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Comparison of Techniques for the Extraction of Lipids and PCBs from Benthic Invertebrates

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Reliable extraction techniques for organic contaminants in biological samples are needed both for monitoring studies and for fundamental research. Because many contaminants are mainly associated with the lipid fraction, concentration levels are frequently expressed on a lipid basis. Therefore, both lipids and contaminants should be efficiently extracted.

A comparison between saponification followed by liquid-liquid extraction and soxhlet extraction of fish samples dried with sodium sulfate was made by de Boer (1988). He showed that non-polar solvents were less efficient in extracting PCBs and lipids than polar solvents (dichloromethane) or mixed polar/non-polar solvents (pentane/dichloromethane, hexane/acetone). He also showed that saponification was the most efficient method for extraction of PCBs. Lipids were most efficiently extracted using the total lipid extraction according to Bligh and Dyer (1959). In this method, the lipids are extracted with a one-phase mixture of water, methanol, and chloroform. De-mixing is induced by adding a volume of chloroform. The addition of water causes a nearly complete transfer of the lipids to the chloroform phase. The volume ratios are critical (see below). Other mixed solvents used in slurry extractions of PCBs and lipids are hexane/acetone (Jensen et al. 1983), and dichloromethane/methanol (Storr-Hansen and Cederberg 1992).

The methods for PCB extraction described above suffer from several disadvantages. The soxhlet method includes drying of the sample with anhydrous sodium sulfate. Although one gram of water can be bound by 0.8 g of sodium sulfate, the amount needed to obtain a free flowing powder is much larger (approximately 5 g per gram tissue). As a result, the solvent demand is quite large. In our experience, the blank values of the method are often problematic, and the results are sometimes erratic. Saponification suffers from the disadvantages that a separate lipid determination has to be made, and that many organic contaminants are decomposed. The slurry extraction method with mixed solvents must be applied several times before the extraction is complete. In this paper, we describe a modification of the Bligh and Dyer method that uses dichloromethane instead of chloroform. The extraction

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Table 1. Required solvent amounts for the total lipids extraction. The amount of water to be added in the first addition is calculated from the sample mass (m) and the fraction of water in the sample (x).

mass of water in sample	first addition			second addition	third addition
	water	$CHCl_3$ (or CH_2Cl_2)	MeOH	$CHCl_3$ (or CH_2Cl_2)	water
(g)	(g)	(mL)	(mL)	(mL)	(mL)
$m x < 4$	$4 - m x$	5	10	5	5
$m x < 8$	$8 - m x$	10	20	10	10
$m x < 12$	$12 - m x$	15	30	15	15

of lipids and PCBs is nearly complete in one extraction step. Results are compared with soxhlet extraction with pentane/dichloromethane (50/50 v/v) and with saponification.

MATERIALS AND METHODS

The lugworm *Arenicola marina* and the common mussel (*Mytilus edulis*) were used as test species. Homogenised samples were stored at -10 °C. After thawing, the homogenate was stirred for one min with a spoon. Sub-sample amounts were 6.5 g for *A. marina* and 3 g for *M. edulis*. All samples were analysed three times in duplicate, with two week intervals. In addition, a certified reference material (cod liver oil, CRM 349) obtained from the European Community Bureau of Reference was analysed using the dichloromethane/methanol extraction. Concentrations of CB28, CB52, CB101, CB153, CB180 are certified for this reference material (Griepink et al. 1988).

Sodium sulfate was extracted overnight with dichloromethane. After solvent evaporation on a water bath, the powder was heated overnight at 350 °C. Bidistilled water was used without further purification. CB112 was used as an internal standard.

Prior to soxhlet extraction, the tissue was ground with anhydrous sodium sulfate until a free flowing powder was obtained. After addition of the internal standard, the mixture was transferred to a soxhlet apparatus and was extracted overnight with 200 mL of pentane/dichloromethane (50/50 v/v). Extracts were concentrated to 3 mL on a water bath.

After addition of 50 mL of 3% (w/v) KOH in methanol, the samples were saponified for 3 hr in a water bath at 70 °C. The mixture was transferred to a separatory funnel, and 30 mL of pentane was added. After shaking, 50 mL of bidistilled water

Table 2. Lipid contents as a percentage of wet weight (mean ± standard deviation). The number of determinations is indicated between parentheses.

