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Assessment of the sample handling procedures in a labor-saving method for the analysis of organochlorine compounds in a large number of fish samples

Lourdes Berdié, Joan O. Grimalt*

Department of Environmental Chemistry (CID–CSIC), Jordi Girona 18, 08034 Barcelona, Catalonia, Spain

Abstract

A rapid single step clean-up procedure with sulphuric acid oxidation for lipid removal has been assessed by a step-by-step recovery approach for its performance in the analysis of organochlorinated compounds in large numbers of fish samples (muscle). Recovery decreases are essentially due to losses by evaporation but their effect is compensated by correction of the recovery factor of tetrabromobenzene that is used as surrogate. Sample grounding with sodium sulphate provides significant higher concentrations than freeze–drying. Soxhlet extraction for 18 h is sufficient to draw most organochlorine compounds from the samples. Repeatability and reproducibility is smaller than the dispersion between fish of similar length and age from the same lake for all compounds except α-hexachlorocyclohexane.

Keywords: Sample handling; Fish; Food analysis; Environmental analysis; Organochlorine compounds; Pesticides; Hexachlorobenzene; Hexachlorocyclohexanes; Polychlorinated biphenyls; DDT; DDE

1. Introduction

In recent years, increasing concern for the widespread occurrence of organochlorine compounds [1] and their possible health effects in humans [2,3] has prompted the completion of statistically-sound studies to get insight on the environmental and public health implications of these molecules. Analyses of large series of samples are required for this purpose and their completion depends on the availability of procedures in which human work load is minimized. Since instrumental analysis can be easily automated [4], the feasibility of the analytical methods usually depends on the difficulties of the clean-up and extraction steps.

Column chromatography with gel permeation [5], Florisil [6], alumina, silica or a combination of both [7] are too time- and labor-consuming for the analysis of large amounts of samples. In contrast, chemical digestion methods, namely sulphuric acid treatment [8] or saponification [9], are of simpler application. These simple procedures have received renewed interest in view of the need of this type of studies. In any case, the analysis of large number of samples requires a compromise between accuracy, precision and sample handling. The performance of the method of choice can be evaluated by the standard addition approach [10].

In the present paper, a step-by-step evaluation study is carried out for a simple method devoted to the analysis of hexachlorobenzene (HCB), hexachlorocyclohexanes (HCHs), DDTs and polychlorinated biphenyls (PCBs) in large numbers of fish samples (muscle tissue). Sulphuric acid oxidation

*Corresponding author.

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was chosen for lipid removal to avoid HCH destruction as it occurs with saponification. The study is aimed to identify the main error sources and to determine whether the occurrence of systematic bias recommend recovery correction of the results [11].

The procedure evaluated involves the following steps: drying, Soxhlet extraction, vacuum rotary evaporation, sulphuric acid clean-up, vacuum rotary evaporation and concentration under nitrogen stream. Alternative procedures, e.g., sodium sulphate vs. freeze–drying, have also been evaluated.

The overall method considered in the present study (Fig. 1) is adequate for the analysis of organochlorine compounds in large amounts of samples. It has been used for the study of fish collected in high altitude lakes distributed over all Europe in the context of the European Union (EU) sponsored ALPE-II and MOLAR projects.

