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Optimization of a matrix solid-phase dispersion method with sequential clean-up for the determination of alkylphenol ethoxylates in biological tissues

M. Zhao, F. van der Wielen, P. de Voogt*

Department of Environmental and Toxicological Chemistry, Amsterdam Research Institute for Substances in Ecosystems, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands

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Abstract

A modified Matrix Solid-Phase Dispersion (MSPD) method with sequential clean-up has been developed to isolate and purify alkylphenol ethoxylates (APEs) and alkylphenols in biological tissues. Elution profile, sequential clean-up adsorbent and experimental set up were optimized. Octadecylsilica was used as the solid-phase for matrix dispersion. Methanol was found to be the optimal eluting solvent for APEs. Aluminum oxide was quite efficient for removing the coeluting interferences. Quantitative analysis was done by reversed-phase HPLC with fluorescence detection. The optimized procedure was applied to analyze both fish and mussel samples. Average recoveries for all spiked tissue samples were greater than 90%. Typical limits of detection amount to tens of ppbs on a wet weight basis.

Keywords: Matrix solid-phase dispersion; Alkylphenol ethoxylates; Nonylphenol; Surfactants

1. Introduction

Alkylphenol ethoxylates (APEs) are among the most widely used non-ionic surfactants in the world. Their biodegradation products, namely: Alkylphenol (AP), Alkylphenol monoethoxylates (AP1E) and Alkylphenol diethoxylates (AP2E) are found to be relatively persistent and lipophilic, thus having a potential to accumulate in organisms. Due to their estrogenic property, these metabolites may interfere with the reproductive success of fish. Jobling and Sumpter [1] have found that low levels (μg/l) of nonylphenol (NP) and NP2E can induce the production of vitellogenin (a precursor of the egg yolk, the synthesis of which is estrogen-controlled) in cultured rainbow trout hepatocytes. In early studies, Soto et al. [2] reported that 4-NP could possibly induce cell proliferation in MCF-7 human breast tumor cells, which are estrogen-sensitive. Acute toxicities of NP for aquatic organisms range from 0.04 to 5 mg/l. Therefore, development of simple

*Corresponding author. Tel.: +31-20-5256565; fax: +31-20-5256504.

E-mail address: pim@mtc.chem.uva.nl (P. de Voogt)

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and sensitive analytical methods for APEs in biological samples is important.

High-performance liquid chromatography (HPLC) with fluorescence detection (FLU) has been proven to be a successful method for the determination of APEs in water and sediment samples [3]. However, isolation of APEs from biological tissues has been a complicated and laborious task because of the nature of the matrix. Its cellular structure needs to be disrupted and there is a high abundance of proteins and lipids. Classical methods for the extraction of APEs from biological tissues, such as Soxhlet extraction and steam distillation, usually take hours and consume relatively large amounts of solvents, and often require manipulation in many steps, like centrifugation, filtration and evaporation, which may lead to low recovery or poor reproducibility. Classical clean-up methods like adsorption chromatography have been proven to be successful for persistent and lipophilic compounds, but their applicability to APEs needs further positive confirmation.

Matrix Solid-Phase Dispersion (MSPD) is a relatively newly developed extraction-clean-up technique [4]. In this approach, a small amount of sample tissue (0.1–5 g) is blended together with the selected solid-phase. The solid mixture is used as a column packing material. According to the solubility characteristics of the compounds of interest, a proper solvent or solvent sequence are used to elute the MSPD column. The extracts are generally clean for analysis, and if necessary, can also be further purified. This method provides a relatively simple process for sample homogenization, cellular disruption, extract fractionation and purification [5]. Recently, this type of pretreatment has been applied successfully to the determination of another kind of widely used non-ionic surfactants, i.e. alcohol ethoxylates, in fish [6].

This paper reports a modification of the MSPD procedure for the sequential isolation and adsorption chromatography (AC) clean-up of APEs from biological tissue samples, followed by separation and quantification by HPLC with fluorescence detection. Solid-phase material, elution profile and further clean-up adsorbents of the method were optimized. The optimized procedure was applied to analyze fish and mussel tissue samples.

2. Experimental

2.1. Materials

2.1.1. Reagents and standards

The solid-phase material used for MSPD was Bakerbond Octadecyl (C18), 40 μm (Baker, Deventer, The Netherlands). Adsorbents used for clean-up were (1) Aluminum oxide (neutral, 0.063–0.200 mm, 70–230 mesh ASTM); (2) Silica (230–400 mesh ASTM), from E. Merck, Darmstadt, Germany. Silica was first activated (at 180°C, after washing with dichloromethane, DCM), whereas aluminum oxide was subjected to the Brockman activity test before use. Both adsorbents were then deactivated with 5% (w/w) demineralized water before use. HPLC grade methanol (MeOH), dichloromethane and acetonitrile were purchased from Rathburn, Walkerburn, Scotland.

