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High-performance liquid chromatographic–fluorescence determination of aliphatic alcohol polyethoxylates and polye(thylene glycol)s in aqueous samples

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Abstract

New derivatization and separation procedures for the routine determination of detergent alcohol polyethoxylates (AEs) and poly(ethylene glycol)s (PEGs) in aqueous environmental and biodegradation screening test samples are proposed. The AE derivatives were separated both homolog-by-homolog or ethoxymer-by-ethoxymer by reversed-phase HPLC–fluorescence (RP-HPLC–FL) after derivatization with naphthyl isocyanate (NIC) and naphthoyl chloride (NC), respectively. The ethoxymer-by-ethoxymer separation of PEGs was obtained by RP-HPLC–FL after derivatization with NC. The fluorescence response of the various AE homologs and AE, PEG ethoxymers were evaluated. AE, PEG as well as nonylphenol polyethoxylates were cumulatively extracted from biodegradation test liquors and sewage-treatment plant influent and final effluent samples by solid-phase extraction on graphitized carbon black (GCB), and then derivatized and analyzed by RP-HPLC–FL. The recovery was systematically >90%, with relative standard deviations of 3–6%, for all compounds considered. The proposed derivatization, separation and detection procedures showed a sensitivity of 5–10 ng of injected AE or PEG derivatives leading to a *S/N* ratio of 10.

Keywords: Derivatization, LC; Poly(ethylene glycol); Alcohol polyethoxylates; Polyethoxylates; Surfactants

1. Introduction

Aliphatic alcohol polyethoxylates (AEs) are the most extensively used nonionic surfactant. Worldwide, AE production and consumption is estimated at 0.5×10^6 metric tons per year [1]. They are produced by industry via ethoxylation of either alcohols from the reduction of the corresponding naturally occurring fatty acids or petroleum-derived alcohols. The resulting commercially available compounds consist of homologs with an even number of carbon atoms ranging typically from 12 to 18 (so called oleochemical AEs) or a mixture of even-odd linear and α-substituted alkyl chains (so called oxo-AEs) with 11 to 15 carbons (Fig. 1). Highly branched AEs, widely used till the 1960s, are synthesized via ethoxylation of "isotridecanol" (C_{10}–C_{13} mixture) obtained from oligomerization of butene and propene. Each homolog shows an ethoxymer distribution accounting typically for 1–30 ethoxy units with an average ethoxylation number in the range 5–15 (Fig. 1). A significant amount, i.e. 2–10% on a molar basis, of unethoxylated alcohol is systematically present in the commercial blends for each

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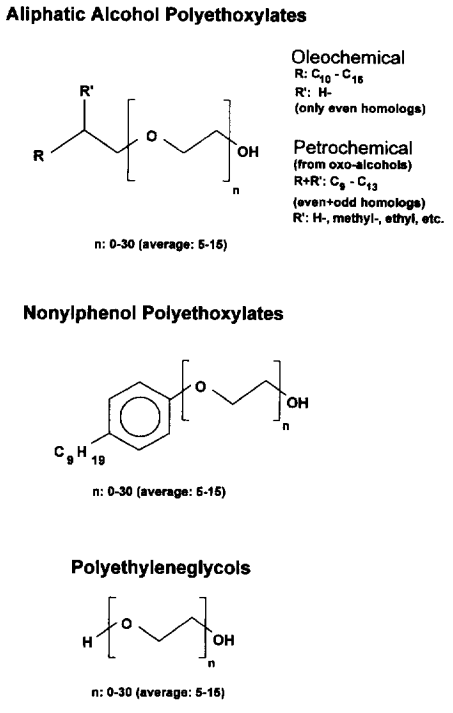


Fig. 1. Structures and acronyms of AE, NPE and PEG.

homolog. In addition to AE, the structures of poly(ethylene glycol)s (PEGs) and nonylphenol polyethoxylates (NPEs) are also presented in Fig. 1. Both PEGs and NPEs are expected to occur in environmental matrices. The former were conclusively demonstrated to be major aerobic biointermediates of AE [2], as well as being originated by other sources. The latter had been by far the most important nonionic surfactants till the late 1970s. In spite of the low biodegradability of their hydrophobic moiety, leading to the release of toxic and persistent 4-alkylphenols, alkylphenol polyethoxylates (APEs) have been recently banned in some countries. Nevertheless, APEs are still produced in large amounts (380 000 tons in 1993 [1]) even though their use is restricted to industry-related cleaning purposes. Thus, significant concentrations of APEs can still be encountered in waters influenced by industrial discharges [3].

The current AE consumption warrants investigation into various areas of interest, such as, for instance, transformation pathways during sewage treatment and in the receiving environmental compartments.

A major prerequisite of any environmental investigation is the availability of adequate analytical procedures providing suitable sensitivity (AEs recorded so far in surface waters are in the 1–40 μg/l range) [4,5] and selectivity (i.e. the dosage of individual components).

