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ARTICLE

Lipid Extraction and Determination of Halogenated Phenols and Alkylphenols as Their Pentafluorobenzoyl Derivatives in Marine Organisms

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A method was developed for the extraction of lipids and analysis of halogenated phenols and alkylphenols in marine organisms. The extraction efficiency was evaluated by comparing the extractable lipid content and the recovery of 13 added phenols from three different marine species (herring, cod, and blue mussel), with the corresponding results from three well-established extraction procedures, the Bligh and Dyer (B&D), the Smedes (S), and the Jensen (J) methods. The J method and the new method, Jensen centrifugation (Jc), gave phenol recoveries of 80−100% for all species, whereas the B&D and S methods gave relatively low recoveries for the most acidic phenols, with recoveries of only 20−50% for pentachlorophenol (PCP) depending on the species. It was concluded that this effect was governed by the dissociation of the phenols and adsorption to the protein tissue during the extraction (due to ionic interactions). To increase the sensitivity of the analysis, the phenols were converted to their pentafluorobenzoyl esters, by using a tetrabutylammonium-catalyzed extractive acylation. The reaction was quantitative within 2 min at room temperature, and the formed derivatives were persistent enough to withstand treatment with concentrated sulfuric acid.

KEYWORDS: Lipid extraction; phenol analysis; halogenated phenols; nonylphenol; derivatization; pentafluorobenzoyl chloride

INTRODUCTION

Since 1967 neutral organic halogenated compounds (OHCs) such as polychlorinated biphenyls (PCB), DDT, and later on polybrominated diphenyl ethers (PBDE) have been analyzed continuously in an ongoing monitoring program of the Baltic biota (*1*). Their acidic counterparts, the halogenated phenolic compounds (HPCs), however, have not been included despite the number of toxic effects they are held responsible for. One physiological property shared by most HPCs is their well-known ability to inhibit oxidative phosphorylation (OXPHOS) in the mitochondria, which disables the production of adenosine triphosphate (ATP) from the phosphorylation of adenosine diphosphate (ADP) (*2*). Disruption of OXPHOS causes the exposed organisms to lose chemical energy in the form of heat, an effect seen when exposed to, for example, elevated concentrations of the widespread pentachlorophenol (PCP) (*2*). Other effects that have been reported for HPCs include altered thyroid and sex hormone status (*3*, *4*).

Reported losses of weight and adipose tissue in Baltic wildlife over the past decades are signs that the ecosystem is out of balance and could indicate the exposure of high concentrations of HPCs causing energy loss. In the above-mentioned monitoring program the fat percentage of herring in the Baltic proper has been recorded over the years, and today the fat percentage is less than half compared to when the program started (*1*). Furthermore, it has recently been reported that the blubber layer of gray seals in the Baltic has gotten thinner (*5*) and that guillemot fledglings have decreased in weight (*6*).

Chlorinated phenols have been used as fungicides in agriculture as well as in wood preservation. Some have been formed unintentionally in the widespread use of chlorine bleaching in the paper and pulp industries (*7*) or formed from aromatic OHCs by metabolization (*8*). Many HPCs (especially brominated phenols) can also be produced by sponges and algae (*9*).

A large number of HPCs (> 100) have been traced in the blood of salmon from the Baltic Sea (*10*). Some of these substances (especially brominated and mixed brominated/chlorinated phenolic compounds) have also been found in other fish species (*11*), red algae (*12*), and blue mussels (*13*) from the Baltic, for example, 6-OH-BDE47, a substance that was recently reported to cause OXPHOS disruption in fish (*14*).

Analytical procedures for HPCs in blood, mother's milk, egg, and some biological tissues have been developed and evaluated during recent years (*15*−*21*). Methods for total lipid extraction, for example, Bligh and Dyer (B&D) (*22*, *23*), Smedes (S) (*24*), and Jensen (J) (*25*), have also been used to extract HPCs from biological tissues (e.g., homogenates of marine organisms or muscle tissue). However, there is still a demand for more efficient methods to analyze HPCs in these types of matrices.

