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Extraction and isolation of linear alcohol ethoxylates from fish

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Abstract

The development of an analytical method for determination of alcohol ethoxylates (AEs), important non-ionic surfactants, in fish is described. Combination of matrix solid-phase dispersion (MSPD) extraction with aluminum oxide clean-up allows for determination of AEs in fish samples with recoveries higher than 75%. The use of internal standards allowed for correction of losses during preparation of the individual samples. Incurred AE constituents could be quantified without integration being compromised by interferences for all compounds except for $C_{14}EO_{14}$. Parallel determinations of the concentrations of ^{14}C-$C_{13}EO_8$ in fish by TLC–RAD and liquid scintillation counting and by HPLC measurements after isolation and derivatization yielded very similar results demonstrating the validity of the isolation and measurement method.

Keywords: Alcohol ethoxylates

1. Introduction

Alcohol ethoxylates (AEs) are non-ionic surfactants used in household and laundry detergents. They are complex mixtures produced by reaction of C_9 to C_{18} alcohols with ethylene oxide to yield alcohol polyoxyethylene ethers [1]. The technical products display statistical distributions of oxyethylene units, ranging from 1 to about 40 and contain some hundreds of individual chemicals [1] which are also encountered in the environment. A commonly used abbreviation of AE structure is C_nEO_m, with *n* and *m* denoting the lengths of the alkyl and the oxyethylene chain, respectively. The average structure of the typical commercial mixtures in Europe is $C_{13.2}EO_{8.3}$ with 70% of the mass composed of alkyl homologs with 12 to 15 C-atoms and 3 to 10 oxyethylene units [2].

In 1993 700 000 t of AEs were produced worldwide [3]. After use, they are discharged with wastewater. It is known that AEs are rapidly degraded by microorganisms [4]. Hence, AEs are efficiently removed during wastewater treatment [5] and the half-life for primary degradation in river water is in the order of 0.2 d [6]. Nevertheless, AEs do occur in surface waters [3] and can be taken up by aquatic organisms. Therefore, assessment of the bioaccumulation behavior is one aspect of the risk assessment of AEs.

However, the lack of parent compound specific analytical methodology for extraction and isolation of AEs from fish has as yet prevented the generation

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of valid bioconcentration data. Therefore, a method for extraction and isolation of AEs from fish at trace levels had to be developed in order to render feasible the investigation of AE bioconcentration behavior. This includes elucidation of the relationship between the structure of the AE constituents and their bioconcentration potential. In view of the complexity of the technical AE mixtures and the resulting analytical difficulties to separate these mixtures in order to generate compound specific data, we decided to perform bioconcentration experiments with simple mixtures of individual AE constituents. The chromatographic tools to be applied had to be able to separate those mixtures. The length of the alkyl as well as of the EO chain of the test compounds are systematically varied (Fig. 1) so that structure bioconcentration relationships can be obtained.

The environmental analytical chemistry of AEs has been reviewed recently [1,7,8]. Reversed-phase high-performance liquid chromatography (HPLC) of AEs derivatized with UV-active or fluorescent reagents has emerged as method for compound specific determination of AEs in environmental samples. It allows for eluting all ethoxymers of one alkyl homolog into one peak, so that the resulting chromatogram can be used to derive the distribution of the alkyl homologs in the sample of interest using an eluent mixture of water, methanol and acetonitrile [9,10]. Zanette and Marcomini [11] demonstrated that a water–acetonitrile gradient can be used to determine the EO distribution of an AE mixture derived from only one alcohol.

m_{EO}	4	8	11	14
$n_{C\text{-atoms}}$				
12		•		
13	•	•		
14	•	•	•	•
16		•		

Fig. 1. Matrix of the AE representatives tested in the bioconcentration experiments. The lengths of the alkyl and the ethoxylate chains are given by $n_{C\text{-atoms}}$ and m_{EO}, respectively.