The separation of the AE complex mixtures was approached by chromatographic techniques such as gas chromatography (GC) after HBr cleavage [5–8], pyrolysis–GC (Py–GC) [9,10], high-temperature GC (HTGC) [11–13], GC–mass spectrometry (GC–MS) [5,11,14], normal- [15–19] and reversed-phase [20–23] high-performance liquid chromatography (HPLC), HPLC–MS [4,24–26] and supercritical fluid chromatography (SFC) [12,13,28].

The application of GC methods is severely limited by the low volatility of the higher-molecular-mass AE oligomers which require adequate derivatization. To the best of our knowledge, only the silyl derivatives with up to 15–17 [11,13] or 20 [12] ethoxy units are amenable to GC, particularly HTGC. Furthermore, the GC separation of the AE derivatives, showing a high extent of peak overlapping, provides a reliable quantitation only by mass spectrometric detection operating in selected ion monitoring (SIM). The MS-SIM detection can be avoided after prior off-line alteration of AEs by HBr leading to the formation of the corresponding alkyl bromides [6,7]. Thus, the homolog composition of AEs is attainable by GC–flame ionization detection (FID) but no information is provided on the ethoxymeric distribution for each homolog. Only qualitative identification was obtained by Py–GC.

SFC does not require derivatization of AEs and shows separation capabilities similar or superior to those of HTGC. Nevertheless, the extent of peak overlapping expected for environmentally occurring AEs is so high that both qualitative and quantitative determination of AEs is not feasible by conventional detection (FID) [12,27]. The current difficulty of coupling SFC and mass spectrometry prevents any application of SFC to the environmental analysis of AE.

Derivatization is also needed by HPLC to allow

the non-absorbing AEs to be detected by absorption or fluorescence. HPLC is, however, more versatile than GC and SFC since both normal- and reversed-phase have the capability of separating the AE derivatives. In particular, the normal phase is best suited to the cumulative ethoxymer-by-ethoxymer separation (i.e. the coelution of ethoxymers with different alkyl chains but the same number of ethoxy units) [19]; the reversed phase is particularly suited to the homolog-by-homolog separation [21], as well as for the elution according to both the alkyl and polyethoxy chain lengths. Thus, reliable information about the ethoxymeric or the homolog distribution of the analyzed AEs can be achieved regardless of the compositional complexity of the sample.

Derivatization of AEs can be avoided only by HPLC–MS. Moreover, the MS operating in SIM does not require the optimization of the homolog-by-homolog, or ethoxymer-by-ethoxymer, separations. Branched and linear AEs of identical molecular mass can be distinguished [24]. In addition to the expensive instrumentation, however, elaborate quantification [24] and response factors dropping down to zero for the lower ethoxymers and the unethoxylated compound [4] are the drawbacks actually affecting the HPLC–MS determination of AEs.

The development of routine procedures is, therefore, necessary and appears more easily attainable by HPLC. The AE derivatizing agents proposed so far were the phenylisocyanate [16], the 1-anthroylnitrile [22] and the 3,5-dinitrobenzoyl chloride [29]. The lack of fluorescence activity of phenyl isocyanate and 3,5-dinitrobenzoyl chloride, on the one hand, and the need for synthesizing through a multistep reaction the fluorescent commercially unavailable 1-anthroylnitrile limited an effective application of these compounds to the environmental analysis of AEs. In fact, only the phenyl isocyanate was used to derivatize AEs in raw and treated sewage and river water [19,30,31].

In this work, we propose new fluorescent agents suitable for the derivatization of AEs and their biointermediates, PEGs, as well as NPEs, the separations (homolog-by-homolog and/or ethoxymer-by-ethoxymer) of AEs and PEGs using reversed-phase HPLC and an overall protocol for the separation of both aliphatic and aromatic nonionic polyethoxylate surfactants, as well as PEGs, in biodegradation test liquors and environmental aqueous samples.

2. Experimental

2.1. Reagents and materials

Different high purity (98 to >99% active material) AE mixtures, kindly provided by the producers, were used as reference materials during the whole investigation. The reported compositions of the mixtures tested are those declared by the manufacturers. Marlipal 28/100 (Hüls, Marl, Germany) and Dehydol LT7 (Henkel, Düsseldorf, Germany) are mixtures of even linear primary C_{12} (48–58%, w/w), C_{14} (19–24%, w/w), C_{16} (9–12%, w/w) and C_{18} (11–20%, w/w) fatty alcohol polyethoxylates characterized by an average number of 10.2 and 7 ethoxy units, respectively. Lorol 124/10EO (Geronazzo, Bollate, Italy) contains only C_{12} (ca. 75%, w/w) and C_{14} (ca. 25%, w/w) oleochemical AE with an $E_{average}$ of 10.2 units. LIALET 125/7 (Enichem Augusta, Milan, Italy) is a mixture of even and odd, linear and 2-alkyl substituted (i.e. mono-branched) primary C_{12}–C_{15} oxo-alcohol polyethoxylates with an average polyethoxy chain of 7 units. LIALET 111/6 (Enichem Augusta) is a mixture of C_{11} linear and 2-alkyl substituted primary oxo-alcohol polyethoxylates with an average of 6 ethoxy units in the polyethoxy chain. The content of 2-alkyl-substituted AE in the LIALET blends ranges from 50% (w/w) for LIALET 111/6 to 59% in LIALET 125/7. Highly pure (>98%) standards of linear primary alcohols C_{11}–C_{18} (Aldrich, Milwaukee, USA) and of the individual C_{12}–$C_{14}E_1$–E_8 AE ethoxymers (Fluka, Buchs, Switzerland) were used for the study of derivatization reactions, chromatographic and detection behaviour of AE derivatives, as well as for recovery studies. The derivatizing agents 1-naphthyl isocyanate (NIC), 1-naphthoyl chloride (NC), 2-naphthoyl chloride (2-NC), 4-nitrobenzoyl chloride (NBC) and benzoyl chloride (BC) (purity, over 98 or 99%) were supplied by Aldrich.