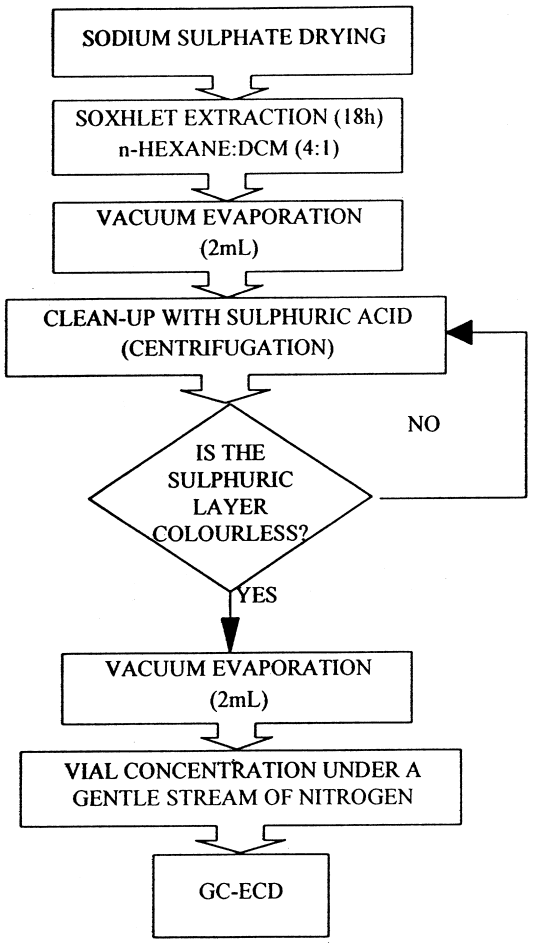

Fig. 1. Analytical protocol of the method considered in the present study. DCM=Dichloromethane.

2. Experimental

2.1. Materials

Residue analysis *n*-hexane (Ref. 1.04371), dichloromethane (Ref. 1.06054), isooctane (Ref. 1.15440), acetone (Ref. 1.00012) concentrated sulphuric acid 95–97% (Ref. 1.00731), silica gel 40, 70–230 mesh (Ref. 10180), and anhydrous sodium sulphate (analytical-reagent grade) (Ref. 1.06649) were from Merck (Darmstadt, Germany). The purity of solvents was checked by concentration of 100 ml to 50 μl and examination by gas chromatography–electron-capture detection (GC–ECD). No significant peaks should be detected for acceptance. Silica gel and sodium sulphate were Soxhlet-extracted before use. The purity of the cleaned reagents was checked by ultrasonic extraction with *n*-hexane–dichloromethane (4:1; 3×20 ml), concentration to 50 μl and analysis by GC–ECD. No interferences were detected. Sodium sulphate was activated overnight by heating at 300°C.

γ-HCH and the 1,2,4,5-tetrabromobenzene (TBB) were from Aldrich (Steinheim, Germany), α- and δ-HCHs and PCBs were from Promochem (Wesel, Germany), and *p,p′*-DDE and *p,p′*-DDT from Dr. Ehrenstorfer (Augsburg, Germany). The standard mixtures of HCH isomers, HCB, PCBs (28, 52, 101, 118, 138, 153 and 180), *p,p′*-DDE and *p,p′*-DDT and surrogate solution composed of 1,2,4,5-TBB and PCB 209 were prepared in isooctane. The freeze–drying standard mixture was prepared in acetone.

2.2. Extraction and clean-up

Muscle tissue (5 g) was ground with activated sodium sulphate until a fine powder was obtained. Alternatively, some tissues were freeze dried (16 h, −60°C, 0.1 Torr; 1 Torr=133.322 Pa) for testing purposes. This mixture was Soxhlet-extracted with 100 ml of *n*-hexane–dichloromethane (4:1) for 18 h. The extract was concentrated under vacuum to 2 ml and 2 ml of sulphuric acid were added. After vigorous stirring in a Vortex-mixer (2 min) the

mixture was centrifuged to remove any foam in the interface and the sulphuric acid layer was discarded.

This clean-up step was repeated until a colourless transparent *n*-hexane layer (2 ml) was obtained (4–6 times). The final sulphuric acid mixture was re-extracted with *n*-hexane (2×2 ml) and all *n*-hexane solutions were combined and concentrated by vacuum rotary evaporation (20°C, 20 Torr) to small volumes (ca. 300 μl). The solutions were then transferred to vials and evaporated just to dryness under a gentle stream of nitrogen (10–20°C). The cleaned extract was redissolved in 50 μl of isooctane for instrumental analysis.