Stock and calibration solutions of alkylphenols and APEs were prepared in methanol and preserved at 4°C.

Glass fibre filters of size 0.45 μm (Whatman) and 0.2 μm filter units (acrodisc PVDF, Gelman) were used for column set up (see Fig. 1).

t-Octylphenol (OP) was obtained from Aldrich, Belgium, 4-nonylphenol (NP) from Fluka, NL, and NP4E and NP10E (mixtures of nonylphenol ethoxylate oligomers with an average number of ethox-

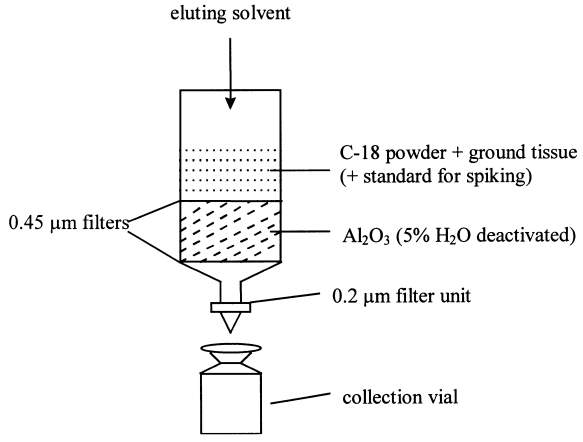

Fig. 1. Schematic drawing of the modified MSPD/sequential clean-up column.

ylate units of four and ten, respectively) were a kind gift from Shell Laboratories, Amsterdam, The Netherlands. The OP, NP and NP10E mixtures were used for blank elution experiments. OP and NP4E were selected for recovery experiments (spiked tissue) to represent early and late eluting standards (in reversed-phase (RP)–HPLC) [7], which are also observed in environmental matrices.

2.1.2. HPLC system conditions

For screening analysis (description given below), the system consisted of a Beckman Model 110A Pump, a Waters 484 Tunable UV Absorbance Detector (at λ=278 nm) and a Shimadzu Chromatopac C-R2AX recorder.

For quantitative analysis, a Waters 600E System Controller, a Waters 717 plus Autosampler and a Waters 474 scanning fluorescence detector were used.

The 125×4 mm analytical column contained 5 μm Lichrospher 100RP-18 (Merck, Darmstadt, Germany) as stationary phase. The mobile phase, operated at a flow-rate of 1.0 ml/min, consisted of an isocratic mixture of methanol–nanopure water (80/20, v/v).

Samples were injected through a Rheodyne 3125 injection valve using a 20 μl loop. The fluorescence detector was operated at the following conditions: excitation λ=225 nm, emission λ=301 nm, gain=100. Since fluorescence detection is much more sensitive than UV detection for the determination of APEs, a lesser amount of standard was added to the tissues for spiking when using FLU.

2.1.3. MSPD Column

A 20-ml glass syringe with a glass plunger was used to construct the MSPD Column. The matrix dispersed solid-phase and sequential clean-up adsorbents were packed into the syringe barrel, where the extraction and purification were carried out, as shown in Fig. 1.

2.1.4. Biological Tissues

Rainbow trout (*Oncorhyncus mykiss*) without innards was purchased at the local commercial food market. The skin was stripped off and the muscle was homogenized, wrapped in aluminum foil and stored deep-frozen (−20°C) until analysis.

Zebra mussels (*Dreissena polymorpha*) were a kind gift from the Laboratory of Aquatic Ecotoxicology, Faculty of Biology, University of Amsterdam, The Netherlands. All soft tissues of the mussel were taken out to make up the tissue sample.

2.1.5. MSPD/sequential AC clean-up operating procedure

2.1.5.1. Pretreatment of octadecylsilica (C18) powder

The C18 material was washed prior to use to remove contaminants that may interfere with the analysis. Twenty grams of C18 powder was put into a clean syringe-barrel (50 ml) with a filter at the bottom. The powder was eluted with about 60 ml of pure methanol, and the solvent exhausted by the plunger. Next, the wet material was transferred into a dry and clean beaker and purged with a gentle nitrogen stream until a free flowing powder was obtained.

2.1.5.2. Preparation of matrix dispersed solid-phase

Between 0.5 and 1 g of biological tissue was weighed and transferred into a coated mortar. For spiking, 100 μl of the standard solution was added with a pipette and the solvent was allowed to evaporate. Two to three grams of prewashed C18 powder was added and the mixture was ground with a coated pestle until a free flowing powder was obtained.