Both NPEs (nonylphenol polyethoxylate characterized by an average of 10 ethoxy units) (Carlo Erba, Milan, Italy) and the individual PEG ethoxymers

E_1–E_6 (Fluka) were >99% pure. The polyethyleneglycol mixtures PEG 400 and PEG 600 ($E_{average}$ of ca. 9 and 13, respectively) were technical grade from Fluka. The pyridine, used for the reaction of AEs, NPEs and PEGs with the above-mentioned acyl chlorides, was of ACS-spectrophotometric grade (Fluka), as well as the dimethyl formamide (DMF) (Aldrich) and the acetonitrile (Baker, Deventer, Netherlands) utilized as derivatization solvents.

All the organic solvents employed for the preparation of standard solutions (acetone, acetonitrile), in the solid-phase extraction (SPE) (methylene chloride, methanol) and for the chromatographic separations (acetonitrile, methanol) were supplied by Baker and were of HPLC grade. Water for SPE and chromatographic purposes was purified by a Milli-Q system (Millipore, Bedford, USA). Aqueous 30% (w/w) HCl used for SPE sorbent conditioning was ultrapure grade from Merck (Darmstadt, Germany).

The sorbent material, graphitized carbon black (GCB; grain size of 120–400 mesh) used for the SPE was purchased from Alltech Associates (Deerfeld, USA). The SPE polypropylene tubes (6 ml volume), polyethylene frits, reservoirs (30–60 ml) and fittings, as well as the Visiprep-SPE Manifold extraction apparatus, were from Supelco (Bellefonte, USA).

All the derivatizations were performed in 1.8- or 3.0-ml glass vials (Supelco) with PTFE-lined screw caps.

2.2. Instrumental equipment

The chromatographic apparatus consisted of a quaternary pump liquid chromatograph series 1050 (Hewlett-Packard) equipped with a Model 1046 fluorescence detector (Hewlett-Packard) and a variable-wavelength UV-Vis absorption detector (flow cell volume: 8 μl) connected in series for simultaneous detection purposes. A Chemstation HP 3365 Series II data system (Hewlett-Packard) was used for the acquisition and handling of chromatograms. The samples (20 μl) were injected with a 100-μl syringe (Hamilton) in a manual 7125 injector (Rheodyne) equipped with a 20-μl loop (Rheodyne). ^{1}H NMR instrumentation used for the characterization of some of the adducts synthesized and for measures of conversion in derivatization products was a Varian Unit 400 spectrophotometer operating at 399.919 MHz.

2.3. Derivatization reactions

2.3.1. NBC and BC derivatives

The reaction conditions applied were those already reported [15,22,29,32] after minor modifications. Triplicate series of 1-ml aliquots of a standard solution of Dehydol LT7 in acetonitrile (ca. 400 μg/ml) were placed into screw PTFE-capped glass vials. The derivatization with the acyl chlorides BC and NBC was carried out by adding 10 μl of pyridine and a high excess (50 μl) of pure derivatizing agent, capping and heating at 80°C for 30 min. The reaction times that ensured complete conversion of the substrate were determined by batch experiments where four 1-ml aliquots of Dehydol LT7 standard solution underwent derivatization with BC and NBC for 15 min, 30 min, 1 h and 2 h, respectively, while the conversion was monitored using HPLC coupled with UV detection.

2.3.2. NIC and NC derivatives

The preliminary reaction conditions applied for the derivatization of AEs by NC (1-NC and 2-NC) were similar to those of BC and NBC. In the case of NIC, the procedure was the same except for the prior removal of the solvent (acetonitrile) containing the AE standard, and the heating at 35°C for 30 min.

The fluorescence capability of the NC and NIC derivatives of AEs prompted the optimization of the two derivatization reactions which led to the following refined procedures which were used throughout the whole subsequent work.