1.1. Extraction and clean-up of AEs in solid samples

Wastewater solids are Soxhlet-extracted with MeOH and the extract obtained is diluted with water and sublated with ethyl acetate [10]. The clean-up of extracts of water or solid samples is performed by either anion-exchange clean-up with silica based ion-exchange columns [12,13], or with Al_2O_3 columns [7,14,15]. No method for extraction or clean-up of AEs in fish has been described yet.

1.2. Choice of methods

From the reported methods for detection, derivatization and separation we chose naphthoyl chloride as derivatizing reagent and reversed-phase HPLC as separation method as our tools to determine ''cold'' AEs. This combination allowed one to separate the test compounds and to detect their derivatives selectively and sensitively. Matrix solid-phase dispersion (MSPD) extraction has been employed to extract analytes of a broad range of polarity from polar penicillins [16], sulfonamides [17,18] and tetracycline antibiotics [19] to hydrophobic organochlorine pesticides [20] from tissue samples. In a previous investigation [21] we found MSPD extraction a selective extraction method for the anionic surfactant linear alkylbenzenesulfonate (LAS). Therefore, we evaluated this method with regard to its suitability for extracting AE from fish tissue. From the clean-up methods reported for AEs in sewage sludge samples the Al_2O_3 adsorption chromatography was selected because its suitability had been proven recently [10,15].

2. Materials and methods

2.1. Chemicals

All salts (Merck, Amsterdam, The Netherlands) and sorbents were of analytical grade. Octadecylsilica (ODS, 40 μm) was supplied by Mallinckrodt–Baker, Deventer, The Netherlands. Al_2O_3

(Brockmann I, standard grade, neutral, 150 mesh, Aldrich) and 1-methyl-imidazole (analytical grade, Fluka) were purchased from Sigma–Aldrich–Fluka (Zwijndrecht, The Netherlands). Solvents (Mallinckrodt–Baker) were of HPLC-grade or equivalent quality except for hexane (Mallinckrodt–Baker) which was purchased in technical quality and glass distilled in our laboratory. HPLC-grade water was prepared using an ELGASTAT system (Elga, Buchs, Switzerland). Thin-layer chromatography (TLC) plates (LK6D silica gel 60A) were supplied by Whatman. Octaethylene glycol monotridecyl ether and tetraethylene glycol monotridecyl ether, ^{14}C-labeled in the C_1-position of the alkyl chain, ^{14}C-$C_{13}EO_8$ and ^{14}C-$C_{13}EO_4$, were custom synthesized by Roy Sheppard from Unilever Research Labs. (Bebington, UK). Prior to the bioconcentration experiment purity was checked by TLC–RAD to exceed 98%. Both compounds were available to us as ''cold'' chemicals, also synthesized by Roy Sheppard (Unilever, Port Sunlight Research Lab.). $C_{14}EO_{11}$ and $C_{14}EO_{14}$ were synthesized in our laboratory. The remaining AE constituents to be studied ($C_{12}EO_8$, $C_{14}EO_4$, $C_{14}EO_8$, $C_{16}EO_8$) were supplied by Fluka (Sigma–Aldrich–Fluka). HPLC chromatograms of standards of the naphthoyl chloride (Merck, Darmstadt, Germany) derivatives of the individual AE constituents indicated that all compounds were of more than 97% purity.