	CH_2Cl_2/MeOH	Soxhlet	Bligh & Dyer
A. marina	0.85 ± 0.04 (4)	0.94 ± 0.15 (6)	0.87 ± 0.02 (6)
M. edulis	1.34 ± 0.02 (6)	1.38 ± 0.08 (6)	1.50 ± 0.10 (6)

Table 3. PCB concentrations (ng/g wet weight) in *A. marina* (average ± standard deviation). The coefficient of variation is indicated between parentheses.

compound	CH_2Cl_2/MeOH (n=6)		Soxhlet (n=5)		Saponification (n=6)	
CB 28	0.11 ± 0.01	(7%)	0.10 ± 0.01	(10%)	0.16 ± 0.10	(61%)
CB 52	0.15 ± 0.01	(10%)	0.26 ± 0.13	(51%)	0.32 ± 0.25	(77%)
CB 101	0.64 ± 0.06	(9%)	0.79 ± 0.19	(24%)	0.62 ± 0.09	(15%)
CB 118	0.76 ± 0.04	(6%)	0.85 ± 0.18	(21%)	0.63 ± 0.17	(27%)
CB 153	2.29 ± 0.13	(6%)	2.28 ± 0.36	(16%)	1.69 ± 0.53	(31%)
CB 180	0.26 ± 0.03	(10%)	0.27 ± 0.07	(26%)	0.15 ± 0.06	(41%)

Table 4. PCB concentrations (ng/g wet weight) in *M. edulis* (average ± standard deviation). The coefficient of variation is indicated between parentheses.

compound	CH_2Cl_2/MeOH (n=6)		Soxhlet (n=6)		Saponification (n=6)	
CB 28	0.29 ± 0.01	(4 %)	0.30 ± 0.02	(5 %)	0.35 ± 0.04	(10 %)
CB 52	0.71 ± 0.12	(16 %)	0.77 ± 0.09	(12 %)	0.63 ± 0.21	(34 %)
CB 101	3.45 ± 0.20	(6 %)	3.30 ± 0.22	(7 %)	2.84 ± 1.40	(49 %)
CB 118	2.67 ± 0.11	(4 %)	2.55 ± 0.15	(6 %)	2.21 ± 1.10	(50 %)
CB 153	9.08 ± 0.50	(5 %)	8.55 ± 0.56	(7 %)	7.07 ± 3.54	(50 %)
CB 180	0.59 ± 0.07	(12 %)	0.56 ± 0.04	(7 %)	0.46 ± 0.23	(50 %)

Table 5. Blank values (average ± standard deviation) expressed as ng/g wet weight for a hypothetical sample mass of 5 g. (ND = not detected.)

compound	CH_2Cl_2/MeOH (n=3)	Soxhlet (n=3)	Saponification (n=5)
CB 28	0.002 ± 0.003	0.011 ± 0.004	0.10 ± 0.17
CB 52	0.06 ± 0.05	0.14 ± 0.09	0.12 ± 0.07
CB 101	ND	0.25 ± 0.29	0.02 ± 0.02
CB 118	ND	0.16 ± 0.22	0.02 ± 0.02
CB 153	0.005 ± 0.004	0.12 ± 0.11	0.03 ± 0.04
CB 180	ND	0.026 ± 0.002	0.007 ± 0.006

was added. The aqueous phase was extracted again with 30 mL of pentane. The combined extracts were shaken twice with 50 mL of bidistilled water. After addition of 1 mL of 2,2,4-trimethylpentane the extract was concentrated to 1 mL.

For the total lipids determination solvents were added to the samples in order to obtain a water:chloroform:methanol volume ratio of 8:10:20 (first addition, Table 1). The water content had been determined by drying at 60 ^{o}C until constant weight. The mixture was stirred in an Ultra Turrax for 2 min. Then a volume of chloroform was added (second addition, Table 1), and the mixture was stirred for 2 min. Finally, water was added (third addition, Table 1), and the mixture was stirred for 30 s. After centrifugation for 20 min at 3000 min^{-1}, the upper (water/methanol) layer was removed with a syringe. The lower (chloroform) layer was transferred with a capillary pipette to a round bottom flask. The residue was rinsed twice with 3 mL chloroform on a vortex mixer. Provided that the aqueous layer was removed almost completely, no centrifugation was necessary to establish phase separation in this step. The combined extracts were concentrated to 3 mL on a water bath.

Samples were also extracted with dichloromethane, methanol, and water, following the total lipids extraction procedure, but using dichloromethane instead of chloroform. Prior to extraction of a new sample, the Ultra Turrax was cleaned by rinsing with a few mL of bidistilled water and acetone in a measuring cylinder that just fitted around the shaft.