As for NC derivatization, 0.1–1 ml aliquots of an acetonic solution of tested standards (concentration: 100–600 μg/ml) were placed in 1.8-ml reaction vials and the solvent was removed by gentle heating under a mild nitrogen stream. The dry residue was redissolved in 100 μl acetonitrile where 10 μl of pyridine and 20 μl of NC were added. The vial was capped and heated at 80°C for 15 min. The reaction products were then diluted with 1 ml of acetonitrile–water (60:40, v/v), sonicated for 10 min and centrifuged for 5 min at 2500 rpm in order to remove a white precipitate produced by the reaction of the exceeding derivatizing agent with water.

In the case of derivatization with NIC, the dry AE residue was redissolved in 100 μl of DMF, 10 μl of NIC were added, and the PTFE-capped vial was left to react at 35°C for 30 min. After reaction, DMF was removed by evaporation, and the residues were dissolved in 1 ml of acetonitrile–water (60:40, v/v) or methanol–acetonitrile (90:10, v/v), depending on the composition of the HPLC mobile phase used for the chromatographic separation. The addition of the acetonitrile–water mixture led to a white precipitate resulting from the reaction of water with the excess of NIC, which was separated by sonication–centrifugation, as reported above for the NC.

The assessment of the quantitative conversion as well as the characterization of the NIC and NC derivatives were attained by ^{1}H NMR following the change of the chemical shift of the protons attached to the terminal carbon of the polyethoxylic chain. The detailed conditions are reported elsewhere [33].

The reaction conditions for the derivatization of AEs by NC and NIC were successfully extended to PEG, NPE, and to C_{11}–C_{18} linear primary alcohols.

The reproducibility of NC and NIC derivatizations was evaluated by at least triplicate reactions.

Blank determinations were carried out for each derivatizing agent by subjecting 0.1–1 ml aliquots of acetone or acetonitrile to the derivatization procedure described above.

2.4. RP-HPLC separations of the NIC and NC derivatives of the tested compounds

The chromatographic separations were carried out on LiChrocart columns (Merck) 250×4 mm I.D. and 125×4 mm I.D. containing LiChrospher 100 RP-18, 5 μm and LiChrospher 100 RP-18 endcapped, 5 μm, respectively. The former was equipped with a 4×4 mm pre-column (Merck) of the same stationary phase. Mixtures of methanol–acetonitrile and acetonitrile–water were used as mobile phase. Because of the time-consuming conditioning required by the shift from one mixture to the other one, the availability of a dedicated column for each eluent mixture is recommended. The separations were obtained at room temperature and at flow-rates in the range 1–2 ml/min.

Both NIC and NC derivatives of a C_{11}–C_{16} and C_{18} linear primary alcohol mixture and of individual $C_{14}E_1$–E_6 and $C_{14}E_8$ ethoxymers, synthesized with the refined procedures to determine the variability of the fluorescence response factors, were separated by either LiChrospher 100 RP-18 endcapped or Lichrospher 100 RP-18. In the first case, elution was performed by isocratic acetonitrile–water containing 90.6% (v/v) phase modifier at 1.25 ml/min flow-rate. One or 2 ml/min isocratic methanol–acetonitrile (90:10, v/v) was instead used to elute AE derivatives from the LiChrospher 100 RP-18 column. In each case the mobile phase was prepared by pre-mixing accurately weighed volumes of the two solvents which ensured chromatographic reproducibility.

The homolog-by-homolog elution of both NIC and NC derivatives of the commercial AE mixtures Marlipal 28/100, LIALET 111/6, LIALET 125/7 and Lorol 124/100 was achieved on LiChrospher 100 RP-18 by using pre-mixed methanol–acetonitrile (90:10, v/v) at a flow-rate of 2 ml/min.

The elution of the NC derivatives of Lorol 124/10 and LIALET 111/6 according to both the alkyl and polyethoxy chain lengths was attained by the following acetonitrile–water gradients at a flow-rate of 1 ml/min (Table 1). The derivatives of the individual PEG ethoxymers (E_1–E_6) and mixtures (PEG 400 and PEG 600) with NIC and NC were eluted on a dedicated LiChrospher 100 RP-18 250×4 mm I.D., 5 μm column using pre-mixed isocratic acetonitrile–water (40:60, v/v) and (60:40, v/v), respectively, at a flow-rate of 1 ml/min. Under these conditions, AEs and NPEs, whenever present in the injected

Table 1
Elution program for the NC derivation of Lorol 124/10EO and LIALET 111/6

t (min)	Acetonitrile (%)	Water (%)
Lorol 124/10EO		
0	60	40
20	80	20
60	80	20
80	90	10
200	90	10
LIALET 111/6		
0	60	40
20	80	20
60	95	5

extracts, are strongly retained. They were periodically washed out from the column by ca. 95% acetonitrile.