2.3. Instrumental analysis

Samples were analyzed in a Hewlett-Packard gas chromatograph Model HP-5890 equipped with an electron-capture detector and an HP-7673-A autosampler. The separation was achieved with a 30 m×0.25 mm I.D. DB-5 column (J&W Scientific, Folsom, CA, USA) coated with 5% diphenyl-polydimethylsiloxane (film thickness 0.25 μm). The oven temperature was programmed from 90°C (holding time 2 min) to 150°C at 15°C/min and finally to 280°C at 4°C/min, keeping the final temperature for 10 min. Injector and detector temperatures were 270°C and 310°C, respectively. Injection was performed in the splitless mode, keeping the split valve closed for 35 s. Helium was the carrier gas (50 cm/s).

3. Results and discussion

The overall recoveries for the method considered in this study have been determined by addition of standards at the low ppb level (3–15 ng/g wet mass; Table 1). Most compounds are recovered below the target of 100±20%. Nevertheless, the internal standards introduced before sample extraction parallel the behaviour of the analytes. Thus, TBB essentially reflects evaporation losses.

3.1. Evaporation

Several tests for the assessment of evaporation losses have been performed. Thus, 100-ml volumes of *n*-hexane–dichloromethane (4:1) were spiked with a standard mixture of organochlorine compounds to obtain sub-ppb solutions and were used to perform the following tests: (1) rotary vacuum evaporation to 2 ml, vial transfer and evaporation to dryness under a gentle stream of nitrogen; (2) Soxhlet reflux for 18 h, rotary vacuum evaporation to a small volume, vial transfer and evaporation to dryness under a gentle stream of nitrogen; and (3) rotary vacuum evaporation to dryness, vial transfer and evaporation to dryness under a gentle stream of nitrogen. These three tests were performed in triplicate. All extracts were redissolved in 50 μl of isooctane for instrumental analysis.

As shown in Table 1, evaporation losses significantly affect the more volatile compounds such as HCHs, HCB, PCBs 28 and 52. As indicated above, these losses are also reflected in the TBB surrogate, which can be used as correction factor.

The volatilization losses due to Soxhlet extraction can be calculated by comparison of the results from tests 1 and 2. As shown in Table 1, lower recoveries are observed when the Soxhlet step is added. However, the differences are not significant when the mean recoveries are compared by reference to the standard deviation values (student $t_{cal}=0.2–1.14<t_{tab}=1.25$; 4 degrees of freedom, 0.70 confidence level). The dispersion values of the two tests had previously been compared and no significant differences in precision were found at 0.95 confidence level.

A critical step for possible evaporation losses is rotary evaporation under vacuum. About 2 ml should be left in the balloon to avoid the losses derived from the pressure drop when the system goes to dryness. However, analyst slips are not unusual at this step. Test 3 was planned to quantify for these losses. Evaporation was stopped immediately after reaching solvent dryness in the balloon. Comparison of tests 1 and 3 show significantly lower concentrations in the latter case. Thus, the recoveries of the volatile compounds, e.g., α-HCH, HCB, γ-HCH and PCB 28, are significantly lower when solvent went to dryness ($t_{cal}=2.7–7.5>t_{tab}=2.13$, 3 degrees of freedom, 0.90 confidence level). Again previous comparison of the dispersion values between these two tests showed no precision differences for most compounds at 0.95 confidence level.

Significant evaporation losses when the solvent

Table 1
Recoveries for organochlorinated compounds in diverse alternative steps of the analytical procedure including those with possible analyte evaporation

