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Reversed-phase high-performance liquid chromatographic determination of linear alkylbenzene sulphonates, nonylphenol polyethoxylates and their carboxylic biotransformation products

Antonio Marcomini*

Department of Environmental Sciences, University of Venice, Calle Larga S. Marta 2137, I-30123 Venice (Italy)

Antonio Di Corcia and Roberto Samperi

Department of Chemistry, University La Sapienza, P.le A. Moro 5, I-00195 Rome (Italy)

Silvio Capri

Water Research Institute (CNR–IRSA), via Reno 1, I-00198 Rome (Italy)

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ABSTRACT

The possibility was investigated of simultaneously determining linear alkylbenzene sulphonates, nonylphenol polyethoxylates and their respective biotransformation products, namely sulphophenyl carboxylates (SPC) and nonylphenoxy carboxylates, by reversed-phase high-performance liquid chromatography with UV and fluorescence detection. Both the ion-suppression and the ion-pair techniques were taken into account for separating the compounds of interest. Each technique exhibited peculiar characteristics of resolution and sensitivity. Differences in the selectivity of the chromatographic system on using either methanol or acetonitrile as organic modifier were also considered. Liquid–solid extraction by an octadecyl-bonded silica (C_{18}) cartridge was employed to isolate all the compounds considered from a sewage treatment plant effluent sample. The recovery of SPC with up to six carbons in the carboxylate chain was unsatisfactory. The SPC distribution appeared to be dominated by the homologues having 5–8 carbon atoms in the carboxylate chain.

INTRODUCTION

Linear alkylbenzene sulphonates (LAS) and alkylphenol polyethoxylates (NPEO) are anionic and non-ionic surfactants widely used in domestic and industrial detergents, respectively. The annual world production was 1.8 million tons of LAS in 1987 [1] and *ca.* 0.3 million tons of NPEO in 1990 [2]. The structures of LAS, NPEO and their biotransformation products are shown in Fig. 1. LAS are present in commercial formulations as complex mixtures of C_{10}–C_{13} homologues and of positional isomers resulting from the attachment of the phenyl ring to the carbon atoms (from the second to the central one) of the linear alkyl chain. Commercial NPEO are mixtures of oligomers and isomers, the latter involving different degrees of branching of the alkyl chain.

Laboratory biodegradation studies showed sulphophenyl carboxylates (SPC) to be the only

* Corresponding author.

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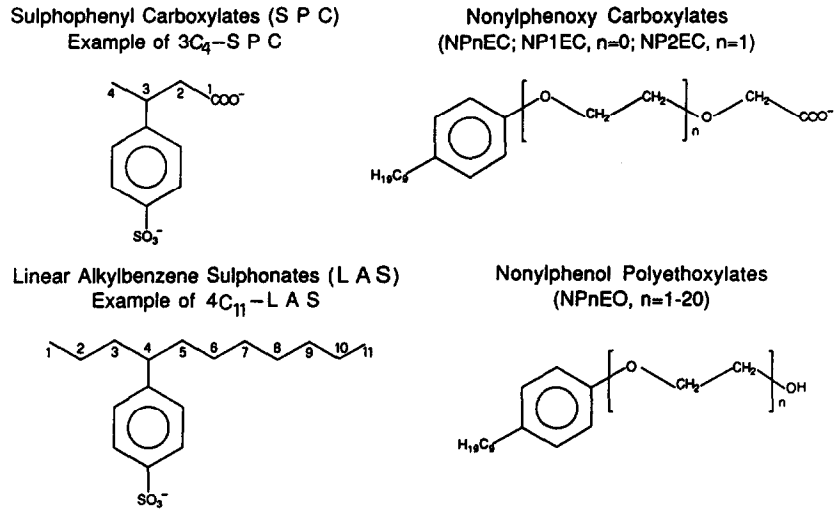

Fig. 1. Structures and abbreviations of sulphophenyl carboxylates (SPC), nonylphenoxyacetic acid (NP1EC) and nonylphenoxyethoxyacetic acid (NP2EC), linear alkylbenzene sulphonates (LAS) and nonylphenol polyethoxylates (NPEO).

biodegradation intermediates of LAS [3,4]. From these studies, it was believed that short-chain SPC could be the most probably occurring LAS metabolites in aqueous environmental samples. A recent investigation, however, identified in treated sewage and groundwater samples SPC containing 3–10 carbon atoms in the alkyl chain [5].

Nonylphenoxy carboxylic acids (NPEC) are formed by biodegradation of NPEO under aerobic conditions [6], especially during activated sludge treatment in sewage treatment plants (STPs) [7]. They constitute the majority of NPEO biodegradation products in the secondary effluent of STPs, together with lower NPEO oligomers (NP1EO and NP2EO) and the completely de-ethoxylated product nonylphenol (NP) [8]. The only NPEC detected in environmental samples were NP1EC and NP2EC [7]. In contrast to SPC, NPEC retain surface-active properties.

High-performance liquid chromatography (HPLC) [9–19] has been preferred to gas chromatography (GC) or GC–mass spectrometry (GC–MS) [20–24] for the specific routine determination in environmental samples of both LAS and NPEO, as it does not require derivatization of LAS and of the higher NPEO oligomers. Another great advantage shown by RP-HPLC over the GC techniques is that the simultaneous determination of both LAS and NPEO using octyl- or octadecylsilica columns is possible [25,26]. In this instance, the LAS components are eluted according to the alkyl chain length and the attachment position of the phenyl ring in the alkyl chain, whereas the NPEO oligomers are eluted as a single peak as the chromatographic process is mainly governed by non-specific interactions occurring between the branched alkyl chain and the stationary phase. So far, few analytical procedures have been developed for determining SPC [27–30] and NPEC [7,24,31]. Most of them involved the use of GC or GC–MS techniques. The SPC were derivatized to methyl [29] or trifluoroethyl esters [30] or altered by desulphonation [28,29]. The NPEC were esterified by methylation [7]. Unaltered SPC were determined by ion-pair RP-HPLC with UV detection [32]. NPEC were analysed by normal-phase HPLC after derivatization to methyl esters [7]. The lack of commercially available standards is presumably the main cause of the scarce information on the environmental behaviour of individual SPC and NPEC.