The mass of the lipids was determined by transferring the concentrated extract to a pre-weighed petri dish, and rinsing twice with 2 mL of dichloromethane. Care was taken to prevent entrainment of sodium sulfate grains and other solids. Whenever a large amount of solids was present, the extract was filtered over anhydrous sodium sulfate contained in a capillary pipette. Solvents were evaporated by placing the petri dishes on a water bath. Finally, the extracts were dried for 10 min at 60 ^{o}C in an oven. Samples were weighed after cooling down for at least 20 min in a dessicator.

For the determination of PCB concentrations, the lipids were dissolved in 3 mL of dichloromethane, and were transferred to a graduated test tube. One mL of 2,2,4-trimethylpentane was added. The dichloromethane was evaporated on a water bath. The lipids could not be dissolved in trimethylpentane directly, due to slow dissolution kinetics. Sample cleanup and fractionation was done according to Duinker and Hillebrand (1983). Samples were analysed on a Carlo Erba HRGC 5160 equipped with an AS200 autosampler, a CP-Sil 8 capillary column (60 m length, 0.15 mm id), and an electron capture detector (constant current mode).

RESULTS AND DISCUSSION

The lipid content of the samples as determined by three different methods is shown in Table 2. For *M. edulis*, the Bligh and Dyer extraction yielded significantly higher

Table 6. PCB concentrations (ng/g wet weight) in cod liver oil (CRM 349) as determined using the CH_2Cl_2/MeOH method (average ± standard deviation), and the certified value (average ± 95% confidence interval).

compound	CH_2Cl_2/MeOH (n=3)	certified value
CB 28	64 ± 3	68 ± 7
CB 52	140 ± 7	149 ± 20
CB 101	330 ± 19	370 ± 17
CB 118	412 ± 34	454 ± 31
CB 153	970 ± 37	938 ± 40
CB 180	309 ± 31	280 ± 22

lipid contents than soxhlet extraction or CH_2Cl_2/MeOH extraction (95% confidence level). The difference was smaller than 6%, however. For *A. marina*, no statistically significant differences between the three methods were observed.

Repeated extraction of all samples with the CH_2Cl_2/MeOH method showed that the lipids are quantitatively extracted in the first extraction (97-99%). Decreasing the amount of methanol to 50% of the value listed in Table 1 resulted in lower lipid yields (84-94%, *A. marina*).

Both for the total lipids extraction and for the CH_2Cl_2/MeOH extraction, centrifugation was found to be the only practical way to establish phase separation. Filtering the mixture over glass wool or glass fibre filters caused severe clogging. During mixing with the Ultra Turrax, a significant weight loss was observed (1.1 to 2.1 g) probably due to the evaporation of chloroform. This amount corresponds to 4 to 7% of the chloroform added. When, after centrifugation, this phase is sub-sampled to determine the lipid content, the weight loss has to be accurately known. Therefore, the weight of each solvent addition must be measured and laborious corrections must be applied. Transferring the complete chloroform layer proved to be more practical. An additional advantage of removing the entire chloroform layer is that the residue can be extracted again with chloroform in order to remove the final 4% of the lipids, as recommended by Bligh and Dyer (1959). The same observations were made when chloroform was replaced with dichloromethane.

Results for PCB concentrations in *A. marina* and *M. edulis* are shown in Tables 3 and 4. One result for the soxhlet extraction of *A. marina* was omitted, because the concentrations were higher by a factor of 3. For the average concentrations, no significant differences between the three methods are observed (95% confidence level). Repeatabilities of the methods differed widely, however. The highest coefficients of variation (CV) were observed for saponification (40%), indicating that the procedure used was not suitable for the extraction of PCBs from these organisms. The lowest variation was observed for the dichloromethane/methanol method for both organisms (8%). CV values for the soxhlet method were 25% and

7% for *A. marina* and *M. edulis*, respectively. Blank values were highest for the soxhlet method (Table 5). The lowest blank values were observed for the dichloromethane/methanol method. Results of the analysis of CRM 349 are in close agreement with the certified values (Table 6).

For the total lipid extraction, chloroform can successfully be replaced with dichloromethane. For the extraction of PCBs, the repeatability of the dichloromethane/methanol method compares favourably with soxhlet extraction and with saponification. The organic solvent demand of this method (~ 40 mL) is smaller than for soxhlet extraction (~ 200 mL) or saponification (~ 110 mL).

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