Compounds	Accumulated recovery losses in fish[a]		Critical steps for loss of volatile compounds (standards)					Sulphuric acid (standards)	
	Spiked range (ng/g)	% Recovery (n=4)	ng spiked	Test 1 (n=3)	Test 2 (n=2)	Test 3 (n=3)	Freeze–drying (n=3)	μg spiked	% R (n=3)
α-HCH	4–12	49[b]±6[c]	5.4	47[b]±8[c]	31[b]±10[c]	12[b]±0[c]	–	1.3	82[b]±9[c]
HCB	6–15	52±5	6.8	30±11	23±11	3±0	57[b]±4[c]	0.7	80±8
γ-HCH	4–10	52±6	4.1	70±10	58±13	22±6	–	2.8	87±9
PCB 28	4–10	67±6	4.5	69±16	53±8	35±13	66	0.65	95±11
PCB 52	4–10	36±1	4.5	67±13	61±8	39±20	81±23	0.85	97±12
PCB 101	5–13	51±7	5.8	87±12	83±1	58±12	82±22	0.85	103±12
p,p'-DDE	9–25	73±4	10.8	104±14	91±1	71±18	109±20	1.2	101±10
PCB 118	3–10	61±7	4.0	95±9	95±5	86±20	100	0.70	105±11
PCB 153	5–14	28±4	6.3	97±6	94±6	82±10	68±12	1.1	104±11
p,p'-DDT	3–8	81±12	3.2	136±24	118±2	105±17	–	0.42	104±12
PCB 138	6–15	59±6	6.8	100±10	96±2	89±11	91±11	0.95	105±11
PCB 180	3–9	66±7	4.0	100±7	101±1	92±12	106±7	0.75	104±11
Surrogates									
TBB	3–9	61±3	4.5	34±10	28±12	8.1±5.1	–	4.8	90±8
PCB 209	5	64±15	–	–			–	1.7	97±11

Test 1: Rotary evaporation (20°C, 20 Torr) to 2 ml and concentration until dryness under a gentle current of nitrogen (10–20°C).
Test 2: Soxhlet reflux (18 h), rotary evaporation to 2 ml and concentration until dryness under a gentle current of nitrogen.
Test 3: Rotary evaporation until dryness and concentration until dryness under a gentle current of nitrogen.
Freeze–drying (−60°C, 0.1 Torr, 16 h) recoveries were determined from a different standard dissolved in acetone. Recovery for PCB 30 (gas chromatographic elution just after HCB) of this standard was 72%. The final extract was not brought to dryness when concentrating under nitrogen.
[a] Test 1+sulphuric acid treatment+emulsion.
[b] Mean.
[c] Standard deviation.

solutions are brought to dryness are not only produced in vacuum rotary evaporation. Concentration under nitrogen stream to dryness is another source of important recovery decreases. Thus, evaporation of the vial solutions to a small volume (ca. 1–2 μl) and not to dryness is reflected in recovery of 92±10% (n=10) for tetrabromobenzene whereas recoveries of 61±3% (n=4) are found in the current case (Table 1). Evaporation to these small volumes only involves dilution errors of 1–0.5% which are largely compensated by the dramatic recovery improvement. In any case, the recovery losses can be compensated by the surrogate.

Freeze–drying is another potential source for evaporation losses. These have also been tested using a standard solution dissolved in acetone (Table 1). The losses due to this step are minor than those observed by rotary vacuum evaporation and nitrogen stream concentration.

3.2. Clean-up

Standard solutions of the target compounds have been treated with sulphuric acid in order to evaluate for possible losses. As shown in Table 1, the recoveries obtained in this test range between 80–105%. Since the recoveries of the less volatile compounds are in the interval of 103–105%, the lower values of HCHs, HCB and PCBs 28 and 52 are likely related with evaporation losses.

Examination of the GC–ECD traces have not shown the formation of any derivative of the standards included in the test mixture. In other controls, the standard mixtures were repeatedly treated with sulphuric acid several times (1–4) and no significant concentration changes were observed as consequence of further oxidation.

However, it must be indicated that organochlorine compound losses may be produced at this step as consequence of the formation of emulsions which may difficult the complete recovery of the *n*-hexane phase. The possible formation of these emulsions depends on the lipid content of the fish tissue digested with sulphuric acid. Recovery losses due to this effect will also be reflected in the surrogate.