The extraction procedure described in this paper, Jensen centrifugation (Jc), is an improvement of the J method that has

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been modified to be faster and easier. Furthermore, the extraction method has been complemented with suitable workup and derivatization procedures to enable analysis of phenolic compounds.

For GC analysis of phenols, derivatization is still a necessity for enhanced chromatographic properties and sensitivity. However, to avoid the risks associated with handling the commonly used diazomethane, a fast and simple, catalyzed acylation with pentafluorobenzoyl chloride is presented.

The aim of the present paper has been to develop an efficient experimental tool with high yields for phenols and lipids that could be used to analyze phenolic contaminants in marine organisms and other biological samples.

MATERIALS AND METHODS

Chemicals and Reagents. An aqueous buffer solution containing tetrabutylammonium (as a phase-transfer catalyst) was made for the derivatization of phenols by adding sodium hydrogen carbonate (6.3 g) to tetrabutylammonium hydroxide (0.5 M, 100 mL), thus yielding a 0.25 M solution of tetrabutylammonium carbonate, sodium carbonate, and sodium hydrogen carbonate respectively (pH ~11.6); both reagents were of pro analysis quality (Fluka Chemie AG, Buchs, Switzerland). The derivatization reagent, pentafluorobenzoyl chloride (PFBCl), of analytical grade, was also acquired from Fluka. Hydrochloric acid (HCl; Fisher Chemicals, Leicestershire, U.K.), pro analysis, was diluted with water 1:1 (v/v) and extracted twice with dichloromethane (100 mL/L HCl) prior to use, to remove any impurities. Methanol of HPLC grade (Merck, Darmstadt, Germany) was distilled prior to use to remove impurities. Silica gel (0.063−0.200 mm) from Merck was heated at 300 °C overnight and deactivated with water (5% w/w) before use. 2,2′,4,5,5′-Pentachlorobiphenyl (CB101; Larodan Fine Chemicals AB, Malmö, Sweden) was used as a volumetric standard for the phenol fraction. 2,2′,5,6′-Tetrachlorobiphenyl (CB53; Larodan Fine Chemicals AB) was used as internal standard for the neutral fraction. Phenol standards and physical data are listed in **Table 1**; structures can be seen in **Figure 1**.

Instrumentation. The phenol analysis was performed on a HP 5890 gas chromatograph with electron capture detector (GC-ECD) (Hewlett-Packard, Avondale, PA). The GC separation was conducted on a DB-5 (5% phenyl, 95% methylpolysiloxane) fused-silica capillary column (J&W Scientific, Folsom, CA), 30 m × 0.25 mm i.d. × 0.25 μm film thickness, utilizing helium as carrier gas (0.7 mL/min), and argon−methane (30 mL/min) as the makeup gas. The sample (1 μL) was injected in splitless mode (250 °C). The oven temperature was programmed from 80 °C (held for 2 min) to 300 °C at a rate of 10 °C/min (held for 6 min), and the detector temperature was set at 325 °C. The analysis of neutral compounds was performed on a 60 m DB-5 column, 0.25 mm i.d. × 0.25 μm film thickness.

Samples. North Atlantic cod (*Gadus morhua*) fillet was bought deep frozen from a local market. Blue mussels (*Mytilus edulis*) were cultivated at the Swedish west coast and bought at the local fish market. All mussels were alive when received and stored deep frozen until use. Herring (*Clupea harengus membras*) was caught in the outer part of the archipelago of Stockholm and bought as fillet at the local fish market. After the skin was removed, the fillets were deep frozen until use. After thawing, all samples were homogenized in a food processor and divided into 10 g portions; the shells were removed from the blue mussels before homogenization.

To assess the possibility for simultaneous determination of neutral compounds, such as PCBs, two homogenates from an intercalibration study conducted by the Quality Assurance of Information for Marine Environmental Monitoring in Europe (Quasimeme) was analyzed, one Greenland halibut sample (*Reinhardtius hippoglossoides*) (QOR097BT round 54, 2008) and one Mediterranean mussel sample (*Mytilus galloprovincialis*) (QOR093BT round 50, 2007).