2.5. Extraction of AEs, NPEs and PEGs from aqueous samples by solid-phase extraction (SPE)

Cumulative extraction of AEs, NPEs and PEGs from laboratory biodegradation screening test liquors and sewage-treatment plant (STP) aqueous samples was achieved by SPE. The SPE cartridges were prepared by placing 1 g of Carbopack into 6 ml polypropylene tubes. The sorbent bed was only slightly compressed in order to avoid excessive resistance to sample passage. Cartridges were conditioned by flowing 8 ml of methylene chloride–methanol (8:2, v/v), followed by 4 ml of methanol and 20 ml of 10^{-2} *M* aqueous HCl at a flow-rate of 2–4 ml/min. Aliquots of 10 ml biodegradation test liquors, 10 ml STP influent or 100 ml final effluent, sampled according to reported conditions [3,4], were passed through the cartridge at a flow-rate of ca. 2–4 ml/min. Sample bottles with volumes of 10, 100 and 1000 ml were rinsed with 5, 10 and 100 ml methanol–water (1:1, v/v), respectively, and a fraction of the washing solution corresponding to that of the sample extracted was passed through the cartridge. Cumulative elution of AE, NPE and PEG was achieved by washing the cartridge with 2 ml of methanol, followed by 8 ml of methylene chloride–methanol (8:2, v/v). The eluted fraction was evaporated to dryness by heating at 50–60°C under a mild stream of nitrogen, and the residue was recovered by a two-step (2+2 ml) addition of acetone, each one followed by a 5-min treatment in an ultrasonic bath. The acetonic solution was then divided into two fractions, one to be used for derivatization with NIC and detection of AEs and NPEs, the second one to be derivatized with NC for PEG determination.

The performance of the SPE procedure was evaluated by quadruplicate recovery of ca. 60 μg of Marlipal 28/100 and 66 μg of PEG 400 from 10, 100 and 1000 ml of tap water. Additional recovery experiments were carried out on 10 ml influent, 100 ml effluent and 1000 ml of river water spiked with 10 μg of AEs and 12 μg of PEGs.

2.6. Detection and quantitation

The AE derivatives were detected simultaneously by both UV absorption and fluorescence. The detection conditions were those providing the maximum UV-absorption or fluorescence activity under the tested experimental conditions (Table 2). After the comparative evaluation of the UV-absorption and fluorescence behaviour of all AE derivatives, only detection by fluorescence was used throughout the whole work. The dependence of the instrumental response on the amount of injected analyte was studied for both NIC and NC derivatives of $C_{14}E_8$ and PEG E_6. No deviation from linearity was found for injected amounts up to 2.5 nmoles.

As far the homolog-by-homolog separation, the AE elution order was inferred from the chromatograms of NIC and NC derivatives of a standard mixture of C_{11}–C_{16} and C_{18} linear primary alcohols eluted under the same chromatographic conditions.

As for the ethoxymer-by-ethoxymer separation of AE mixtures, the identification of the order of the elution was achieved by the concurrent analysis of the same AE mixtures where 10 μl of the acetonic standard solutions of C_{11} linear primary alcohol (for LIALET 111/6) or of C_{12}, C_{14} linear primary alcohols, $C_{14}E_1$–E_6 and $C_{14}E_8$ (for Lorol) were spiked prior to derivatization. According to the typical ethoxymeric distribution of commercial AEs, the concentration of co-eluting ethoxymers was assumed to be the average of those of the neighbouring ethoxymers (i.e. the previous and the next eluting ones) of the same homolog.

AE quantitation in biodegradation test liquors was attained by adding 10–100 μl of acetonic standard solutions of $C_{14}E_8$ and PEG E_5 pure ethoxymers

Table 2
UV absorption and fluoresence detection conditions

Derivatizing agent	UV absorption	Fluorescence	
	λ (mm)	λ_{ecc} (mm)	λ_{em} (mm)
NIC	228	232	358
NC	228	228	365
2-NC	228	228	365
BC	228	228	295
NBC	268	228	295

(concentration: 500 μg/ml) to the sample before undertaking the SPE. The fluorescence molar response factor, calculated by using the area of the internal standard, was applied to the quantitation of the residual AE substrate. The consistency of the initial ethoxylation degree during the biodegradation was assumed for the residual substrate. NPEs and PEGs were quantified by means of external calibration curves obtained by analysing different aliquots of NP10E and PEG 400 standard solutions after derivatization with NIC and NC, respectively. The resulting concentrations were corrected by the recovery of the AE and PEG internal standards. External calibration curves obtained by using $C_{14}E_8$ and PEG 400 were also used for the quantitation of AEs and PEGs in the environmental extracts. The amounts of each analyte injected were typically in the 100–200 ng interval.

3. Results and discussion

3.1. Derivatization reactions

The following new reagents were tested to derivatize AEs and the corresponding unethoxylated primary alcohols: 1- and 2-NC, BC and NBC. In addition, the recently proposed [32], but little investigated, NIC, was thoroughly examined. The derivatization reactions were monitored by RP-HPLC so that the reaction time, yield and reproducibility could easily be evaluated from the intensity variation of the peak(s) corresponding to each derivative. The reproducibility was inferred from at least triplicate derivatizations.

In addition to the oleochemical and petrochemical typical commercial mixtures (i.e. Dehydol, Lialet etc.), individual AE homologs and ethoxymers were derivatized in order to assess their individual response factors.