2.2. Fish and MSPD extraction of fish

Exposure as well as spike recovery experiments were performed with 6–8-month-old fathead minnows (*Pimephales promelas*) reared in the hatchery of Utrecht University weighing 0.66 ($\pm$0.21) g. The content of hexane extractable lipids in the fish was five percent in earlier experiments [21]. A freshly killed fish was weighed and put into the mortar and 1 ml of MeOH was added to the mortar. The fish was homogenized with a pestle and spiked with known amounts of a mixture of AEs in recovery experiments or internal standard dissolved in 50 or 100 μl CH_3CN. Then 4 g ODS per 1 g of fish was added, mixed with the fish homogenate until a homogeneous paste was obtained. The paste was allowed to fall dry and upon renewed grinding with the pestle a powder was obtained. The powder was transferred to a column prepared from the barrel of a 20-ml injection syringe (Becton & Dickinson). Glass or quartz wool was filled into the bottom of the column, a disk of filter paper was put on top of the quartz wool and the obtained column was rinsed with hexane and ethyl acetate. Subsequently, the powder was transferred to the column and eluted with solvents to yield the following fractions: 1 hexane (20 ml), 2 ethyl acetate (4 ml) and ethyl acetate–MeOH (1:1) (12 ml per g fish), and 3 MeOH (8 ml per g fish).

2.3. Al_2O_3 clean-up of MSPD extracts of fish

Two clean-up procedures described in the literature were evaluated with respect to their suitability to isolate AE from MSPD extracts of fish. Procedure 1 had been developed by Kiewiet et al. [10]. The dried MSPD extracts were resuspended in 1 ml of hexane–CH_2Cl_2 (1:1) and applied to a 1 cm I.D. glass column fitted with quartz wool and filled with 7 g Al_2O_3 (Brockmann I, standard grade, neutral, 150 mesh) deactivated with 5% (w/w) HPLC-grade water. Firstly, the column was eluted with 90 ml of hexane–CH_2Cl_2 (1:1). The eluate was discarded. Secondly, 90 ml of CH_2Cl_2–MeOH (100:1) was used as eluent. In the development of the clean-up method the CH_2Cl_2–MeOH (100:1) eluate was collected in different fractions to identify the AE elution window. In procedure 2, outlined by Cassani et al. [15] the dried MSPD extract was resuspended in 1 ml hexane–CH_2Cl_2 (1:1) and allowed to drain through prepacked Al_2O_3 columns (1 g, Supelco, Sigma–Aldrich–Fluka). AE constituents were eluted with 15 ml CH_2Cl_2–MeOH (95:5). The eluate is evaporated to dryness, derivatized, evaporated to dryness again, dissolved in 1 ml cyclohexane–CH_2Cl_2 (1:1), and passed over prepacked Al_2O_3 columns (Supelco, Sigma–Aldrich–Fluka). After washing the column with 15 ml cyclohexane–CH_2Cl_2 (1:1), the naphthoic acid derivatives of AEs are eluted with 15 ml CH_2Cl_2–MeOH (95:5).

2.4. Derivatization of AEs for HPLC analysis

Extracts and standards were blown to dryness under a stream of N_2 and treated as described by Lux and Schmitt [9]. The dried residue was redissolved in 900 μl CH_3CN by sonication for 15 min and

transferred to glass stoppered tubes. After addition of 25 μl 1-methyl-imidazole and 10 μl naphthoyl chloride, the tubes were closed and allowed to react at 60°C for 30 min. The reaction was quenched by addition of 65 μl CH_3OH.

2.5. HPLC separation of the AE derivatives

AE constituents were separated on a C_{18} reversed-phase column (100×3 mm, 5 μm particle diameter, Chromsphere, Chrompack, Bergen op Zoom, The Netherlands). The gradients were delivered by a Gynkotek M480 pump (Separations, Alblasserdam, The Netherlands), the samples were injected by a Spark Holland Basic Marathon autosampler (Separations) fitted with a 20-μl injection loop. A Jasco 920F fluorescence detector (Separations), set at 300 nm and 385 nm excitation and emission wavelength, respectively, was employed for detection. Chromatographic data were collected and processed with Chromcard software (Interscience, Breda, The Netherlands). A gradient using CH_3CN and water exclusively (program 1) was employed if the primary goal was to separate the ethoxymers of one alkyl homolog. If separation of alkyl homologs was the primary goal program 2 was used. Both are detailed in Table 1.