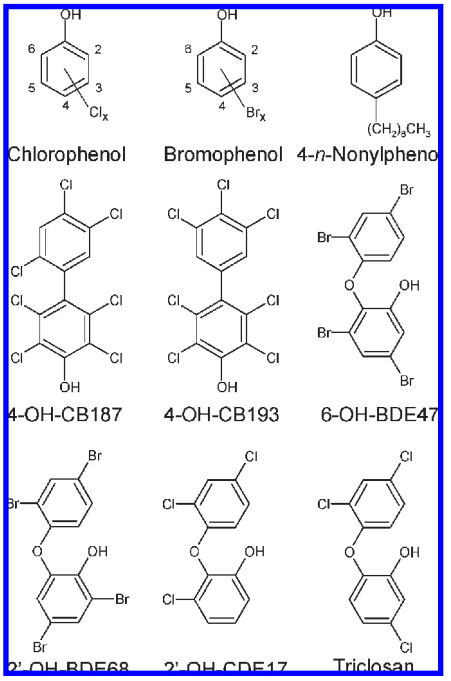

Figure 1. Chemical structures for phenols used in this study.

Table 1. Phenols Used in This Study, Sources, and Calculated pK_a and log K_{ow} (ACD/LABs Software V8.14)

class and individual compound	pK_a	log K_{ow}	source
chloro- and bromophenols			
2,6-dichlorophenol (DCP)	7.0 ± 0.1	2.6 ± 0.2	Aldrich Chemie (Steinheim, Germany)
2,4,6-trichlorophenol (TrCP)	6.6 ± 0.2	3.6 ± 0.3	Aldrich Chemie (Steinheim, Germany)
2,4,6-tribromophenol (TrBP)	6.3 ± 0.2	4.3 ± 0.5	Aldrich Chemie (Steinheim, Germany)
2,3,4,6-tetrachlorophenol (TeCP)	5.6 ± 0.3	4.2 ± 0.3	Aldrich Chemie (Steinheim, Germany)
pentachlorophenol (PCP)	4.7 ± 0.3	4.8 ± 0.4	Aldrich Chemie (Steinheim, Germany)
pentabromophenol (PBP)	4.4 ± 0.3	6.1 ± 0.6	Aldrich Chemie (Steinheim, Germany)
chlorobiphenyls			
4-OH-CB187	4.1 ± 0.5	6.9 ± 0.4	synthesized as described elsewhere (*26*)
4-OH-CB193	4.1 ± 0.5	6.9 ± 0.4	synthesized as described elsewhere (*26*)
bromodiphenyl ethers			
6-OH-BDE47	6.8 ± 0.4	7.2 ± 0.8	synthesized as described elsewhere (*27*)
2′-OH-BDE68	6.6 ± 0.5	7.1 ± 0.7	synthesized as described elsewhere (*27*)
chlorodiphenyl ethers			
2′-OH-CDE17	7.8 ± 0.4	5.0 ± 0.4	unpublished synthesis[a]
triclosan	7.8 ± 0.4	5.2 ± 0.4	Fluka Chemie AG (Buchs, Switzerland)
alkylphenols			
4-*n*-nonylphenol (4-*n*-NP)	10.1 ± 0.2	6.2 ± 0.2	Larodan Fine Chem. (Malmö, Sweden)

[a] A gift from Göran Marsh (Stockholm University, Stockholm, Sweden).

Extraction. A 10 g sample was homogenized for 1 min in a 100 mL glass centrifuge tube with a mixture of 2-propanol (IPR; 25 mL) and *n*-hexane/diethyl ether (Hx/DEE; 3:1 v/v, 20 mL). After centrifugation for 5 min at 2300 rpm (900*g*), the liquid phase was decanted into a 150 mL separatory funnel (i.d. 30 mm). The procedure was repeated with a mixture of IPR (10 mL) and Hx/DEE (3:1 v/v, 40 mL). The combined extracts were washed twice by inverting the funnel 30 times with HCl (0.2 M) in aqueous sodium chloride (0.9% w/v, 50 + 20 mL). The aqueous phases were discarded, and the organic phase was decanted into a preweighed 100 mL round-bottom flask and evaporated to dryness in a rotary evaporator (Büchi, Flawil, Switzerland) at reduced pressure (~360 mmHg) and 60 °C. Any remaining solvents in the sample were evaporated with a gentle stream of nitrogen, and the flask was reweighed until constant weight.