The reaction conditions, tested during the screening step and similar to those previously reported for the 3,5-dinitrobenzoyl chloride [22], were found to be already suited to obtaining quantitatively the benzoyl and 4-nitrobenzoyl derivatives of AEs.

The further optimization of the AE derivatization by NC and NIC allowed the increase of the reaction reproducibility and the improvement of the chromatographic separation of the AE derivatives by reducing the excess of added derivatizing reagent and removing the unreacted reagent after completion of the derivatization.

The derivatization of PEGs and NPEs could also be attained by NIC and NC because of the structural similarity of these compounds to AEs, consisting of the terminal −OH groups in the polyethoxylic chains (Fig. 1). The reaction conditions were the same as for AEs and both PEGs and NPEs were exhaustively and reproducibly derivatized.

The reproducibility of the derivatization reactions, expressed as the relative standard deviation of the peak areas corresponding to each derivative, was in the range 0.5–2.2% for each type of derivatizing reagent.

Since only the NIC and NC are amenable to fluorescence detection, most of the work was focused on the fluorescence behaviour and the chromatographic separation of the AE, NPE and PEG derivatives of these compounds.

3.2. Detection of the AE and PEG derivatives

The detectability of the various AE and PEG derivatives was investigated by using isocratic elutions of methanol–acetonitrile and acetonitrile–water on octadecylsilica (C_{18}) and C_{18} endcapped columns, respectively.

Fig. 2 shows the relative intensities of the maximum molar UV absorption and fluorescence of derivatized C_{12}–C_{18} AEs after the RP-HPLC separation by aqueous acetonitrile. Both the NIC and NC derivatives of AEs exhibited an approximately twofold molar UV absorption compared to that of the benzoyl and 4-nitrobenzoyl chlorides (Fig. 2A). The fluorescence molar intensity of the NIC derivatives was significatively higher than that of NC derivatives (Fig. 2B).

The possible influence of the alkyl chain length and the mobile phase composition on the fluorescence response was estimated by examining the NC derivatives of the C_{11}–C_{18} linear primary alcohols (the unethoxylated compounds) (Fig. 3). From the inspection of this figure, it follows that the fluorescence molar responses attained with acetonitrile–

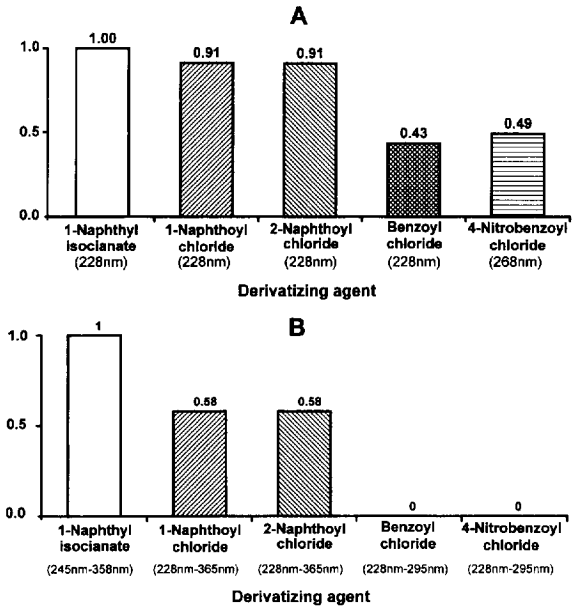


Fig. 2. Relative absorption and fluorescence molar intensities of the C_{12}–C_{18} AE mixture after derivatization with 1-NIC, 1- and 2-NC, benzoyl chloride and 4-nitrobenzoyl chloride. Stationary phase: C_{18} column; mobile phase: isocratic and gradient acetonitrile–water.

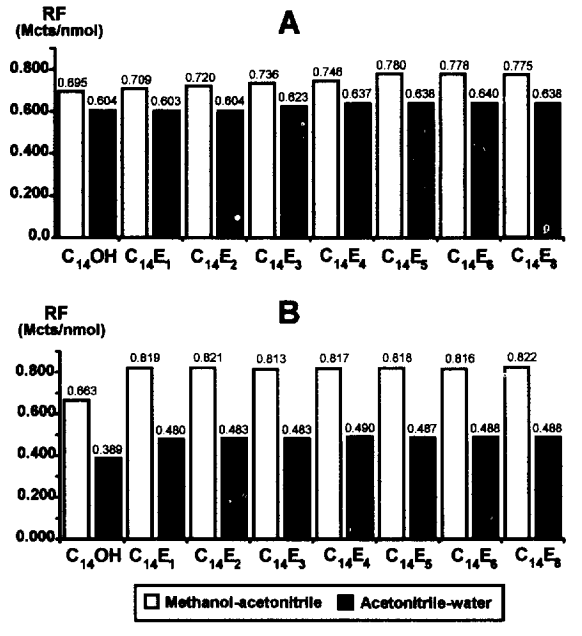


Fig. 3. Fluorescence responses of NC derivatives of the C_{11}–C_{18} primary alcohols. Stationary phase: C_{18} column; mobile phase: acetonitrile–water.