2.6. Quantitation

Calibration curves for all analytes as well as the internal standards (at least four concentrations) were constructed in the linear concentration range using linear regression. The recovery of the internal standard was used to correct for losses during sample workup. The recovery of the analytes relative to that of the internal standard was established in recovery experiments.

2.7. Radiochemical analysis

Total radioactivity was measured by liquid scintillation counting (LSC) (Packard TriCarb 2300 TR, Packard Research Instruments, Groningen, Netherlands) using Emulsifier Safe (Packard Research Instruments) as scintillation liquid. Samples were chromatographed with $CHCl_3$–MeOH–NEt_3–water (85:25:5:1) and ethyl acetate–acetone–water (7:3:1) on TLC plates for ^{14}C-$C_{13}EO_4$ and ^{14}C-$C_{13}EO_8$, respectively. After drying, plates were scanned (Berthold LB 2842 automatic linear TLC analyzer, EG&G, Nieuwegein, The Netherlands) for 5 or 10 min. Scan results were processed and evaluated with Linear Analyzer Chroma software (EG&G).

2.8. Validation of the extraction and isolation scheme for "cold" AEs

Fish were exposed to a constant concentration (200 μg l^{-1}) ^{14}C-$C_{13}EO_8$ for 42 h during a bioconcentration experiment. Fish specimen with incurred ^{14}C-$C_{13}EO_8$ were sampled and were MSPD extracted and the amount of parent compound was determined by measuring the total radioactivity in a 200-μl aliquot of the sample by LSC and determining the fraction of ^{14}C-$C_{13}EO_8$ by TLC–radiochemical detection (RAD) (50 μl aliquot). The remaining

Table 1
HPLC gradient programs 1 and 2 used throughout the bioconcentration experiments performed with AEs (the flow-rates in both programs were 600 ml/min)

Program 1			Program 2			
Time (min)	Water (%)	CH_3CN (%)	Time (min)	Water (%)	CH_3CN (%)	CH_3OH (%)
0–1	40	60	0–2	40	45	15
1–20	20	80	2–40	0	85	15
20–60	0	100	40–45	0	100	0
60–65	0	100	45–50	0	100	0

750 μl were subjected to an Al_2O_3 clean-up, derivatization and HPLC analysis.

3. Results and discussion

For separation of AE constituents we tested gradient program 1 and found out that $C_{13}EO_8$ and $C_{14}EO_{14}$ coelute with each other while the remaining test compounds are baseline separated (not shown). Since $C_{13}EO_8$ was to be used as reference compound in all experiments, another solvent system had to be selected for use in the experiment in which the bioconcentration of $C_{14}EO_{14}$ was tested. A gradient similar to that described by Lux and Schmitt [9] (program 2) was found to separate $C_{13}EO_8$ from $C_{14}EO_{14}$ and was therefore used when appropriate.

3.1. MSPD extraction of AEs from fish samples

Fractionated elution of spiked fish (Table 2) demonstrated that ^{14}C-$C_{13}EO_4$ is almost quantitatively extracted in the EtOAc fraction. In contrast, the solvent strength of EtOAc is insufficient to elute ^{14}C-$C_{13}EO_8$ quantitatively from the MSPD column. Therefore, the MSPD elution scheme was modified. ^{14}C-$C_{13}EO_8$ was eluted by first 5 ml EtOAc followed by 10 ml EtOAc–MeOH (1:1) per g fish. In subsequent experiments the amount spiked and the sample size were varied. The recovery of ^{14}C-$C_{13}EO_8$ and ^{14}C-$C_{13}EO_4$ was higher than 93% in all trials and highly reproducible (data not shown).