The MSPD column was installed as shown in Fig. 1. A 0.45 μm filter was inserted at the bottom of the syringe barrel. Three grams of deactivated aluminum oxide was transferred into the barrel and another filter was put on top of the Al_2O_3. A 0.2 μm filter unit was attached to the tip of the syringe to filter all the eluate from the column. The Al_2O_3 and filters were washed with 10 ml of methanol before the MSPD elution, and the collected washout was discarded. Then the mixture powder of C18-tissue was transferred onto the column and the syringe was tapped slightly to remove the air pockets inside the material.

2.1.5.3. APEs elution

Pestle and mortar were rinsed three times with a total of 10 ml of methanol and the rinse solution was transferred onto the column. Another 8 ml of methanol were added and the eluate was pressed out with the plunger into a preweighed clean vial at a flow-rate of about 0.5 ml/min. This is the first fraction of APEs elution. Next, the column was eluted with another 5 ml of methanol and the eluate was collected into a second preweighed clean vial. This is the second fraction of APEs elution, used to check if all the APEs compounds had been eluted.

The two fractions of APEs elution were concentrated to about 1 ml on a 60°C water bath under a gentle stream of nitrogen and stored at 4°C in the dark until analysis.

2.2. Optimization of the MSPD procedure

2.2.1. Elution profile screening experiments

The optimization of the elution scheme was conducted stepwise. First, a screening experiment was carried out with NP to select the proper solvents. This experiment involved two elution programs employing different solvents and using recoveries of NP as a criterion to evaluate efficacy and consumption of solvents. The screening experiment was carried out in duplicate, with the objective to quickly find the optimal elution volume. The elution profiles were recorded by collecting several, relatively small fractions. Table 1 lists the two elution programs performed with the spiked blank (i.e. without tissue) samples.

Next, methanol was used (employing the optimal elution volumes found for NP in the screening experiment) to evaluate this elution regime for OP and NP10E, in order to derive the final optimal values relevant for both AP and APE.

2.2.2. Cleanup adsorbents

At first silica was used as the clean-up adsorbent. However, extracts obtained from the silica column turned turbid upon concentration, thereby revealing the presence of possibly interfering substances in the cleaned extracts. Several experiments were done with aluminum oxide as an alternative.

2.2.3. MSPD

At the beginning of this MSPD experiment, uncoated mortars and pestles were used to grind the samples with the C18 material. This contributed to significant losses (up to ~20%) of compounds before transfer onto the column. Later, enameled ones substituted them, which improved the overall performance.

Both fish and mussel tissue samples were analyzed by the modified MSPD-AC method. Recoveries for the standards were determined from the ratio of the data obtained from MSPD extraction-AC clean-up and the data obtained by direct analysis of each standard at the respective concentrations without extraction and clean-up.

3. Results and discussion

3.1. Optimization results

Both C18 and Al_2O_3 need to be washed prior to use. Fig. 2 a and b present chromatograms of

Table 1
Comparison of the performance of the MSPD-AC column using two elution programs, expressed in the recoveries (in %) of a spiked amount (0.9 μg) of 4-NP[a]

Eluate fraction	Program 1			Program 2		
	Solvent	Volume (ml)	Recovery (%)	Solvent	Volume (ml)	Recovery (%)
1	Methanol	5		Acetonitrile	5	
2	Methanol	5	89[b]	Acetonitrile	5	72[b]
3	Methanol	5	9	Methanol	5	20
4	Dichloromethane	10	0	Dichloromethane	10	0
Total		25	98		25	92

[a] Figures represent the average from experiments performed in duplicate using RP–HPLC with UV detection (λ=278 nm).
[b] Recovery of fractions 1+2 combined (for further explanation, see text).