The main objective of this work was to develop a "tailor-made" RP-HPLC system able to

yield the best fractionation of a complex mixture, such as that containing SPC, NPEC and their respective LAS and NPEO precursors. For this purpose, we evaluated the selectivity characteristics offered by the ion-suppression and the ion-pair HPLC techniques. Ion-pair HPLC was employed by adding to the mobile phase either a tetraalkylammonium salt or an inorganic salt, such as sodium perchlorate. The addition of this salt to the mobile phase has been widely adopted for fractionating LAS mixtures. A secondary objective was to evaluate the ability of an octadecyl-bonded silica (C_{18}) cartridge to extract from both treated and untreated wastewaters the compounds considered. This was done by suitably modifying previously reported methods [33,34].

EXPERIMENTAL

Materials and reagents

The LAS and NPEO standards were purchased from Carlo Erba (Milan, Italy). The average alkyl chain length of the C_{10}–C_{13} LAS mixture was $C_{11.2}$. The average polyethyoxylic chain length of NP*n*EO ($n = 1$–20) was 8.8. Pure C_{14} LAS homologue was kindly supplied by EniChem (Milan, Italy). Technical nonylphenol (NP) was obtained from Fluka (Buchs, Switzerland). A 75:25 (w/w) mixture of nonylphenol mono- (NP1EO) and diethoxylate (NP2EO) was obtained from Kolb (Hedingen, Switzerland). Most of the phenylalkanoic acids (phenylacetic, 2-phenylpropionic, 4-phenylbutyric, 5-phenylvaleric and phenylmalonic) were obtained from Fluka; 2- and 3-phenylbutyric and 3-phenylglutaric acid were purchased from Aldrich (Milwaukee, WI, USA). The solvents used for the syntheses were purchased from Carlo Erba and those for HPLC from Riedel-de Haën (Seelze, Germany). The HPLC mobile phase modifiers trifluoroacetic acid (TFA) and tetrabutylammonium dihydrogenphosphate (TBA-H_2PO_4) were supplied by Aldrich; sodium perchlorate was from Merck (Darmstadt, Germany). C_{18} extraction cartridges (1 g) were purchased from Supelco (Bellefonte, PA, USA). The cartridge was preconditioned with 5 ml of acetonitrile and 5 ml of methanol followed by 10 ml of distilled water.

Synthesis and characterization of carboxylic biodegradation intermediates

Synthesis of sulphophenyl carboxylates (SPC). The SPC were synthesized by sulphonation of the corresponding commercially available phenylalkanoic acids according to conditions reported by Taylor and Nickless [32]. Briefly, 20 ml of sulphuriç acids were added to 5 g of phenylcarboxylic acid and heated at 60°C with gentle stirring for 24 h. Unreacted starting material was removed with 2×100 ml of diethyl ether and the resulting solution was neutralized with NaOH. Three volumes of 2-propanol were added to precipitate the sodium sulphate and the mixture was left in a refrigerator overnight. The supernatant solution was filtered and evaporated to dryness on a rotary evaporator. The solid white residue was dissolved in hot methanol and reprecipitated by adding diethyl ether. Recrystallization was carried out in triplicate. The yields obtained were about 50% for monocarboxylic SPC and about 10% for bicarboxylic SPC. The sulphophenyl monocarboxylates $2C_2$-SPC, $3C_3$-SPC, $2C_4$-SPC, $3C_4$-SPC, $4C_4$-SPC and $5C_5$-SPC, and the bicarboxylates C_3-SP2C and $3C_5$-SP2C were synthesized.

Synthesis of nonylphenoxyacetic acids (NPEC). Both nonylphenoxyacetic acid (NP1EC) and nonylphenoxyethoxyacetic acid (NP2EC) were prepared by oxidation with Jones reagent (method A, see below) [23]. In addition, NP1EC was synthesized by reaction of chloroacetic acid with nonylphenol (NP) in an alkaline aqueous solution (method B) according to suitably modified conditions reported previously [35]. A common procedure was followed for the product purifications.

In method A, 11 g of NP1EO–NP2EO (75:25) mixture were added with stirring at 60°C to 100 ml of Jones reagent during 6 h. Heating of the solution was continued for about 10 h, then the mixture was left at room temperature. The desired products were recovered from the mixture by dilution with water (*ca.* 400 ml) followed by extraction with diethyl ether (8×100 ml). The combined green extracts were washed first

with 5% aqueous sulphuric acid (5 × 50 ml) and then with water. A green viscous oil was obtained after evaporation to dryness.

In method B, 22 g of nonylphenol were dissolved in 10 ml of ethanol. To this solution, 20 ml of aqueous NaOH and 20 ml of chloroacetic acid were added dropwise three times, at 1-h intervals. The pH during the reaction was maintained at *ca.* 10 with aqueous sodium hydroxide. The resulting solution after acidification to pH 4 and cooling was extracted with diethyl ether. The extracts obtained were washed with water, evaporated to dryness on a rotary evaporator and dried over magnesium sulphate.