Table 2
Results of fractionated elution of ^{14}C-$C_{13}EO_4$ and ^{14}C-$C_{13}EO_8$ from fish spiked with 20 μg g^{-1} and 10 μg g^{-1}, respectively, by MSPD (RSDs are in parentheses, $n=3$)

Solvent	Fraction of applied amount recovered			
	Volume (ml)	^{14}C-$C_{13}EO_4$ (%)	Volume (ml)	^{14}C-$C_{13}EO_8$ (%)
Hexane	8	<1 (4)	8	1 (5)
EtOAc	8	97 (1)	4	75 (9)
			4	5 (43)
CH_3OH	8	1 (6)	8	13 (20)
Sum		98		94

Therefore, the above MSPD scheme is suitable for extraction of AEs from fish tissues.

3.2. Evaluation of two different Al_2O_3 isolation procedures

Two different Al_2O_3 clean-up procedures were compared to each other with regard to their suitability for isolation of $C_{12}EO_8$, $C_{14}EO_8$ and $C_{16}EO_8$ from fish tissues. Both procedures yield analyte recoveries of >90% with a maximum relative standard deviation (RSD) of <6% (not shown) demonstrating that recovery is independent of alkyl chain length. In addition, both isolation procedures produce extracts with little interferences in the retention time region of interest. Hence, both are equally suitable for isolation of the above homologous series of octaethylene glycol monoalcoholethers from MSPD extracts of fish samples.

Prior to the bioconcentration experiment with $C_{13}EO_8$, $C_{14}EO_4$, $C_{14}EO_8$ and $C_{14}EO_{11}$ the recovery was determined with clean-up procedure 2. The recovery of $C_{14}EO_4$ in a triplicate experiment was 20, 101 and 89% while it was 82, 110 and 99% for $C_{14}EO_{11}$. Given that radiolabeled $C_{13}EO_4$ was quantitatively recovered from spiked fish we concluded that $C_{14}EO_4$ had been lost during one of the two steps of the clean-up procedure. Upon further investigation we found that the retention of the naphthoic acid derivative of $C_{14}EO_4$ was poorly reproducible in the second Al_2O_3 clean-up step (data not shown) indicating that the solvent strength was too high. Therefore, this step was modified by replacing the hexane–CH_2Cl_2 (50:50) solvent mixture with cyclohexane–CH_2Cl_2 (50:50). As a result the recoveries of all four test compounds and of the internal standard $C_{12}EO_8$ were quantitative (Table 3).

Similarly, when testing the recovery of the mixture $C_{12}EO_8$, $C_{13}EO_8$, $C_{14}EO_{14}$ and $C_{16}EO_8$ with clean-up procedure 1, we observed that the recovery of $C_{14}EO_{14}$ was reduced to 34.5 (±5.3)%. It appeared that CH_2Cl_2–MeOH (100:1) (70 ml) is of insufficient solvent strength to elute $C_{14}EO_{14}$ from the Al_2O_3 column. Elution of the Al_2O_3 column with firstly 50 ml CH_2Cl_2–MeOH (100:1) followed by 20 ml CH_2Cl_2–MeOH (100:3) improved the

Table 3
Recoveries of $C_{12}EO_8$, $C_{13}EO_8$, $C_{14}EO_4$, $C_{14}EO_8$ and $C_{14}EO_{11}$ in spiked fish (3.8 to 4.5 μg g^{-1} per individual compound) after modifying clean-up procedure 2 by replacing hexane–CH_2Cl_2 (50:50) with cyclohexane–CH_2Cl_2 (50:50) (n=3) (RSDs in parentheses, n=3)

Compound	Recovery (%)
$C_{12}EO_8$	90 (3)
$C_{13}EO_8$	90 (3)
$C_{14}EO_4$	84 (1)
$C_{14}EO_8$	88 (2)
$C_{14}EO_{11}$	84 (6)

recovery of $C_{14}EO_{14}$ to 96.5% without eluting more interferences (Table 4). Hence, while the clean-up does not discriminate between alkyl homologs the recovery of the different ethoxymers is dependent on the polarity of the eluent.