methanol were systematically higher than those recorded with acetonitrile–water. The values reported in Fig. 3, the mean of triplicate measurements, are affected by relative standard deviations varying between ±0.75% for $C_{12}OH$ and ±1.4% for $C_{18}OH$ eluted by methanol–acetonitrile and between ±0.86% for $C_{12}OH$ and ±1.5% for $C_{18}OH$ eluted by acetonitrile–water. The fluorescence molar response factors rose with an increase in the alkyl chain length from C_{11} to C_{18} by 21% (acetonitrile–methanol) and 38% (acetonitrile–water), respectively, but only by 8% (acetonitrile–methanol) and 12% (acetonitrile–water) if the C_{11} and C_{18} homologs are not included (Fig. 3).

The influence of the polyethoxylic chain length on the fluorescence response factors of AE after triplicate derivatization by NC (Fig. 4A) and NIC (Fig. 4B) was assessed by considering the ethoxymeric series of the C_{14} homolog eluted by both acetonitrile–methanol and acetonitrile–water (Fig. 4). The NC derivatives confirmed the approximately 40% systematic difference of the response factors obtained by the two tested elution mixtures (Fig. 4A). In contrast, the fluorescence response of the NIC derivatives were less sensitive to the mobile-phase composition and a systematic difference of only approximately 20% was found between the acetonitrile–methanol and acetonitrile–water mixtures. The values of the molar response factors were very similar for each AE ethoxymer derivatized either by NC or NIC. The main difference between the two series of derivatives is represented by the primary alcohol ($C_{14}OH$) exhibiting a fluorescence response approximately 20% lower than the various AE ethoxymers after derivatization by NC (Fig. 4A), and quite similar to the AE ethoxymers in the case of NIC derivatization (Fig. 4B).

Finally, the fluorescence response factors of the PEG derivatives of NC and NIC were investigated by testing the individual oligomers with 1–6 ethoxy units as well as the mixtures with an average ethoxy units figure of 9 (PEG 600) and 13 (PEG 600), respectively. Fig. 5 shows that the response factors vary remarkably up to the ethoxymer with 6 ethoxy units (E_6) and then no significant difference was found for E_6 and the mixtures PEG 400 and PEG 600 containing the lower ethoxymers only as minor components. Notably, E_1 and E_2 did not give

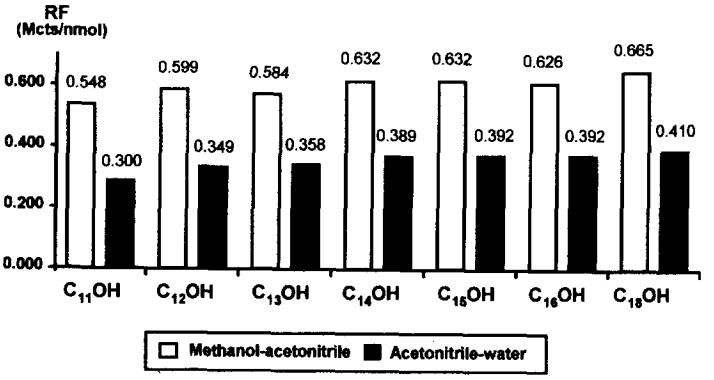


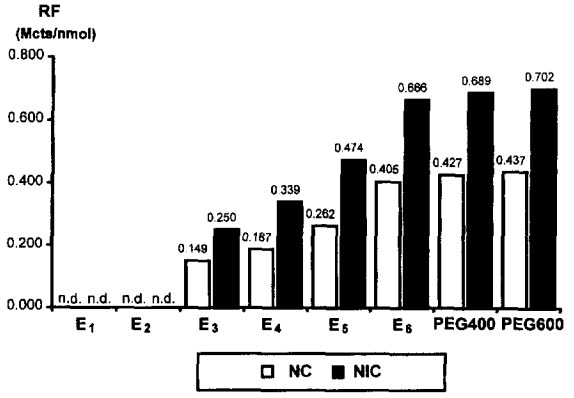
Fig. 4. Fluorescence molar responses of the NC (A) and NIC (B) derivatives of the C_{14} individual AE ethoxymers, as well as $C_{14}OH$. Stationary phase: C_{18} and C_{18} endcapped columns with methanol–acetonitrile and acetonitrile–water, respectively, as the mobile phase.

detectable derivatives despite several attempts and quite satisfactory (0.87–2.1%) relative standard deviations of triplicate derivatization of the E_3 and E_5 ethoxymers by both NC and NIC.

3.3. Reversed-phase HPLC separation

It was already reported that the 3,5-dinitrobenzoyl AE derivatives could be separated on octadecylsilica [22,29] according to the length of both the alkyl and the ethoxylic chains by using a broad variety of mobile-phase solvent composition (acetone–, acetonitrile–, tetrahydrofuran–water). A major disadvantage of these separations was the overlap of ethoxymers with different alkyl chain so that they are of little interest for environmental applications.