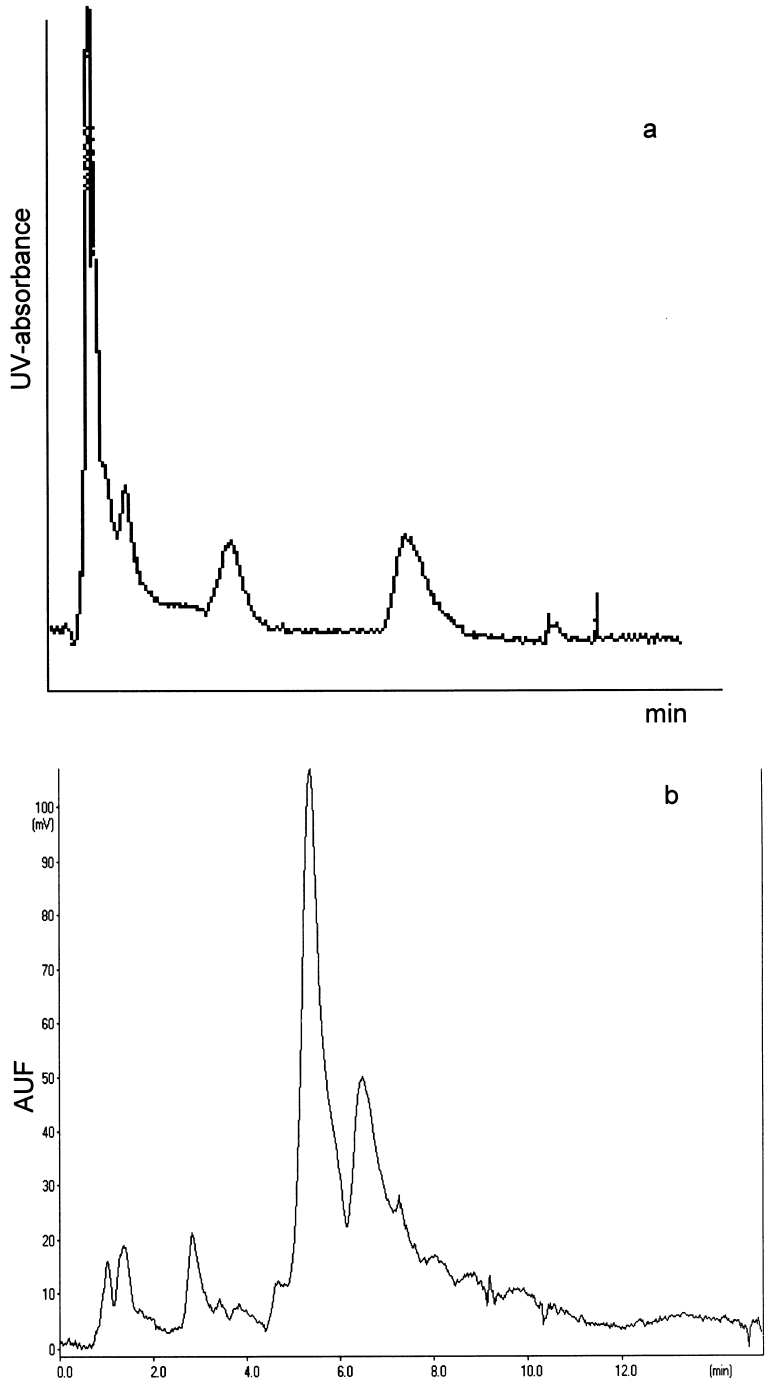

Fig. 2. RP–HPLC chromatograms of blank extracts from unwashed Al_2O_3 and C18 powder showing presence of interferences in unwashed adsorbents. (a) Methanol extract from unwashed Al_2O_3, recorded by UV detector at λ=278 nm; (b) methanol extract from unwashed C18 powder, recorded by FLU detector at Ex λ=225 nm and Em λ=301 nm. For HPLC conditions, see experimental section.

methanol extracts from unwashed Al_2O_3 and C18 powder, respectively, concentrated to the same extent as in the sample treatment. They show the presence of coeluting interferences in non-washed solid-phase and AC materials.

Methanol turned out to be the preferred eluting solvent for APEs in MSPD column. The recovery data for a NP standard mixture using the two elution programs are compared in Table 1. On average about 90% of the spiked NP (0.9 μg) eluted in fractions 1 and 2 when methanol was used. With acetonitrile, when an equal volume had passed, still only 72% of the spiked amount were recovered. Fractions 1 and 2 are reported combined because the elution of the first 5 ml (fraction 1) results in a collected volume of only 1 ml of eluate due to the dead volume of the MSPD column.

After about 15 ml of methanol in program 1, all NP had eluted from the column. The overall performance of elution program 1 turned out to be more efficient than program 2 as is reflected in the recovery of the spike (98 vs. 92%, cf. Table 1). With program 2, in general an initial fraction of up to 2 ml more than in program 1 appeared to be necessary to recover the maximum of spike. This would correspond to an increase in solvent consumption of between ~10 and ~20%.

Using program 1 and collecting an initial fraction of 15 ml, an average recovery of 97% for OP (triplicate measurement) and 100% for NP10E (duplicate) was found. Therefore, the final elution profile was decided to be: Analyte fraction, methanol 18 ml. A volume of 18 ml (instead of 15 ml) was chosen to cope with variations in column dead volume. When analyzing the spiked tissue samples, a second fraction of 5 ml of methanol was also collected to ensure that all compounds of interest had been collected in the analyte fraction. Invariably this second fraction appeared to contain none of the analytes of interest.