Fig. 2 provides a scheme of the sample preparation procedure followed in analyzing fish samples obtained in bioconcentration experiments in which fish were exposed to mixtures of individual AE constituents. AEs were isolated from the MSPD extracts by the modified procedure 1 and excess derivatization reagent was removed by an additional Al_2O_3 clean-up analogous to procedure 2. The aqueous concentrations ranged between 0.03 μ*M* and 0.6 μ*M* and the range of resulting concentrations in fish extended from below the limit of detection ($C_{14}EO_{14}$) to 12 μ*M* ($C_{16}EO_8$). The chromatogram in Fig. 3 represents a fish that was exposed to $C_{13}EO_8$, $C_{14}EO_8$ and $C_{16}EO_8$ and that was extracted and cleaned up following the procedure depicted in Fig. 2. It demonstrates that the analytical method produces chromatograms which allow for quantitation of AEs.

Table 4
Recoveries of $C_{12}EO_8$, $C_{13}EO_8$, $C_{14}EO_{14}$ and $C_{16}EO_8$ from fish spiked with 3.3 to 4.6 μg g^{-1} of the individual compounds employing the clean-up procedure 2 after modification (RSDs in parentheses, n=3)

Compound	Recovery (%)
$C_{12}EO_8$	95 (8)
$C_{13}EO_8$	84 (3)
$C_{14}EO_{14}$	97 (22)
$C_{16}EO_8$	75 (4)

3.3. Limits of quantitation

The limit of quantitation (LOQ) for the individual AE test compounds was estimated as the amount of compound required to increase the signal to ten-times the noise of the baseline. The resulting amount per 20 μl injection was estimated to be 2 pmol. Since the same fluorophore was employed for detection, the LOQ for the individual compounds can be expected to be rather similar. Given the final sample volume of 1 ml and an average mass of the fish of 0.65 g the LOQ amounts to 150 nmol kg^{-1}. For $C_{13}EO_8$, the reference compound used throughout our study this molar concentration translates into 80 μg kg^{-1} for fish. In fish exposed to $C_{14}EO_{14}$ at 170 μg l^{-1}, however, the analyte coelutes with an interference. We used standard addition to approximate the LOQ of this AE constituent (930 nmol kg^{-1} or 800 μg kg^{-1}).

3.4. Confirmation of "cold" method

After having worked out the method for isolation of AEs from fish we confirmed the performance of the procedure by first determining the concentration in three fish that had been exposed to ^{14}C-$C_{13}EO_8$ for 35 h under flow through conditions by LSC and TLC–RAD for 35 h under flow-through conditions. Subsequently, ^{14}C-$C_{13}EO_8$ was isolated from the fish extract by procedure 2 and the concentration was determined by HPLC–fluoresence (FL) detection. Comparison of the radioactivity and the fluorescence chromatograms demonstrated that the predominant peaks in both chromatograms have the same retention time. Since the retention time is identical to that of the standard of $C_{13}EO_8$, it can be concluded that the peak in the fluorescence trace of the fish sample corresponds indeed to $C_{13}EO_8$. The ratio of the concentrations determined by HPLC–FL detection divided by TLC–RAD was calculated in triplicate. The average ratio was 1.02 ± 0.07 and therefore close to unity with little variation, indicating that the methods yield results that agree well with each other.

