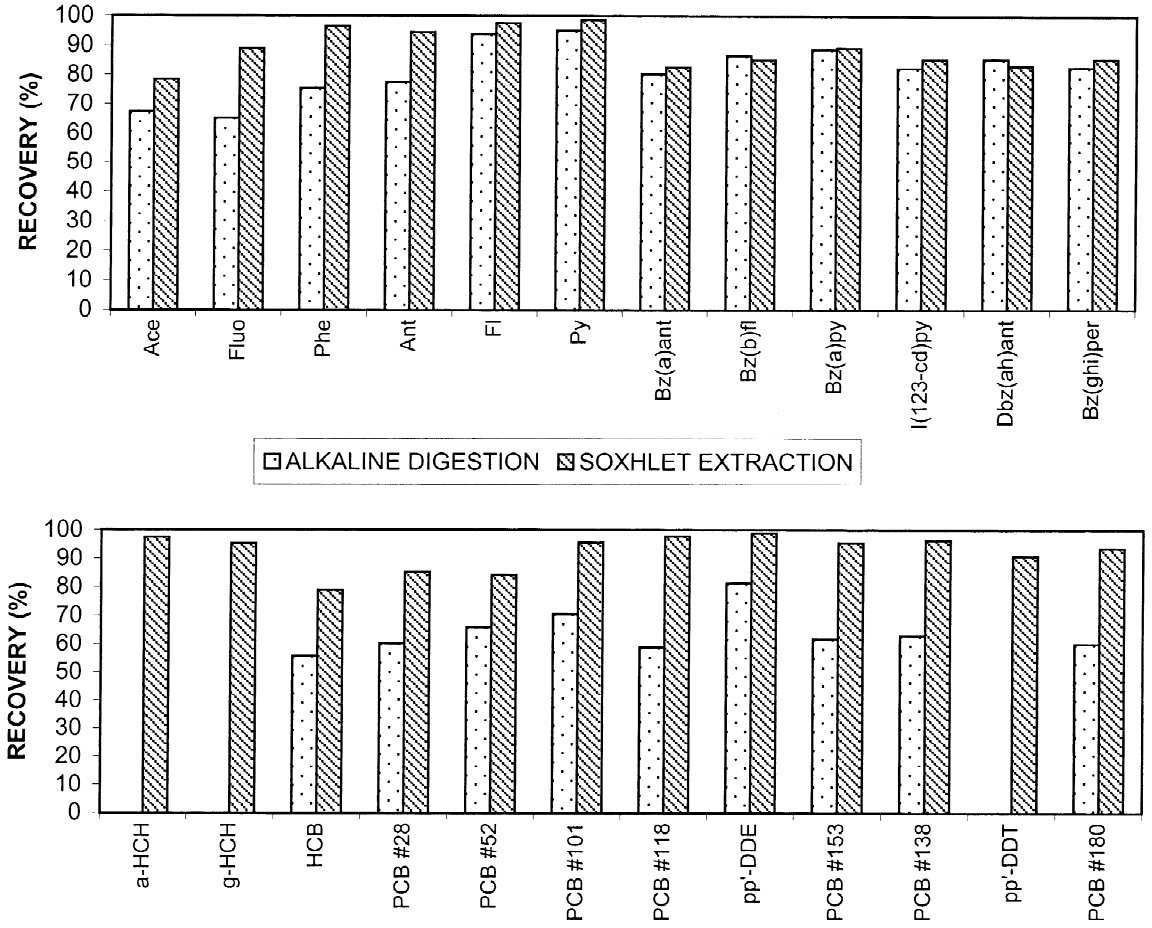

Fig. 2. Recovery values of alkaline digestion and Soxhlet extraction.

3.2. Development of chromatographic fractionation

The column chromatography method allowed the separation of the contaminants of interest in two fractions, the former containing all organochlorine compounds except HCH and the latter HCH and PAH (Fig. 3). The small dimensions of the column selected for clean up afford an easier and quicker method of preparation and sample treatment than in previous studies [23,24]. Thus, previously reported alumina/silica columns involve elution volumes of 65 ml for the recovery of PAH and additional 45 ml for the complete recovery of organochlorine compounds [14,23]. In the case of florisil columns, the complete elution of both compound groups is achieved with 45 ml [23]. In addition, the column chromatography method reported in the present study affords a better separation between the two compound groups than in these previous studies since all PAH are collected in the second fraction (together with HCH) and most organochlorine compounds, including HCB, DDTs and PCBs, in the former.

3.3. Repeatability, detection and quantification limits

The relative standard deviation (RSD) of the method was obtained from three replicates of one fish sample from the harbour of Barcelona. All compounds had levels higher than the limits of quantification. For PAHs, the RSD were around 16% or lower, except for indeno(1,2,3-cd)pyrene, dibenzo-

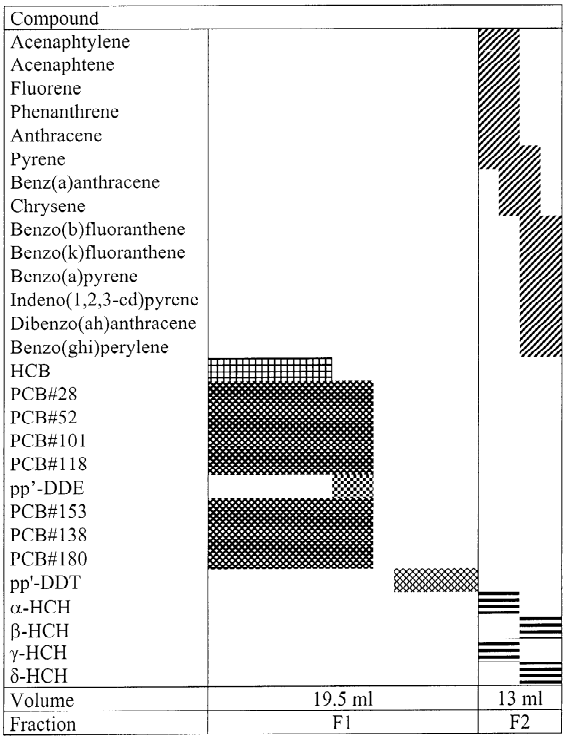

Fig. 3. Elution of PAH and organochlorine compounds in the column chromatography clean up developed in the present study.