Although the selection of methanol is based on the criterion of recovery mainly (and to a minor extent on solvent reduction), one might argue that acetonitrile would be preferable since it is a non-protic solvent which may improve peak shape. However, the tailing observed in some chromatograms is thought to be due to the complex nature of the mixtures rather than to the use of methanol.

The present experimental results showed that sequential clean-up by aluminum oxide was more efficient in removing interferences from biological tissue than silica. Fig. 3 a and b show the UV-chromatograms of extracts from spiked fish samples that had been cleaned up by silica and aluminum oxide, respectively. The extracts were usually concentrated to about 1 ml for HPLC analysis. The extracts from SiO_2 clean-up, however, if concentrated to less than about 1.5 ml on the water bath, would become turbid. Although a similar phenomenon was observed in extracts from Al_2O_3, albeit only after concentration to less than about 300 μl, the difference was that the precipitate in the extracts from the silica column could not be redissolved in any of methanol, dichloromethane, acetonitrile or hexane, whereas the precipitate in the aluminum oxide clean-up solution redissolved easily upon adding more methanol to the solution. The eluted extracts from fish samples were clear and colorless, while those from mussel were clear but yellow.

The chemical composition of the white precipitate in concentrated extracts has not been elucidated yet. The relatively large peak still appearing in the initial part of the chromatogram obtained with UV detection (Fig. 3b) did not appear in chromatograms obtained with FLU detection (Fig. 3c), which meant that the interferences could absorb UV but were non-fluorescing substances. Similar phenomena were also observed in an experiment of isolation of drug residues from bovine muscle [4]. The interferences observed in those extracts were found to consist mainly of proteins by testing with ninhydrin. It should be emphasized here that these substances probably only marginally interfere with the determination of AP(E), since the UV chromatogram (Fig. 3b) shows that they elute before the first eluting AP, viz. OP.

3.2. Recovery study results

Representative chromatograms of non-spiked and spiked fish and mussel tissue samples are shown in Figs. 4 and 5. For fish muscle (0.5 g) spiked with 108 ng of 4-*t*-OP and 300 ng of NP4E, mean recoveries of 91 ± 12 (OP, five replicates) and 100 ± 5 (NP4E, $n = 5$) were observed. For mussel tissue (0.9 g) spiked with the same amounts of OP and NP4E,