3.3. Extraction

Sample grounding with sodium sulfate is one of the tasks requiring more work load among those outlined in Fig. 1. Since the main objective of this step is drying for efficient solvent extraction, the alternative choice of freeze–drying has also been evaluated. Matrix effects are the main aspects of concern in this case. Thus, the test has been performed by replicate analysis of fish sample aliquots and not standard mixtures.

As shown in Table 2, a systematic difference is observed between the two methods. In all cases the concentrations obtained by sodium sulphate grounding are higher than with freeze–drying. The differences are significant at >0.80 confidence level. Only in the case of HCB and PCB 180 the concentration differences are not significant. The precision of the two methods had been previously compared and no significant difference at 0.95 level was found in most cases.

Soxhlet extraction efficiency after 18 h reflux time has also been evaluated. Four sodium sulphate-dried fish samples extracted with the regular procedure have been extracted for an additional period of 18 h. A representative example of the GC–ECD profiles obtained in the analysis of these four replicates is shown in Fig. 2. No significant amounts of organochlorine compounds are left after the 18 h regular extraction period.

3.4. Analytical precision vs. environmental dispersion of concentrations

A mandatory aspect of any analytical procedure for environmental studies concerns its precision which should be higher than the dispersion of results in the system under study. Thus, the repeatability of the present method, e.g., the value under which the absolute difference between two results obtained by the same operator with the same instrument in the same laboratory and in a short period of time is expected to lie with a probability of 95%, has to be evaluated in terms of the environmental dispersion of organochlorine concentrations. For this purpose, five fish from Lake Stavsvatn (Norway) encompassing similar lengths (25–33 cm) and ages (2–3 years) have been selected for this purpose. This example constitutes a population of fish encompassing low dispersion values since all them were collected in the same lake and their length and ages fall within narrow ranges.

Table 2
Concentrations (pg/g wet mass) of organochlorine compounds in fish muscle of four trouts collected in Jörisee Lake

Compound	Sulphate drying		Freeze–drying	
	Individual measurements	Mean±S.D.	Individual measurements	Mean±S.D.
α-HCH	0.07[a]±0.01[b]		0.04	
	0.04±0.001		0.04±0.002	
	0.07±0.003		0.05±0.001	
	0.09±0.001	0.07±0.02	0.07±0.005	0.06±0.02
HCB	0.10±0.01		0.02	
	0.06±0.01		0.09±0.02	
	0.11±0.02		0.13±0.06	
	0.13±0.08	0.10±0.03	0.09±0.02	0.11±0.02
PCB 28	0.05±0.02		<0.01	
	0.01±0.001		<0.01	
	0.04±0.002		<0.01	
	0.05±0.005	0.04±0.02	<0.01	<0.01
PCB 101	0.40±0.20		<0.01	
	0.17±0.06		<0.01	
	0.27±0.03		<0.01	
	0.29±0.02	0.28±0.09	<0.01	<0.01
p,p′-DDE	2.68±1.3		1.16	
	1.97±0.33		1.19±0.03	
	1.54±0.17		1.23±0.12	
	2.24±0.24	2.10±0.48	0.86±0.007	1.29±0.43
PCB 118	0.33±0.18		0.02	
	0.16±0.04		0.01±0.001	
	0.31±0.05		0.01±0.02	
	0.30±0.04	0.27±0.08	0.03±0.02	0.09±0.14
PCB 153	0.73±0.37		0.36	
	0.55±0.11		0.41±0.01	
	0.39±0.07		0.27±0.06	
	0.79±0.13	0.61±0.08	0.33±0.01	0.40±0.14
p,p′-DDT	1.28±0.67		0.56	
	0.54±0.08		0.19±0.02	
	0.58±0.12		0.46±0.15	
	0.79±0.23	0.79±0.34	0.39±0.04	0.43±0.20
PCB 138	0.72±0.38		0.36	
	0.52±0.11		0.31±0.01	
	0.41±0.08		0.33±0.12	
	0.74±0.13	0.60±0.16	0.36±0.001	0.39±0.12
PCB 180	0.20		0.19	
	0.29±0.10		0.20±0.01	
	0.18±0.04		0.18±0.04	
	0.40±0.07	0.27±0.10	0.23±0.03	0.22±0.05

[a] Mean.
[b] Standard deviation.