(ah)anthracene and benzo(ghi)perylene in which it was between 20 and 24%. These values are similar to those reported in reference materials [18] and lower than those described in other studies on fish PAH [14]. All organochlorine compounds show good repeatability, with RSDs lower than 12% (Table 1). Previously reported RSD repeatabilities for organochlorine compounds were about 23% in whale tissues (including liver) [26] and between 0.1 and 22% for reference materials [18].

Detection and quantitation limits (Table 1) were calculated as described in Ref. [19]. They range between 8–30 pg/g and 1–25 pg/g for PAH and organochlorine compounds, respectively. In the case of the organochlorine compounds, these values are similar to those reported for fish muscle [19].

3.4. Standard addition method

The standard addition method has been used to evaluate the linearity (r^2) of the overall methodology, not specifically calibration. The good linearity observed, higher than 0.90 for most PAHs and higher than 0.98 for all the organochlorine compounds (Table 1), indicates that the method is adequate for liver samples containing a wide range of concentrations of the studied compounds. Some higher molecular mass PAH exhibit lower regression coefficients, 0.73–0.82. Nevertheless, these regression coefficient values also show the feasibility of the method for these compounds.

The spiked samples used in this test have also been used to calculate the recoveries of the whole method. They are all higher than 70% for PAHs and 80% for organochlorine compounds, except in the case of HCB whose recovery is 68% (Table 1). These recoveries are similar or higher to those reported elsewhere, both in the case of organochlorine compounds [17–19,25] and PAH [14,17,18]. Similar results have also been reported in the analysis of sewage sludges by supercritical fluid extraction [27]. However, in one of these studies recoveries of the order of 50% were obtained for the three-ring PAH [18].

3.5. Case studies

Examples of the applicability of the method described in the present study are shown in Table 2. Fish livers from a remote high altitude lake in the Pyrenees (Redó Lake) and a polluted site (Barcelona harbor) have been analyzed. The concentrations of organochlorine compounds found in the fish liver from Redó Lake are similar to those found in fish muscle from the same lake [28,29]. RSD of the organochlorine compound concentrations in liver and muscle [30] of fish from this lake are similar. Method repeatability (RSD in Table 1) is significantly lower than the observed within lake and harbor variabilities of the fish analyzed individually, PAH RSD 44–230% and 35–140%, respectively, and organochlorine compound RSD 30–100% and 27–76%, respectively.

In the case of PAH, exposure experiments with benzo(a)pyrene have shown that concentrations in liver are about 100 times higher than in muscle [30,31]. The method is therefore useful for the analysis of organochlorine compounds and PAH

Table 2
Representative examples of the concentrations of organochlorine compounds and PAH in fish liver (ng/g wet weight) from remote high altitude lakes (Redó; $n=11$) and polluted sites (Barcelona harbour; $n=3$)

Compound	Redó lake		Barcelona harbour		Compound	Redó lake		Barcelona harbour	
	Mean	Standard deviation	Mean	Standard deviation		Mean	Standard deviation	Mean	Standard deviation
Acenaphthylene	0.65	0.46	2.3	2.2	Indeno(1,2,3-cd)pyrene	0.19	0.18	4.9	3.2
Acenaphthene	0.39	0.30	15	21	Dibenzo(ah)anthracene	0.12	0.19	5.5	3.8
Fluorene	1.5	0.66	9.0	8.7	Benzo(ghi)perylene	0.087	0.14	2.6	2.9
Phenanthrene	8.8	5.9	19	17	HCB	0.5	0.15	3.4	2.6
Anthracene	0.60	0.37	2.0	1.5	PCB≠28	0.38	0.16	13	3.5
Fluoranthene	1.8	1.1	6.9	3.4	PCB≠52	1.2	0.93	31	14
Pyrene	1.6	0.83	6.5	3.0	PCB≠101	2.0	1.1	62	33
Benz(a)anthracene	0.17	0.095	1.1	0.38	PCB≠118	1.3	0.74	43	12
Chrysene	0.49	0.36	1.1	0.96	pp′-DDE	11	9.8	52	36
Benzo(b)fluoranthene	0.62	1.4	2.5	1.9	PCB≠153	3.4	3.0	120	48
Benzo(k)fluoranthene	0.40	0.64	1.5	1.8	PCB≠138	3.4	2.4	110	43
Benzo(a)pyrene	0.16	0.18	3.5	3.2	PCB≠180	1.8	1.8	110	46

from the same samples avoiding combination of several clean up steps [17,18].

4. Conclusions

The method selected for study (Fig. 1) is based on one single clean up step and useful for the analysis of PAHs, organochlorine pesticides and PCBs in large numbers of fish liver samples. Soxhlet extraction of liver tissues ground with anhydrous sodium sulphate provides better recoveries than alkaline digestion avoiding the degradation of pp′-DDT and HCH. The column chromatography fractionation methods allows the separation of all PCBs and DDTs from PAH. Repeatability tests show RSDs under 23%, with average values of 7% and 15% for organochlorine compounds and PAH. Good linearity and recoveries are obtained after evaluation of the method by the standard addition test.

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