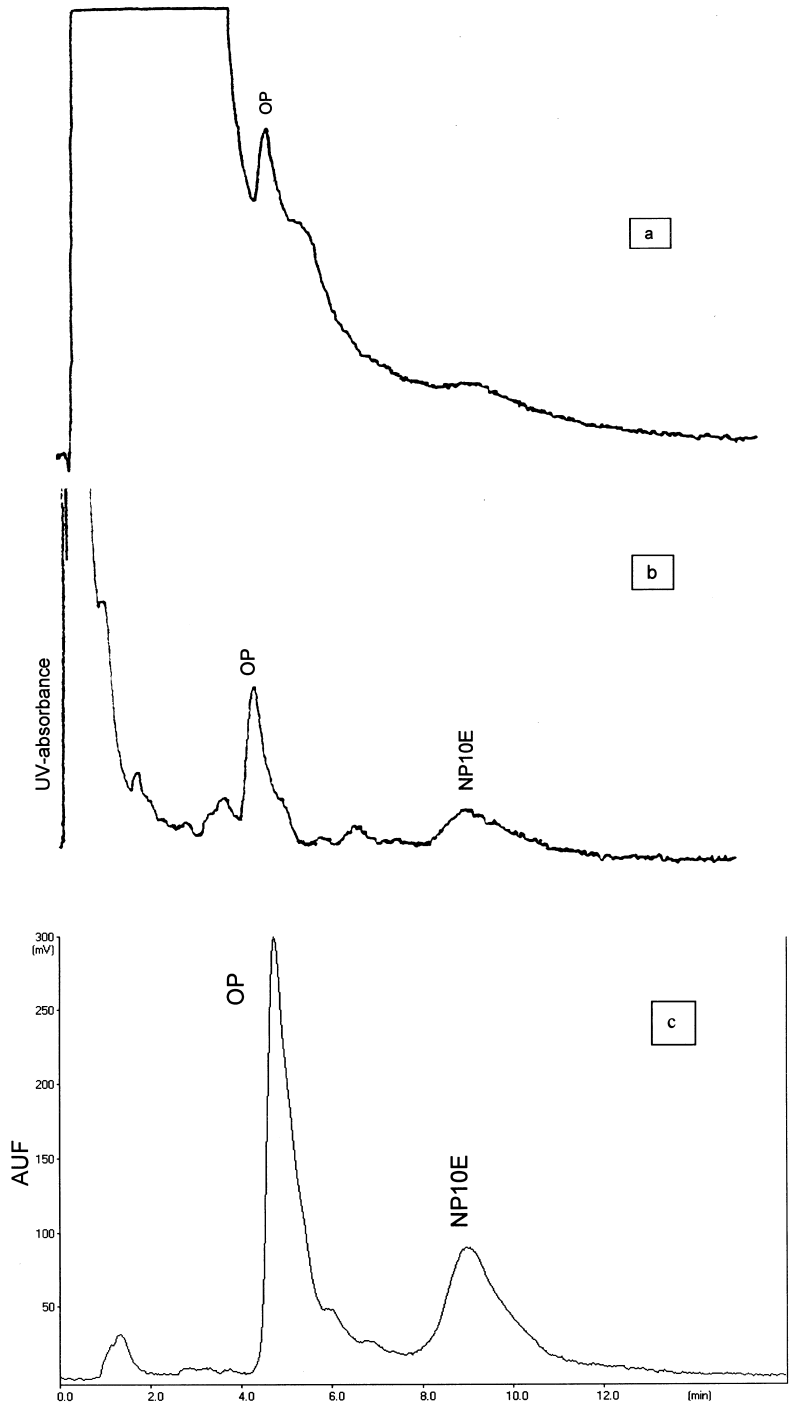

Fig. 3. Comparison of silica and aluminum oxide as sequential clean-up adsorbents after MSPD extraction. Sample: fish muscle 0.5 g, mixed with 2 g of C18 material. (a) UV chromatogram (λ=278 nm) of fish spiked with 5.4 μg of 4-*t*-OP and 18.6 μg of NP10E, MSPD extract cleaned up on silica (2 g); (b) UV chromatogram (λ=278 nm) of fish spiked with 5.4 μg of 4-*t*-OP and 18.6 μg of NP10E, extract clean-up on aluminum oxide (3 g); (c) FLU chromatogram (Ex. λ=225 nm, Em. λ=301 nm) of fish spiked with 1.1 μg of 4-*t*-OP and 1.9 μg of NP10E, clean-up on Al_2O_3 (3 g). For RP–HPLC conditions, see experimental section.