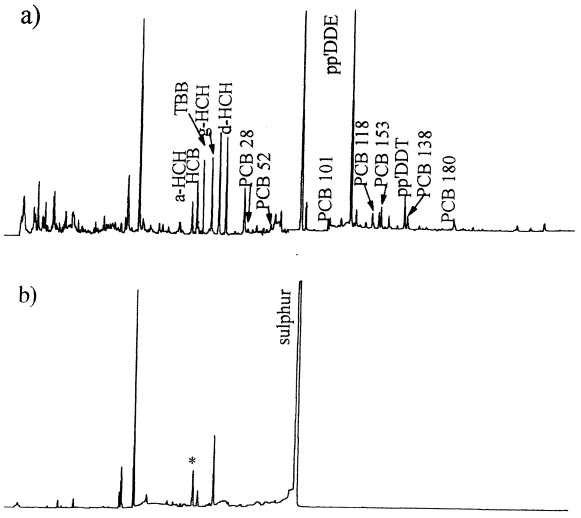

Fig. 2. GC–ECD profiles showing: (a) the organochlorine compounds in a Stavsvatn fish muscle obtained by sodium sulphate drying and Soxhlet extraction (18 h), (b) the GC–ECD amenable compounds after a further 18 h period of Soxhlet extraction (*) peak eluting close to HBB.

In Table 3, the organochlorine concentrations of this population are compared with the repeatability obtained from three replicates of one fish sample. In all cases except α-HCH the dispersion of the method (±0.01–0.1 ng/g) is lower than the dispersion of the analyses of fish from the same lake. The means and standard deviations of these two groups of samples are represented in Table 4.

Table 3
Comparison of repeatibility and reproducibility of the method described in the present study with the dispersion of concentrations between fish of similar length and age from the same lake (n=5)

Compound	Repeatibility 1.96 σ_P $\sqrt{2}$	Reproducibility 1.96 σ_R $\sqrt{2}$	Dispersion between similar fish
α-HCH	0.72	1.3	0.019
HCB	0.25	0.25	0.39
γ-HCH	0.14	0.09	0.22
PCB 28	0.28	0.06	0.19
PCB 52	0.22	0.12	0.75
PCB 101	0.05	0.33	0.42
p,p'-DDE	1.9	3.5	8.6
PCB 118	0.19	0.36	2.0
PCB 153	0.75	1.1	4.0
p,p'-DDT	0	0.18	2.1
PCB 138	1.1	0.78	4.2
PCB 180	0.50	1.3	4.1

Units in ng/g.

Another aspect to be considered is method reproducibility, e.g., the values under which the absolute difference between two results obtained by different personnel, different instruments and different laboratories or between long periods of time is expected to lie within a probability of 95%. According to this definition, reproducibility was calculated by repeated analysis of the same sample by another analyst eight months after the initial determinations. The results show again a smaller dispersion than the variability

Table 4
Comparison of the dispersion of the analytical method (replicate analysis of one fish collected in Stavsvatn Lake) with the dispersion of a fish population (similar mass and length) from the same lake

Compound	Replicates (same fish)		Different fish (same lake)	
	n=3	% S.D./Mean	n=5	% S.D./Mean
α-HCH	0.40[a]±0.16[b]	40	0.10[a]±0.04[b]	40
HCB	0.22±0.08	36	0.35±0.14	40
γ-HCH	0.12±0.04	33	0.21±0.08	38
PCB 28	0.06±0.01	17	0.14±0.07	50
PCB 52	0.06±0.05	83	0.28±0.27	96
PCB 101	0.08±0.02	25	0.24±0.15	62
p,p'-DDE	4.38±0.67	15	5.05±3.09	61
PCB 118	0.31±0.07	23	0.83±0.71	86
PCB 153	1.01±0.27	27	1.67±1.44	82
p,p'-DDT	<0.02		0.55±0.80	150
PCB 138	1.12±0.39	35	1.63±1.50	94
PCB 180	0.88±0.18	20	1.67±1.48	88

[a] Mean.
[b] Standard deviation.