3.5. AEs in feral fish

AE concentrations in effluents of Dutch waste-

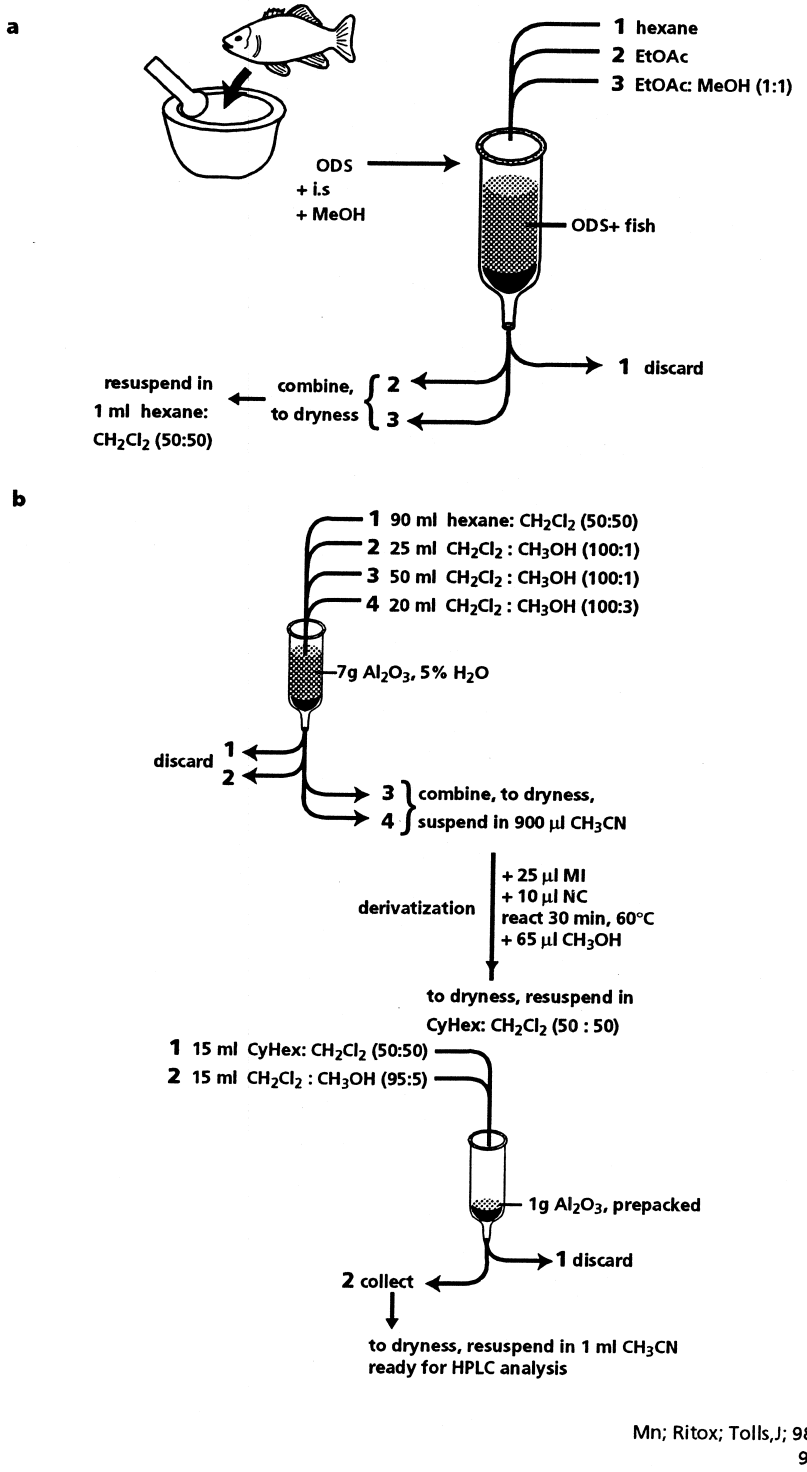

Fig. 2. Scheme of the MSPD extraction of AEs from fish samples (a). The volumes used were (1) hexane (20 ml), (2) ethyl acetate (4 ml) and (3) ethyl acetate–MeOH (1:1) (12 ml per gram fish). Fractions 2 and 3 were combined and contained the AEs. The analytes were isolated from the sample matrix by column chromatography (b).