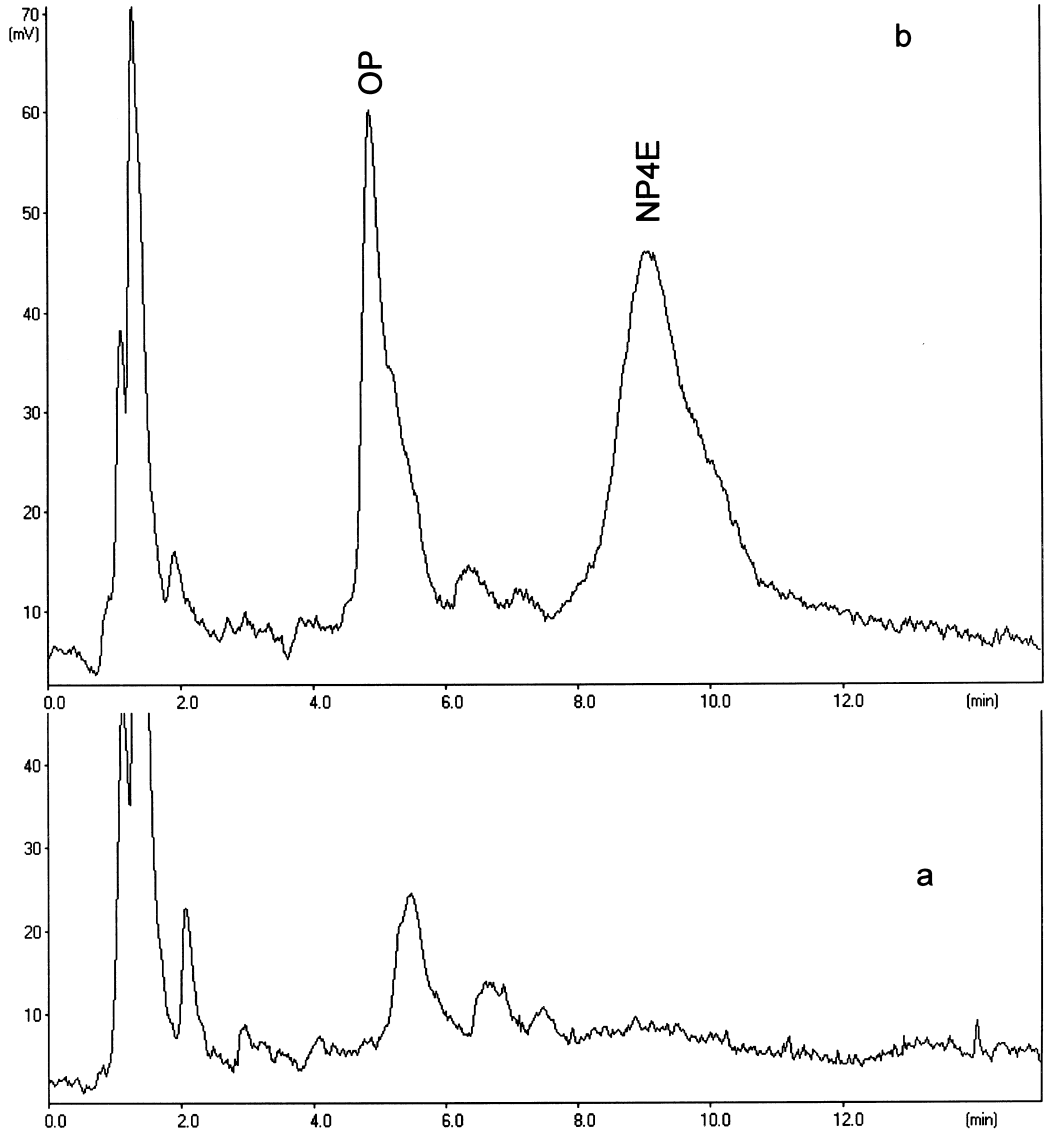

Fig. 4. RP–HPLC–FLU chromatograms of extracts from fish tissue obtained with the sequential MSPD-AC procedure. (a) Extract from non-spiked fish tissue sample (0.5 g of fish muscle+2 g of C18); (b) extract from spiked fish tissue sample (0.5 g of fish muscle+2 g of C18 +0.1 μg of 4-*t*-OP+0.3 μg of NP4E). Conditions: FLU detection as in Fig. 3c. For RP–HPLC conditions, see experimental section.

the recoveries amounted to 108±10% (n=2) and 101±1% (n=2), respectively.

Although many applications of NPE involve mixtures of oligomers with an average of eight to ten ethoxylate units or more, the actual mixtures one can observe in environmental samples are usually mixtures with shorter average chain lengths (see, e.g. Ref. [7]). Since NP4E is much more reflective of such environmentally relevant mixtures, the actual fish and mussel spiking experiments were conducted with NP4E rather than, e.g. NP10E. We have no reasons to believe that the results of the MSPD-AC method would be much different for NP10E, because the screening experiments with NP10E discussed