Table 5
Detection and quantitation limits of the method described in the present study

Compound	Limit of detection (pg/g)	Limit of quantitation (pg/g)
α-HCH	6.9	7.6
HCB	7.4	8.1
γ-HCH	9.1	10
PCB 28	11	12
PCB 52	8.8	9.7
PCB 101	9.1	10
p,p′-DDE	11	12
PCB 118	13	14
PCB 153	8.9	9.8
p,p′-DDT	22	24
PCB 138	12	13
PCB 180	12	14

between the group of fish from the same lake (Table 3).

3.5. Limits of detection and quantitation

Calibration curves of the compounds included in Table 1 were generated by progressive dilution of standard mixtures and subsequent instrumental analysis. The regression lines of the curves closer to the lowest detectable concentration range were used to determine the ordinate at the origin and its standard deviation. The abscissa corresponding to this ordinate value+three-times the standard deviation was taken as detection threshold. The quantitation threshold was calculated as the abscissa corresponding to the ordinate+ten-times the standard deviation. These thresholds were transformed into detection and quantitation concentration limits by reference to 5 g of muscle tissue using the dilution steps indicated in Section 2. The resulting limits (Table 5) exhibit about the same values for all compounds (in the order of 10 pg/g).

4. Conclusions

The method described in Fig. 1 is useful for the analysis of trace organochlorine compounds in large numbers of muscle fish samples providing higher precision than that observed in the most uniform fish populations.

Among all sample handling steps, evaporation losses has been observed to constitute the main aspect of recovery decrease. They can be minimized to less than 10% when it is avoided that the extract go to dryness. In any case, the effect of these losses can be compensated by correction by the TBB surrogate.

Sample grounding with sodium sulphate provides significant higher concentrations than freeze–drying. Soxhlet extraction for 18 h is sufficient to draw most organochlorine compounds from the muscle samples, no significant peaks representing measurable concentrations of these compounds have been found by further extraction after this period.

Repeatability and reproducibility is smaller than the dispersion between fish of similar length and age from the same lake for all compounds except α-HCH.

Acknowledgements

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References

[1] S.L. Simonich, R.A. Hites, Science 269 (1995) 1851.
[2] B. Hileman, Chem. Eng. News 19 (1993) 11.
[3] J.O. Grimalt, J. Sunyer, V. Moreno, O.C. Amaral, M. Sala, A. Rosell, J.M. Antó, J. Albaigés, Int. J. Cancer 56 (1994) 200.
[4] L. Berdie, M. Santiago-Silva, R. Vilanova, J.O. Grimalt, J. Chromatogr. A 778 (1997) 23.
[5] D.W. Kuehl, M.J. Whitaker, R.C. Dougherty, Anal. Chem. 52 (1980) 935.
[6] B. Bush, J. Snow, R. Koblintz, Arch. Environ. Cont. Toxicol. 13 (1984) 517.
[7] M. Aceves, J.O. Grimalt, J. Albaigés, F. Broto, L. Comellas, M. Gassiot, J. Chromatogr. 436 (1988) 503.
[8] E.B. Ofstad, G. Lunde, K. Martinsen, Sci. Total Environ. 10 (1978) 219.
[9] L.G.M.Th. Tuinstra, W.A. Traag, H.J. Keukens, J. Assoc. Off. Anal. Chem. 63 (1980) 952.
[10] BCR Certificates of chlorobiphenyls in waste mineral oil, Protocol for Reporting Results, Institute for Environmental Studies. Amsterdam, 1995, pp. 1–14.
[11] W. Horwitz, in: M. Parkany (Ed.), Proceedings of the 7th International Harmonization Symposium on a Protocol for Recovery Factors, Royal Society of Chemistry, Cambridge, 1996, pp. 1–6.