The NP1EC–NP2EC mixture and NP1EC synthesized by the above two methods were purified by silica gel chromatography using a 40 × 2 cm column packed by suspending the sorbent in chloroform. The sorbent material (30–70-mesh silica; Merck) was activated at 110°C for 24 h. Aliquots of the dry residues were dissolved in a minimum volume of chloroform, transferred to the top of the column and eluted with the same solvent (500 ml). The NP1EC–NP2EC mixture obtained by method A and NP1EC obtained by method B were found in the third fraction (last 100 ml), after those containing a yellow side-product (first 100 ml) and unreacted 4-nonylphenol (next 100 ml).

Characterization of the synthesized compounds. Both SPC and NPEC were characterized by elemental analysis (Carlo Erba NA 1500 C, H, N analyzer), UV (Perkin-Elmer Lambda 5) and IR (Perkin-Elmer Model 683) spectrophotometry and ^1H NMR spectroscopy (Varian FT 80 A). Based on elemental analysis results, the purity of each compound, calculated from the difference between the observed recovery and the calculated recovery averaged for each element, was between 92% and 98%. The UV maxima were between 215 and 225 nm, with molar absorptivities in 1:1 water–acetonitrile of 9550–12 200 l mol^{-1} cm^{-1} for SPC and 9150–9360 l mol^{-1} cm^{-1} for NP1EC and NP2EC, respectively. The proton NMR spectra of SPC in ^2H_2O, showing a pseudo-quartet of the four aromatic protons at δ 7.60 (reference tetramethylsilane), confirmed that sulphonation had occurred at the *para* position.

HPLC apparatus and conditions

The method development work was performed on a Hewlett-Packard Model 1050 liquid chromatograph consisting of a quaternary pump, a four-bottle solvent-delivery system and a Rheodyne syringe-loading sample injector (Model 7125). The data were collected and treated by an electronic integrator (Hewlett-Packard Model 3396A). Fluorescence detection was performed by a Hewlett-Packard Model 1046A instrument (flow cell volume 10 μl). The detector was operated with an excitation wavelength of 225 nm and an emission wavelength of 295 nm, with a spectral band pass of 10 nm.

The chromatographic separation was performed in the reversed-phase mode by using an octadecylsilica column (LiChrospher RP-18, 250 × 4 mm I.D., 5-μm particle size, from Merck) equipped with a guard column and operated at room temperature.

Elution was carried out in the gradient mode, at a flow-rate of 1.0 ml min^{-1}, using aqueous acetonitrile and aqueous methanol containing alternatively $NaClO_4$, TFA and $TBAH_2PO_4$ as phase modifiers. In presence of $TBAH_2PO_4$, the pH was adjusted to 5.5 with 0.1 *M* NaOH.

The following elution programmes were used depending on the type of mixture and phase modifier.

Water–acetonitrile containing $NaClO_4$:
solvent A, acetonitrile; solvent B, water containing 14 g l^{-1} $NaClO_4$:
0– 3 min 40% A–60% B
3–23 min linear gradient to 30% B
23–26 min linear gradient to 10% B
26–30 min linear gradient to 60% B
30–32 min 40% A–60% B

Water–acetonitrile containing TFA:
solvent A acetonitrile; solvent B, water containing 0.14 g l^{-1} TFA; solvent C, water:
0 – 0.5 min 5% A–40% B–5% C
0.5–10 min linear gradient to 60% A and 40% C
10 –20 min linear gradient to 90% A and 10% C
20 –23 min 90% A–0% B–55% C
23 –28 min linear gradient to 5% A–40% B–55% C

Water–acetonitrile containing $TBAH_2PO_4$:
solvent A, acetonitrile; solvent B, water containing 3.4 g l^{-1} $TBAH_2PO_4$; solvent C, water:
0 – 0.1 min 35% A–32.5% B–32.5% C
0.1– 8 min linear gradient to 70% A–15% B–15% C
8 –15 min linear gradient to 90% A–5% B–5% C
15 –19 min linear gradient to 75% A–12.5% B–12.5% C
19 –23 min linear gradient to 35% A–32.5% B–32.5% C

Water–acetonitrile containing both $NaClO_4$ and TFA:
solvent A, acetonitrile; solvent B, water containing 0.14 g l^{-1} TFA; solvent C, water containing 14 g l^{-1} $NaClO_4$; solvent D, water:
0 – 0.5 min 5% A–40% B–0% C–55% D
0.5–10 min linear gradient to 60% A–0% B–20% C–20% D
10 –20 min linear gradient to 90% A–0% B–0% C–10% D
20 –28 min linear gradient to 5% A–40% B–0% C–55% D

Water–methanol containing $NaClO_4$:
solvent A, methanol; solvent B, water containing 14 g l^{-1} $NaClO_4$:
0–15 min 60% A–40% B
15–20 min linear gradient to 20% B
20–23 min 80% A–20% B
23–26 min linear gradient to 10% B
26–30 min linear gradient to 40% B

Water–methanol containing TFA:
solvent A, methanol; solvent B, water containing 0.14 g l^{-1} TFA; solvent C, water:
0–10 min 5% A–95% B–0% C
10–20 min linear gradient to 60% A and 40% C
20–30 min linear gradient to 70% A and 30% C
30–32 min linear gradient to 85% A and 15% C
32–37 min linear gradient to 5% A–95% B–0% C