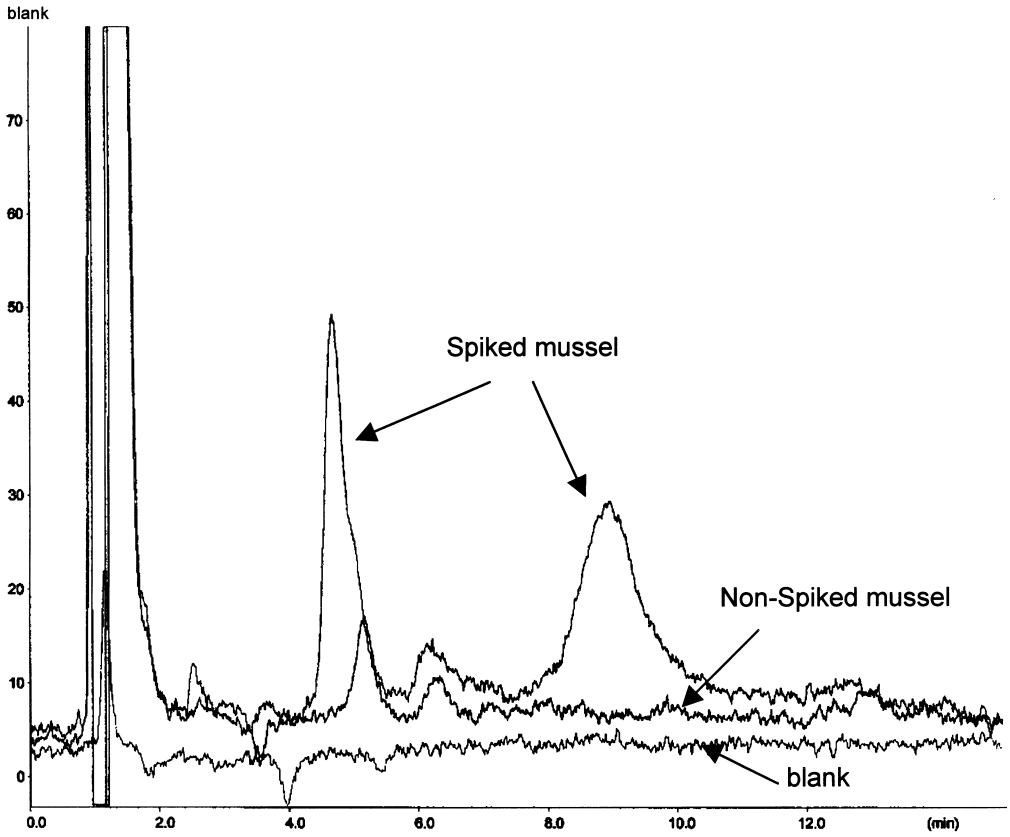

Fig. 5. RP–HPLC–FLU chromatograms of extracts from mussel tissue obtained with the sequential MSPD-AC procedure. Shown are: extract from non-spiked mussel tissue sample (0.9 g of mussel tissue+3 g of C18); extract from spiked mussel tissue sample (0.9 g of mussel tissue+3 g of C18+0.1 μg of 4-*t*-OP+0.3 μg of NP4E); and extract of reagent blank. Detector conditions as in Fig. 3c. For RP–HPLC conditions, see experimental section.

above, resulted in a quantitative recovery. Admittedly, the results presented here are only relevant for certain mixtures of oligomers, and do not indicate how separate ethoxymers behave on the MSPD-AC column. To that end, normal-phase HPLC or HPLC–MS must be used, or alternatively, separate single compounds must be tested. The latter are not available commercially. Both items will be the subject of our future work.

The peaks in the chromatograms of non-spiked fish tissue samples are appearing at retention times slightly different from those in mussel tissue sample, indicating the different composition of the two extracts. Although the peaks are all within the retention time range of APEs under our experimental conditions, the available data are not sufficient to identify all of them. Further identification and confirmation of these compounds will be done by LC–MS later.

3.3. Advantages and limitations of the method

The MSPD method has been applied for the treatment of several kinds of biological samples since it was developed, e.g. for the successful determination of alcohol ethoxylates (AEs) [6] – another type of widely used non-ionic surfactants – and ivermectin [8] in fish. In the latter study, where silica was used for clean-up purposes, interferences similar to the ones found in the present study were observed. Aluminum oxide may help to remove these. The AE study [6] also successfully applied

two different ways of Al_2O_3 clean-up, following a procedure originally developed by Kiewiet et al. [9].

While preparing the matrix-dispersed solid-phase, the water present in the sample is reduced due to sorption to the silica carrier material and the tissue structure is totally opened up for extraction. Thus, high recoveries could be obtained with only a small amount of solvent through a rather simple procedure at room temperature. This is a major advantage of MSPD technique compared with classical solid/liquid extraction methods, leading to a large reduction of solvent consumption.

Another remarkable advantage of the presented modified MSPD method is that the isolation and purification are combined and accomplished within one sequential step. This reduces the total sample pretreatment time from many hours in classical extractions (e.g. Soxhlet or steam distillation) to several tens of minutes. Moreover, it also avoids possible bias in analyte concentrations due to a separate clean-up procedure, which may involve risks of loss (due to evaporation) or contamination (due to several handling steps).

A typical detection limit can be calculated from the results of the spiked tissue samples. Adopting a criterion of three times the standard deviation in the noise level, such limits of detection would be equal to about 10 ng of OP and 30 ng of NPE per gram of wet tissue.

The limitation of this procedure is that the column-packing process, elution profile and eluting speed have to be very carefully manipulated to ensure good reproducibility of the elution profile and an optimal recovery.

4. Conclusions

Sample pretreatment is a critical step in the analysis of APEs in biological tissues. A preferable technique should be time-efficient and require minimum amounts of solvents. The presented sequential MSPD-AC method provides a rapid alternative to conventional methods, which makes it a promising approach for the determination of APEs in biological tissues at the ppb level. The method reduces solvent consumption significantly.

Octadecylsilica proved to be an efficient solid-phase for the MSPD column. Aluminum oxide is more efficient than silica as clean-up adsorbent. Methanol was found to be the optimal eluting solvent for APEs. The recoveries for all spiked tissue samples were greater than 90% and limits of detection are typically 10–30 ng per gram of wet tissue.

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