Isolation of Phenols. The extract was resolved and transferred to an 8 mL test tube with *n*-hexane (Hx; 2 + 2 mL). The volume was reduced to 2 mL under a gentle stream of nitrogen and partitioned with a sodium hydroxide solution (0.5 M, in 50% ethanol, 3 + 3 mL). The alkaline fractions were pooled in a 15 mL test tube and washed with Hx (2 mL) (this fraction is pooled with the original 2 mL extract when neutral compounds are analyzed). The alkaline solution was then acidified with HCl (2 M, 2 mL) and extracted with Hx/DEE (3:1 v/v, 4 + 4 mL). The organic fractions (containing acidic components) were pooled in another 15 mL test tube.

Cleanup. The extracts, especially from the cod, contained relatively high amounts of free fatty acids, which contribute to degradation of the derivatization reagent. To minimize the reagent amount needed, the free fatty acids were converted into their methyl esters. The sample volume was reduced to 1 mL under a stream of nitrogen before the addition of methanol (3 mL) containing sulfuric acid (2% w/v) (*28*). After 1 h at 70 °C, water (5 mL) was added and the sample was extracted with Hx/DEE (3:1 v/v, 4 + 4 mL).

Further Cleanup (Mussels). To separate the phenols from interfering polar substances, the sample volume was reduced to 0.5 mL and transferred to a Pasteur pipet (fitted with a plug of silylated glass wool at the bottom) containing silica gel (0.5 g, deactivated with water 5% w/w). The column was washed with Hx (6 mL) before the phenols were eluted with Hx/DEE (3:1 v/v, 6 mL) containing glacial acetic acid (2% w/v). The effluent was collected in a 15 mL test tube and washed with HCl (0.2 M, 2 mL).

Derivatization. Before GC analysis of the phenolic compounds, acylation with PFBCl was carried out. The sample volume was reduced to 3 mL under a stream of nitrogen before the derivatization buffer (2 mL, see Chemical and Reagents) and PFBCl (20% v/v in toluene, 10 μL) were added; the test tube was then shaken for 2 min at room temperature. The aqueous phase was discarded, and the sample was treated with concentrated sulfuric acid (98%, 2 mL), the organic phase was isolated ,and 1 μL was injected on the GC together with the volumetric standard (CB101).

Method Efficiency. To determine the precision of the method and the yields of phenols and lipids, six 10 g samples from each of the three species (herring, cod, and blue mussel) were extracted. Five of the samples were spiked with a mixture of 4-*n*-NP (3 ng/μL) and 12 HPCs (1 ng/μL, respectively) in toluene (100 μL) (see **Table 1**); the sixth sample was used as a blank. By spiking the samples as such, the final concentrations were well within the detector's field of linearity.

The efficiency of the extraction procedure was evaluated by comparing the recoveries with the corresponding results from three well-known cold-solvent extraction methods, making a total of 24 extractions per species. The extractions were performed according to the methods of Bligh and Dyer (B&D), Smedes (S), and Jensen et al. (J) (all phenol recoveries were determined using the cleanup and dervatization described in this paper). Detailed descriptions of the three extraction methods can be found in the Supporting Information.

The limit of detection (LOD) for the detector was defined as a signal with 3 times the height of the chromatographic noise. The limit of quantification (LOQ) for the method was defined as 5 times the height of the noise and was determined by analyzing extracts from spiked 10 g cod samples (which did not naturally contain these substances).