Water–methanol containing $TBAH_2PO_4$:
solvent A, methanol; solvent B, water containing 3.4 g l^{-1} $TBAH_2PO_4$; solvent C, water:
0 – 0.1 min 20% A–50% B–0% C–30% D
0.1–10 min linear gradient to 60% A–0% B–8% C–32% D
10 –20 min linear gradient to 70% A–0% B–6% C–24% D
20 –28 min linear gradient to 85% A–0% B–3% C–12% D
28 –34 min 85% A–0% B–3% C–12% D
34 –39 min linear gradient to 20% A–50% B–0% C–30% D

Water–methanol containing both $NaClO_4$ and TFA:
solvent A, methanol; solvent B, water containing 0.14 g l^{-1} TFA; solvent C, water containing 14 g l^{-1} $NaClO_4$; solvent D, water:
0 – 0.5 min 5% A–40% B–0% C–55% D
0.5–10 min linear gradient to 60 % A–0% B–20% C–20% D
10 –20 min linear gradient to 90% A–0% B–0% C–10% D
20 –28 min linear gradient to 5% A–40% B–0% C–55% D

Samples

Influent and final effluent water were 24-h composite samples. They were taken from mechanical–biological sewage treatment facilities. All specimens were immediately passed through glass-fibre filters (0.2 μm) (Whatman), 1% of formaldehyde (37%; Merck) was added and the mixtures were stored at 4°C.

Isolation and identification procedures

The isolation of SPC and NPEC, and of LAS and NPEO, from filtered (0.2-μm glass-fibre filters; Whatman) aqueous samples was carried out by the following two procedures. One involved the removal of water by rotary evaporation of 10 ml of influent, 50 ml of final effluent and 200 ml of river water. The semi-dried residue was dissolved by ultrasonication for 5 min in 5 ml of methanol containing 0.05 *M* sodium dodecyl sulphate (SDS) that was used to aid the resolubilization of the surfactants. The aqueous methanol extracts were concentrated by evaporation on a steam-bath to *ca.* 0.5–2 ml, depending on the type of sample. The alternative isolation procedure followed in this work em-

ployed commercially available C_{18} extraction cartridges. After acidification of the aqueous sample with HCl (pH 2) and conditioning of the cartridge as described above, 10 ml of influent, 50 ml of effluent and 200 ml of river water were passed through the C_{18} cartridge. After extraction, SPC, NPEC, LAS and NPEO were eluted with 5 ml of methanol. Blow-down of the methanolic eluate was carried out in a water-bath under a stream of nitrogen to 0.5–1.5 ml, depending on the type of sample.

The SPC and NPEC in environmental aqueous samples were identified both from the retention times and by external addition of both the synthesized compounds and a series of SPC which were obtained by LAS biodegradation experiments. The latter were formed during the run of the Modified OECD Biodegradation Screening Test [36] carried out under mild inoculum (fertile soil) conditions [37,38]. Analysis performed by both GC–MS and RP-HPLC provided the identification and quantification of isomers of the C_6–C_8 SPC homologues (*i.e.*, 2,3C_6-SPC, 3,4C_7-SPC and 3,4,5C_8-SPC) and of bicarboxylate SPC not synthesized, namely the succinic (C_4-SP2C) and adipic (C_6-SP2C) compounds. The amounts of biologically generated SPC were sufficiently high to allow standard addition and recovery experiments to be carried out [37,38].

Quantification

SPC, NPEC, LAS and NPEO were determined using calibration graphs. When necessary, the standard addition method was applied to the determination of the compounds considered in environmental samples. The calibration graphs were obtained by dissolving in water–acetonitrile (50:50, v/v) 0.02–0.25 mg ml^{-1} of LAS, 0.005–0.15 mg ml^{-1} of NPEO and 0.002–0.08 mg ml^{-1} of each synthesized SPC and NPEC compound. Recovery experiments and standard additions were performed by adding to the aqueous environonmental sample prior to the extraction or to the final extracts, respectively, 5–125 μg of each compound class. The additions were made according to the criterion of approximately doubling the original concentrations.

By using $NaClO_4$ as phase modifier, SPC were eluted as a single peak. In this instance, the calibration graph was constructed using the biologically generated SPC because their distribution was similar to that found in the environmental samples.

The column injection volumes of both standard solutions and environmental samples were 20–100 μl and contained *ca.* 0.6–12 μg of SPC, 0.4–7.5 μg of LAS, 0.05–1.4 μg of NPEC and 0.1–4.5 μg of NPEO.

RESULTS AND DISCUSSION

The chromatographic behaviours of the components of the mixtures containing the surfactants and some of their synthesized biointermediates were altered by varying both the organic modifier, namely acetonitrile and methanol, and the phase modifier, that is $NaClO_4$, trifluoroacetic acid (TFA) and tetrabutylammonium dihydrogenphosphate ($TBAH_2PO_4$). For the sake of clarity, the effects observed on adding the three phase modifiers, individually or in combination, to water–acetonitrile mixtures will be discussed separately from those obtained with water–methanol mixtures.

With water–acetonitrile mixtures

The chromatograms showing the separations of the considered compounds obtained by gradient elution of aqueous acetonitrile mixtures containing (A) $NaClO_4$, (B) TFA, (C) $TBAH_2PO_4$ and (D) both $NaClO_4$ and TFA are shown in Fig. 2. As already indicated, to determine LAS by RP-HPLC, mobile phases of different compositions containing almost invariably relatively high concentrations of $NaClO_4$ as ion-pair phase modifier have frequently been proposed [9–15]. By considering the nature of the counter ion, this chromatographic system could be called "soft" ion-pair chromatography. By suitably adjusting some chromatographic parameters, the capacity of this technique for fractionating the mixture of interest was evaluated (Fig. 2A). As can be seen, both SPC and the two NPEC were eluted before LAS and NPEO, as expected considering that the first two compound classes are more polar