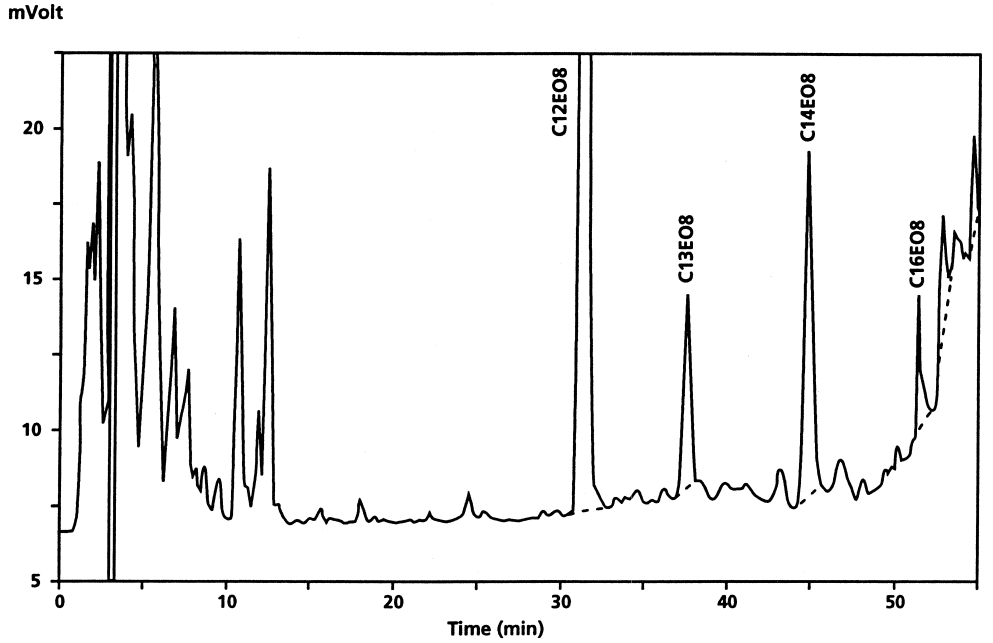

Fig. 3. Chromatogram of a fish exposed via the water phase to $C_{13}EO_8$, $C_{13}EO_4$ and $C_{16}EO_8$. $C_{12}EO_8$ was used as internal standard.

water treatment plants were around 0.01 μM for the sum of all AE constituents. Hence, the sum of the concentration of AE constituents employed in the bioconcentration experiments (0.3 to 1.1 μM) greatly exceeded those in natural waters. Therefore, the analytical method presented here is not likely to be appropriate for determination of AEs in feral fish. It has to be viewed as an initial approach to make biota samples amenable to analysis of the widely used non-ionic surfactant AEs. In the future, the use of HPLC for chromatographic separation of AEs in combination with mass spectrometry for sensitive and specific detection could render those AE constituents with an appreciable bioconcentration potential measurable in fish exposed to surface water.

4. Conclusions

A MSPD extraction scheme has been developed that is suitable to extract AEs quantitatively from fish. Two different Al_2O_3 column chromatographic isolation procedures, developed to clean-up extracts of sewage sludge samples, were modified to isolate AEs from the fish extracts. The resulting samples are sufficiently purified and allow for determination of most AE constituents at concentrations that can be expected in bioconcentration experiments without interferences compromising quantitation of almost all of these compounds. The results with radiolabeled as well as ''cold'' AE constituents demonstrate that these compounds can be extracted quantitatively from spiked fish as well as from fish with incurred AEs. In addition, the use of internal standards allows for correction of losses occurring during preparation of the individual samples.

The tested HPLC gradients allow for separation of either the alkyl homologs or the ethoxymers. $C_{13}EO_8$ can be separated from all other test compounds. Hence, $C_{13}EO_8$ can be employed as reference compound in all bioconcentration experiments. The validity of the analytical approach developed for the determination of ''cold'' AEs was demonstrated in the comparison with the radiochemical determination method. Therefore, the combination of MSPD extraction and Al_2O_3 isolation methods is regarded to be suitable for determination of individual AEs in fish in bioconcentration experiments.

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