The possibility for simultaneous analysis of neutral OHCs was investigated by extracting samples from intercalibration studies. Two matrices were analyzed (halibut and mussel) using the same method as described above, although after the alkaline isolation of phenols, the neutral fraction was treated with concentrated sulfuric acid and analyzed by GC-ECD.

RESULTS AND DSCUSSION

Method Development. The extraction procedure (Jc) described under Materials and Methods is a modification of the J method (*25*). The same solvents and volumes are used, although they are divided in two extractions instead of three. The proportions of the solvents were chosen to give monophasic solvent systems during the extractions. The filtration procedure used in the J method (see Supporting Information) to separate the extract from the tissue residue has been replaced by centrifugation to avoid clotting of the filter by certain matrices, such as liver or mussel. Furthermore, the combined extracts are washed twice with HCl (0.2 M) to remove all of the added IPR, thus facilitating the evaporation and increasing the recoveries of the most volatile compounds (e.g., low-chlorinated phenols). In the predecessor (*25*) the extract is washed only once with phosphoric acid (0.1 M, 50 mL), thus leaving traces (~8 mL) of IPR, water, and phosphoric acid in the organic phase. To further facilitate the evaporation a rotary evaporator was used at reduced pressure (~360 mmHg) and 60 °C, thus lowering the evaporation time to a couple of minutes.

To isolate the phenols, the extracts (dissolved in Hx) were partitioned twice with a sodium hydroxide solution in 50% ethanol. Due to partitioning in the boundary layer of surfactant-like phenols (e.g., nonylphenol and some hydroxylated biphenyls and diphenyl ethers), one extraction was proved often to be inadequate. However, two extractions were proved sufficient for quantitative recoveries in this study. Although the method was intended for HPCs, it was also developed to enable analysis of nonylphenol. This affects the alkaline partitioning to a larger extent than the other steps, as nonylphenol requires a much larger ratio between the volumes of the alkaline phase and the organic phase than the HPCs in this study do.

Acylation with PFBCl was chosen for the derivatization, and the reaction procedure was performed in combination with a buffer containing tetrabutylammonium (TBA) as a catalyst. TBA has previously been used as a phase-transfer catalyst for the extractive alkylation of phenols with pentafluorobenzyl bromide (*29*, *30*). The extractive acylation from an alkaline aqueous solution is much more selective than alkylation, thus generating less background. It is also a much faster reaction, and for most phenols no catalyst is needed. Yet, for relatively polar phenols with two ortho halogens, this reaction does not proceed as rapidly. The usage of TBA as a phase-transfer catalyst proved to be satisfactory in increasing the rate of the reaction, yielding quantitative formations of PFB esters in a few minutes. The introduction of a PFB group to the phenols provides a higher sensitivity in comparison with nonfluorinated derivatives (e.g., methyl ethers), especially for low-chlorinated and nonhalogenated phenols when working with GC-ECD. Furthermore, all phenols in this study were as their PFB esters persistent enough to withstand treatment with concentrated sulfuric acid in a final cleanup step.

Lipid Determination. The newly developed extraction procedure (Jc) showed significantly higher lipid content for all species (**Table 2**) compared to the S method, using analysis of variance (ANOVA) with the level of significance (α) set to 0.05 [herring ($p = 0.002$), cod ($p < 0.001$), and mussel ($p = 0.041$)]. Compared to the B&D and J methods, it does not differ significantly for all of the species individually. However, by applying a multivariable ANOVA ($\alpha = 0.05$) considering all species, the Jc method showed a significantly greater lipid extraction capability than the B&D

Table 2. Extractable Lipids in 10 g Homogenates of Three Different Marine Organisms Expressed in Percent Using Four Different Extraction Procedures[a]

	lipids (%) (*std error*)		
method	herring	cod	mussel
Jc	3.51 (*0.09*)	1.02 (*0.04*)	1.57 (*0.04*)
J	3.74 (*0.04*)	1.03 (*0.04*)	1.63 (*0.03*)
S	3.29 (*0.08*)	0.58 (*0.01*)	1.50 (*0.05*)
B&D	3.40 (*0.08*)	0.73 (*0.01*)	1.45 (*0.06*)

[a] All determinations were performed in six replicates.

method ($p < 0.001$) and a lower capability than the J method ($p < 0.001$). A small decrease in the lipid yield compared to the J method was expected when speed and simplicity were targeted in the development of the Jc method; for example, the amount of extractions were decreased from three in the J to two in the Jc, and the re-extraction of the aqueous phase was omitted.