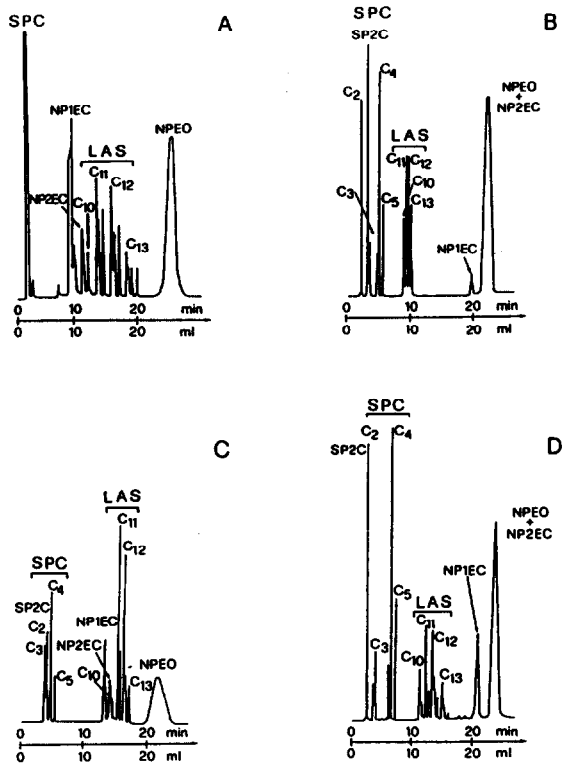

Fig. 2. RP-HPLC-fluorescence detection chromatograms showing the elution of SPC, LAS, NPEC and NPEO with aqueous acetonitrile mixtures containing (A) $NaClO_4$, (B) TFA, (C) $TBAH_2PO_4$ and (D) both $NaClO_4$ and TFA.

than the third [39]. The eight synthesized SPC were only weakly retained by the stationary phase and eluted into two peaks. Several factors suggest that this technique is impracticable for determining SPC. From a qualitative point of view, no information can be obtained about the metabolic pathway of LAS. From a quantitative point of view, there is a high probability that extraneous compounds are co-eluted with SPC, resulting in overestimation of the LAS intermediates. This risk is greatly increased when using a non-selective detector, such as a UV detector. Even when using fluorescence detection, however, the quantification is made difficult if no information on the average distribution of SPC is available, as the signal intensity per unit mass decreases as the alkyl chain length of the SPC homologues is increased.

Concerning the two NPEO metabolites, NP1EC was completely resolved, but the peak for NP2EC was only partially resolved from those for the innermost isomers of the C_{10} LAS.

By comparing chromatographs obtained under the various conditions selected, it appears that the positive feature of using $NaClO_4$ as a phase modifier is that partial separations of positional isomers of LAS, and also the separation of NP1EC and partial separation of NP2EC from the innermost C_{10} LAS isomers, were obtained. The elution order of the positional isomers for each LAS homologue followed that of decreasing distance of the substituent phenyl group from the end of the alkyl chain. Although incomplete, this separation may be of interest when rapid information on the biodegradation rate of the various LAS isomers is desired.

The separation of the SPC components was achieved by using the ion-suppression technique (Fig. 2B). With the TFA concentration selected, the term partial ion suppression should more correctly be used. In fact, by increasing the amount of TFA dissolved in the mobile phase, a continuous increase in the retention times of LAS was noted to the point (corresponding to about 5 g l^{-1} TFA) that the peak for the highest LAS homologue overlapped that for NP1EC. Under the conditions selected, the elution order of SPC was $2C_2$-SPC, C_3-SP2C + $3C_5$-SP2C, $3C_3$-SPC, $2C_4$-SPC + $3C_4$-SPC + $4C_4$-SPC, $5C_5$-SPC.

Compared with the previous situation, this offers several advantages. One is that the resolution of the SPC mixture decreases the probability of overestimation of LAS biointermediates by unknown compounds. The second is that all of the isomers of each SPC homologue are lumped into one peak. This makes the determination of SPC in real water samples easy to perform by using a few synthesized SPC compounds and assuming that all of the isomers of each homologue exhibit the same fluorescence quantum yield. Finally, although not detailed, rapid information on the metabolic pathway of LAS can be achieved.

For the LAS mixture, all of the isomers of each homologue were co-eluted in a single peak. This effect can be advantageously exploited for the routine determination of traces of LAS, as

the peak for each homologue increases in intensity.

Compared with the results obtained by using $NaClO_4$, the most dramatic effect obtained on suppressing the ionization of NPEC was that they were eluted well after LAS and near NPEO with the peak for NPEC overlapped by that for its parent compounds. These effects can be explained by considering that non-ionized NPEC have a higher affinity for the stationary phase than partially dissociated LAS. In addition, interactions occurring between the stationary phase and the branched alkyl chain, common to both NPEO and NPEC, play a dominant role in determining close elutions of the two compound classes.

By classical ion-pair chromatography, achieved by using a tetralkylammonium salt as phase modifier (Fig. 2C), the elution sequence of the four classes of compounds was the same as that obtained with $NaClO_4$ as phase modifier. On the other hand, the TBA^+ naked ion was more effective than Na^+ in forming stable, hydrophobic ion pairs with SPC. As a result, they were retained by the stationary phase and, thus, partially separated from each other. The elution order of SPC was $2C_2$-SPC + $3C_3$-SPC, C_3-SP2C + $3C_5$-SP2C, $2C_4$-SPC + $3C_4$-SPC + $4C_4$-SPC, $5C_5$-SPC. It appears that the addition of an ion-pair-forming agent to the mobile phase did not allow the separation of the C_2 from the C_3 SPC homologue which was achieved by the ion-suppression retention mechanism. This was due in part to the fact that derivative formation, as occurs with the ion-pair technique, tends to obscure small differences in the chemical characteristics of solutes and in part to the low capacity factors of the first two members of SPC that resulted in a low resolving power of the chromatographic system. On the other hand, attempts to increase their retention by increasing the concentration of $TBAH_2PO_4$ caused LAS to be co-eluted with NPEO.