Although the relative yields of the B&D and S methods compared to the Jc are well within 90% for herring and mussel lipids, they are only 71 and 57%, respectively, for cod. An explanation for these results might be found in the fat composition of the sample and one particular step of the extraction procedures. All four methods utilize the same principles by homogenizing the sample in a solvent mixture composed to render a one-phase system with the water content of the sample. Whereas the extracts are separated from the insoluble protein tissue without creating a two-phase system in the Jc and J methods, water is added in the B&D and S methods, resulting in a biphasic solvent system. If the pH is not sufficiently suppressed in this step, acidic substances such as free fatty acids and acidic phenols can be solvated in the aqueous phase. Furthermore, these substances can be adsorbed onto the protein tissue due to the addition of sufficient water to yield a biphasic system, as previously described for acidic phospholipids when using the B&D procedure (*31*). The adsorption can partially be explained by direct ionic interaction between acidic lipids, for example, free fatty acids and oppositely charged proteins, for example, cytochrome *c* (a basic protein) (*31*). These effects are due to the pH as well as the composure and concentrations of ions originating from the sample. Although high concentrations of cations will decrease the interactions with positively charged proteins, divalent cations can work as bridges in the formation of complexes between acidic phospholipids and acidic proteins, for example, albumin (*32*). These interactions can be dramatically reduced by the separation of the extract from the tissue residue before the addition of water (*31*). These effects might explain the low lipid recoveries for the cod when using the B&D and S methods. Still, it is unlikely that 30% of the native lipid content in cod fillet consists of acidic lipids. These results are probably due to hydrolysis of the lipids, which liberates vast amounts of free fatty acids, a sign of aging and decomposing of this sample. This theory is supported by the results from earlier publications (*25*), where the J method had a relative yield of 97% compared to the B&D method for the recovery of cod lipids. In this study the relative yield is 143%, thus indicating a big difference in lipid composition between now and then for the same species. The limitation of the S method for degraded samples is not a surprise because it has already been indicated by Smedes (*33*).

Phenol Recovery. For phenols the Jc method was proved to be most versatile and stands out in this study due to its ability to extract the different phenol classes fairly equally. For the OH-PBDEs, OH-PCDEs, and alkylphenols in this study, all methods showed acceptable recoveries between 75 and 100%, with only minor exceptions (slightly lower recoveries for blue mussel). The Jc method has significantly increased the recoveries of the most volatile substances (e.g., 2,6-DCP) compared to the J method (see **Figure 2**). Furthermore, the Jc method shows significantly higher recoveries ($\alpha = 0.05$) for the most acidic monoaromatic phenols ($pK_a < 6.5$) for all species compared to the B&D and S methods, most noticeably for PCP and PBP.