With respect to the elution sequence obtained by the use of ion-suppression HPLC, the elution of C_3-SPC and the two bicarboxylate forms was reversed by ion-pair formation. This effect can be ascribed to the fact that the latter two compounds form a triple derivative as they contain three negative charges, so that their actual molecular sizes are enhanced.

Compared with the ion-suppression technique, that exploiting the ion-pair retention mechanism caused NPEO to be eluted into a much broader and indented peak. This indicates that the latter technique is more selective than the former for the separation of the various NPEO oligomers, which, however, cannot be achieved owing to the limited efficiency of the HPLC column. This effect was unclear to us, taking into consideration that the two phase modifiers should not affect significantly the chromatographic process of neutral eluates, such as NPEO. From a practical point of view, it is preferable to obtain NPEO lumped into a sharp peak as this improves the detection limit of analytical procedures devoted to monitoring NPEO in natural water samples where these surfactants are present at very low parts per billion levels.

The combined effects of both $NaClO_4$ and TFA as phase modifiers were also evaluated (Fig. 2D). As mentioned above, at the low ion-suppressor concentrations used, only the dissociation of the carboxylic group, common to both SPC and NPEC, was totally enhibited. Under this hybrid situation, the elution process of LAS was controlled by the mechanism of "soft" ion-pair formation, as partial separation of the isomers of each LAS homologue was again achieved. For the other compound classes, the chromatographic process was very similar to that obtained by the addition of the sole ion-suppression agent, with the exception of the SP2C, which co-eluted with C_2-SPC.

With aqueous methanol mixtures

It is known that the nature of the organic modifier in RP-HPLC can affect the separation of a given mixture by peculiar interactions taking place between particular eluates and the organic solvent. These are the so-called "secondary effects" of the organic modifier. In order to establish whether the secondary effects could positively affect the fractionation of the mixture under consideration, acetonitrile was replaced with methanol as organic modifier. Obviously, when using methanol in place of acetonitrile, some parameters of the gradient elution process

were suitably adjusted (see Experimental) to obtain the best time–resolution combination. In Fig. 3, chromatograms are shown for the elution of the compounds considered by using the same phase modifiers as used with aqueous acetonitrile mixtures. The sequence of the chromatograms reported was the same as with the aqueous acetonitrile mixtures.

Apparently, the replacement of acetonitrile with methanol had the effect of eluting all of the NPEO oligomers into a much sharper peak. The less selective elution of the various NPEO oligomers mixture obtained with methanol as organic modifier might be ascribed to the fact that differences in polarity of the various NPEO oligomers arising from differences in the length of the polyethoxylic chain are levelled out by specific interactions taking place with the terminal hydroxyl of these eluates and that of methanol. To substantiate this hypothesis, the mixture composed of all of the NPEO oligomers was simultaneously injected into the HPLC column with that formed only by the first two members of NPEO (*i.e.*, NP1EO and NP2EO). When water–acetonitrile was used as the eluent, NP1EO and NP2EO produced a peak over lapping the final part of the peak for NPEO, whereas the opposite occurred with methanol as organic modifier.

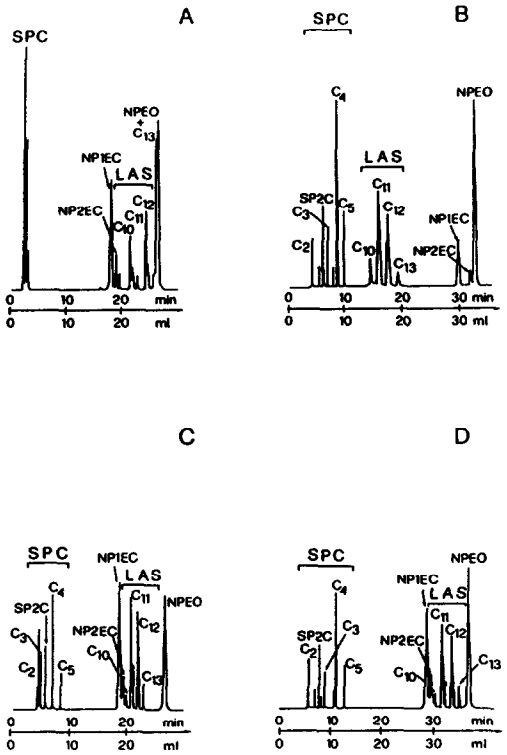

Fig. 3. RP-HPLC-fluorescence detection chromatograms showing the elution of standard solutions of SPC, LAS, NPEC and NPEO, with aqueous methanol mixtures containing (A) $NaClO_4$, (B) TFA, (C) $TBAH_2PO_4$ and (D) both $NaClO_4$ and TFA.

Under the ion-suppression conditions (Fig. 3B), the benefits of using a methanol–water as the eluent were that LAS homologues were well separated from each other and, mostly, we were able to separate NP2EC from NPEO.

Under the conditions of ion-pair formation, the replacement of acetonitrile with methanol succeeded in separating C_2-SPC from C_3-SPC (Fig. 3C).

Interestingly, under the combined ion-pair formation and ion-suppression effects provided by the simultaneous presence in the mobile phase of $NaClO_4$ and TFA (Fig. 3D), NPEC were eluted before LAS. This indicates that more stable ion pairs were formed between LAS and the Na^+ counter ion in the presence of methanol. The reason for this effect was unclear to us.

Effect of phase modifier concentration on fractionation

As is known, charged species can be chromatographed by RP-HPLC provided that either an ion-pair-forming agent or an ion-suppressor is added to the mobile phase. When this is not done, charged compounds are eluted all together with the dead volume of the column. In contrast, within certain limits, the equilibrium of the distribution of ionogenic compounds between the stationary and the mobile phases can be altered by suitably varying the concentration of the phase modifier, so that the best fractionation of a given complex mixture can be achieved.

Fig. 4 shows the effect of varying the concentration of $NACIO_4$ on the retention volumes of SPC and NPEC. The chromatographic behaviour of LAS on varying the salt concentration has already been investigated [26]. We observed that the chromatographic process of the latter com-

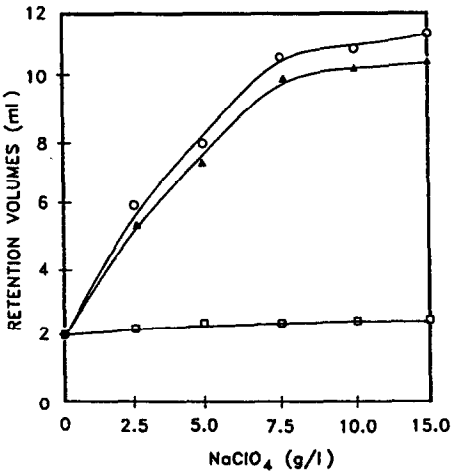

Fig. 4. Dependence of the retention volumes of NP1EC (○), NP2EC (▲) and SPC (□) on the salt concentration in aqueous acetonitrile.

pounds considered was not significantly affected by varying the nature of the inorganic salt. At any salt concentration considered, no effect on the retention of SPC was observed. Probably the ion pairs formed between SPC and the Na^+ counter ion are still so highly polar that no adsorption of them on the stationary phase can take place. In contrast, the retention of NP1EC and NP2EC steadily increased up to a salt concentration of *ca.* 7.5 g l^{-1}, where the two NPEO intermediates were fairly well separated from each other. Further increases in salt concentration up to 32 g l^{-1} did not improve the separation of the compounds considered. The plots of the retention volumes of SPC, NPEC and LAS *versus* the concentrations of the phase modifiers TFA and $TBAH_2PO_4$ were similar to that of NPEC in Fig. 4. Initial concentrations of TFA and $TBAH_2PO_4$ lower than 0.05 and 0.5 g l^{-1}, respectively, prompted an earlier elution of the compounds considered and adversely affected the resolution of the LAS, SPC and NPEC components shown in the Figs. 2 and 3. Raising the initial concentrations of both TFA and $TBAH_2PO_4$ above 0.4 and 8.0 g l^{-1}, respectively, caused a continuous increase in the retention volumes of LAS, SPC and NPEC without any further improvement in the resolution of their components.

Detection by UV and fluorescence methods

The synthesized biotransformation products of LAS and NPEO, namely SPC and NPEC, can be detected by both UV absorption at 225 nm and fluorescence with excitation and emission wavelengths of 225 and 295 nm, respectively, since they retain the spectroscopic properties of the parent compounds. A simple, inexpensive UV detector can be employed for analysing commercial products, while the monitoring in aqueous environmental samples of surfactants and their intermediates is more accurately performed by a selective detection method, such as fluorescence. Under the various chromatographic conditions examined, the detectabilities of the compounds considered were measured by connecting a UV and a fluorimetric detector in series and injecting 10 nmol of each compound.

With UV detection, some variations in the molar absorptivities of the eluates were noted, depending on the particular mobile phase selected. These fluctuations, however, were not significant enough to indicate the superiority of a particular chromatographic system in terms of selectivity.

The fluorescence signal intensities, reported in Table I, show that even the fluorescence quantum efficiency of SPC, LAS and NPEO was to some extent affected by the eluent system employed. On the other hand, an abrupt decrease in the quantum efficiency was observed for NPEC when eluted in the presence of an ion-suppression agent. No particular effect was caused by the presence of TFA in the mobile phase, as its replacement with any other ion suppressor did not lead to an increase in the quantum efficiency of the NPEO intermediates. Evidently, the sharp decrease in the quantum efficiency of the NPEO skeleton is directly related to the introduction into it of an undissociated carboxylic group. When coupled to ion-suppression chromatography, a fluorescence detector is useless for determining small amounts of NPEC.

Application to environmental samples

The RP-HPLC procedures proposed for the concurrent separation of SPC, NPEC, LAS and NPEO were applied to the analysis of aqueous

TABLE I

SIGNAL INTENSITIES WITH FLUORESCENCE DETECTION (EXCITATION AT 225 nm; EMISSION AT 295 nm) OF SPC, LAS, NP1EC AND NPEO DISSOLVED IN BOTH ACETONITRILE AND METHANOL MIXTURES WITH VARIOUS PHASE MODIFIERS

Compound	Peak area (counts × 1000)[a]					
	$NaClO_4$		TFA		$TBAH_2PO_4$	
	CH_3CN	CH_3OH	CH_3CN	CH_3OH	CH_3CN	CH_3OH
SPC	8.6	11.6	4.9	11.2	9.4	9.4
LAS	12.2	17.8	23.3	9.9	14.3	12.0
NP1EC	19.3	18.6	1.6	2.6	17.2	13.5
NPEO	67.3	66.9	67.0	80.1	63.8	69.9

[a] Values recorded by the integration corresponding to 10 nmol of each compound injected under the various chromatographic conditions reported under Experimental. The peak areas of each compound class were calculated by summing the peak areas of the individual components.

samples, such as treated and untreated wastewaters. Two sample preparation procedures were followed. One involved water removal by distillation of suitable volumes of each sample (10 ml of influent, 50 ml of final effluent) followed by dissolution of the semi-dried residue in methanol. When exploiting the selectivity of fluorescence detection, owing to the very low vapour pressure of the considered compounds and their relatively high concentration levels in wastewaters (1–10 mg l^{-1}), this sample preparation procedure is suitable for measuring the presence in the environmental samples of SPC and NPEC, and also of LAS and NPEO.