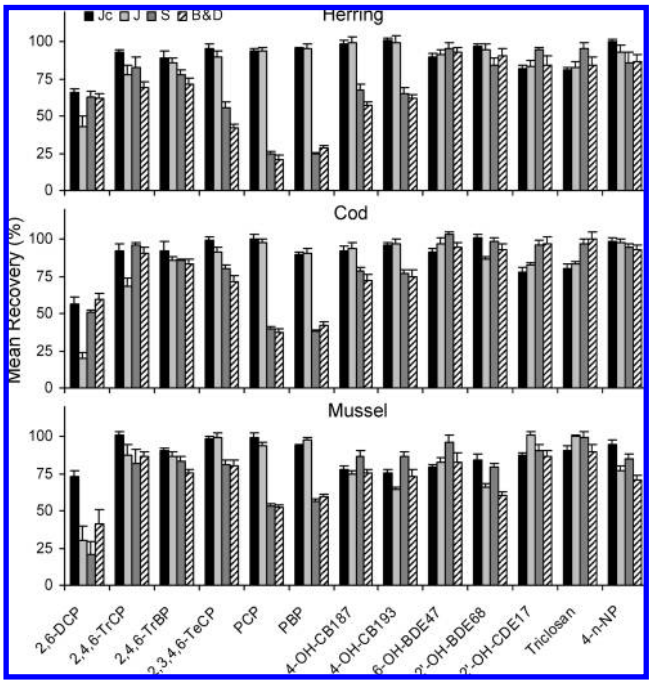

Figure 2. Mean recoveries ($n = 5$) of 13 added phenols from 10 g homogenates of three different marine organisms, expressed in percent using four different extraction procedures.

The low recoveries of the most acidic phenols ($pK_a < 6.5$) when using the B&D and S methods can be explained by the same ionic interactions with proteins as for the acidic lipids, as well as the solubility of the phenolate ions in the aqueous phase. Still, it is obvious that the poor recoveries are not solely due to the pK_a when the results for the highly acidic OH-PCBs are evaluated. Although the recoveries are lower than for the less acidic OH-PCDEs and OH-PBDEs in this study, they are still much higher than for the somewhat less acidic monoaromatic phenols such as PCP and PBP (see **Figure 2**). Considering the likenesses in both pK_a and log K_{ow} between the OH-PCBs in this study and PBP, one would not expect such a vast difference in recovery. Furthermore, the only structural difference between the OH-PCBs in this study and PCP is the para orientated trichlorophenyl group. However, as stated earlier (see Method Development) the second phenyl group gives detergent-like properties to the phenols, resulting in partitioning in the boundary layer of the solvents, which in this case increases the recoveries due to less ionic interactions with proteins.

The results of the lipid content for the cod and the low recovery of the most acidic phenols, for example, PCP and PBP, gained by the B&D and S methods (see **Table 2** and **Figure 2**) emphasize the importance of separating the extract from the protein tissue before the addition of water to yield a two-phase system, as done in the Jc method. Furthermore, a pH reduction of the aqueous phase is necessary when the layers are separated in order to suppress the dissociation of the free fatty acids and phenols, which otherwise renders them freely soluble in the aqueous phase.

Limit of Detection and Limit of Quantification. The LOQ for the method (see **Table 3**) was comparable with those of

Table 3. LOD for the Detector and LOQ for the Method (10 g Cod Sample)

compound	LOD (pg)	LOQ (pg/g)
2,6-DCP	0.08	20
2,4,6-TrCP	0.07	10
2,4,6-TrBP	0.08	10
2,3,4,6-TeCP	0.07	10
PCP	0.04	5
PBP	0.15	40
4-OH-CB187	0.20	50
4-OH-CB193	0.30	60
6-OH-BDE47	0.25	50
2′-OH-BDE68	0.20	40
2′-OH-CDE17	0.20	50
triclosan	0.20	50
4-*n*-NP	0.30	50

other previously described methods for these types of compounds (*15*, *18*). Still, the use of mass spectrometry, for example, electron capture negative ionization mass spectrometry (ECNI-MS), can significantly increase the selectivity and sensitivity of the method. The use of PFB has been proved to be quite affective in combination with ECNI-MS, and for some analytes, for example, triclosan, the relative response factor in comparison with the methyl ether is even 4 times higher than it is on GC-ECD (*15*).

Simultaneous Analysis of Neutral Organohalogen Compounds. The results from the intercalibration samples were well within the margins set by Quasimeme for the seven PCBs analyzed (CB28, CB52, CB101, CB118, CB138, CB153, CB180) as well as the extractable lipids. Thus, the method was proved to be quite capable of simultaneous determination of neutral compounds and phenols.

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Supporting Information Available: Detailed descriptions of the three methods, Bligh and Dyer (B&D), Smedes (S), and Jensen (J), as well as the mean recovery and deviation of all phenols from the recovery study. This material is available free of charge via the Internet at http://pubs.acs.org.

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