The second procedure was based on percolation through a C_{18} extraction cartridge of the same samples as reported above after adjusting their pH to about 2 and adding salt. This sample pretreatment was suggested by the observation of the synergistic effects produced by the simultaneous presence of a salt and an ion suppressor on the retention of the eluates on a reversed-phase stationary phase, as been illustrated above. Fig. 5 shows chromatographs obtained on preparing the samples by the first procedure and injecting the extract into the HPLC apparatus operating in the acetonitrile ion-suppression mode with fluorescence detection. As can be seen, no peak for NPEO and their intermediates was evident. This is because the water samples were collected in a plant treating domestic sewage. As is known, no European household formulation contains NPEO any longer [42]. The identification of SPC was done by external addition to the final extracts of authentic SPC, of both chemical and biochemical origin. In the influent, the small amounts of SPC found were bicarboxylate or

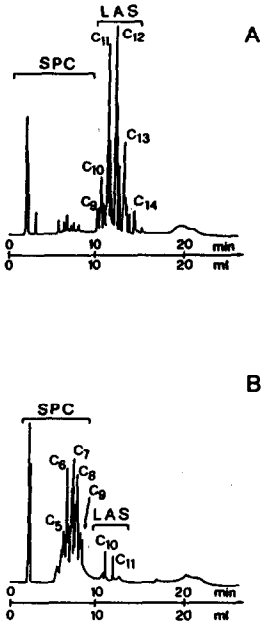

Fig. 5. RP-HPLC traces obtained on injecting the extracts obtained from (A) 10 ml of an influent and (B) 50 ml of an effluent of a plant treating domestic sewage. Fluorescence detection was used.

short-chain monocarboxylate species. In contrast, C_5–C_8 SPC homologue predominated in the effluent. The overall SPC concentrations calculated from triplicate analyses of 10 ml of influent and 50 ml of effluent were 1.4 ± 0.2 mg l^{-1} in the influent and 4.5 ± 0.5 mg l^{-1} in the effluent after water distillation and 1.1 ± 0.3 mg l^{-1} in the influent and 3.2 ± 0.4 mg l^{-1} in the effluent after percolation through the C_{18} cartridge. This suggested that SPC were partly lost on extracting 50 ml of the effluent with the C_{18} cartridge. In these same samples the LAS concentrations were 6.7 ± 0.4 mg l^{-1} in the influent and 0.08 ± 0.03 mg l^{-1} in the effluent, with no statistically significant differences between the results obtained by using the two procedures.

Recovery experiments on SPC and NPEC were conducted by spiking effluent samples and by following the two isolation procedures. The results are reported in Table II. The C_{18} extraction cartridge partially failed to retain SPC, especially those having less than six carbon atoms in the alkyl carboxylate chain. In contrast, the analyte isolation procedure by water evaporation provided quantitative recoveries (96–102%) with a good relative standard deviation (3–9%) for each of the added analytes. The recoveries of NPEC, and also those of LAS and NPEO according to previous work [33], obtained by making use of both isolation procedures were satisfactory.

TABLE II

RECOVERY OF SPC AND NPEC AFTER ADDING THEM TO 50 ml OF A SEWAGE TREATMENT PLANT EFFLUENT SAMPLE AND EXTRACTION WITH A C_{18} CARTRIDGE

Compound	Added (μg)	Recovery (%)
$2C_2$-SPC	72	12.6 ± 3.3
C_3-SP2C	88	30.3 ± 4.2
$5C_5$-SPC	76	38.0 ± 4.5
C_6-SPC[a]	70	57.3 ± 5.0
C_7-SPC[b]	104	72.0 ± 2.9
C_8-SPC[c]	76	82.7 ± 3.4
NP1EC	96	91.0 ± 2.9
NP2EC	24	87.7 ± 2.5

[a] Mixture of $2C_6$-SPC and $3C_6$-SPC.
[b] Mixture of $3C_7$-SPC and $4C_7$-SPC.
[c] Mixture of $3C_8$-SPC, $4C_8$-SPC and $5C_8$-SPC.

CONCLUSIONS

RP-HPLC coupled with fluorimetric detection can allow the simultaneous separation of the surfactants LAS, NPEO and their carboxylate biointermediates, provided that a careful choice of both the organic and phase modifiers is made. The flexibility of this chromatographic technique makes it suitable for laboratory biodegradation studies and also for field monitoring of the compounds of interest in aqueous samples. By using acetonitrile as organic modifier and simultaneously adding $NaClO_4$ and TFA to the mobile phase, the metabolic pathway of LAS, under various laboratory biodegradation test conditions, could be readily understood by following the gradual selective disappearance of peaks for the various homologues and positional isomers of LAS and the simultaneous increase in peaks for the various SPC. For monitoring the target compounds in aqueous environmental samples, the ion-pair retention mechanism with methanol–water mixtures appears to be the most selective, as it is capable of separating the two NPEC from the parent compounds.

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