A recent chromatographic investigation [20] on the amenability of the NIC derivatives of AE to the homolog-by-homolog separation showed the crucial role played by the mobile-phase composition (acetonitrile–water) on the elution order of the various ethoxymers. In particular, the homolog-by-homolog elution could be obtained only in a well-defined narrow range of acetonitrile–water composition. In this work, a new mobile-phase mixture (i.e. methanol–acetonitrile) was tested to improve the homolog-by-homolog separation; we also investigated the chromatographic behaviour of new derivatives (i.e. those obtained with NC), structurally similar to the NIC ones.



n.d.: not detected

Fig. 5. Fluorescence molar response of individual PEG ethoxymers (E_1–E_6) and PEG 400, PEG 600 after derivatization with NIC and NC. Stationary phase: C_{18}; mobile phase: acetonitrile–water.

The chromatograms of the C_{11}–C_{18} mixture of linear primary alcohols (Fig. 6A and Fig. 7A) and a typical oleochemical AE mixture (C_{12}–C_{18} homologs) after derivatization by NIC and NC, respectively, are presented in Figs. 6 and 7. The comparison of the A and B chromatograms in Fig. 6 shows that the elution times of the NIC even primary alcohol derivatives and those of the corresponding AE homologs including all ethoxymers are exactly the same. By comparing the A and B chromatograms in Fig. 7, it follows that the complete coelution of the ethoxymers with the same alkyl chain could not be attained for the NC derivatives, since the lower ethoxymers as well as the unethoxylated primary alcohol are increasingly retained when the poly-

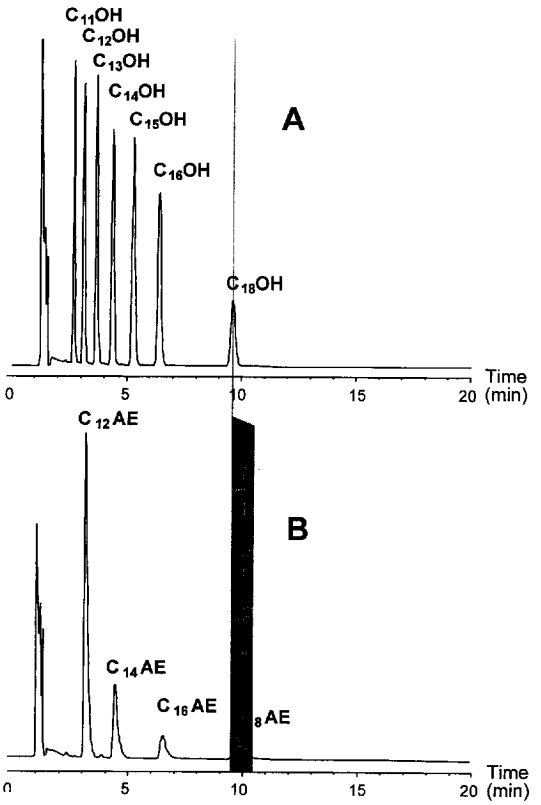


Fig. 6. Chromatograms of the NIC derivatives of the primary C_{11}–C_{18} alcohols (A) and the C_{12}–C_{18}AE oleochemical mixture (B). Stationary phase: C_{18} column; mobile phase: methanol–acetonitrile.

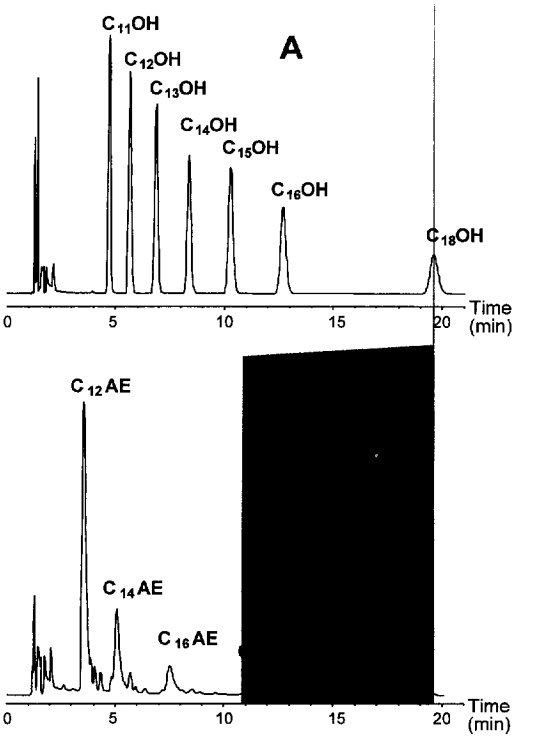


Fig. 7. Chromatograms of the NC derivatives of (A) the primary C_{11}–C_{18} alcohols, and (B) the C_{12}–C_{18}AE oleochemical mixture. Stationary phase: C_{18} column; mobile phase: methanol–acetonitrile.

ethoxylic chain length is decreased. Thus, NIC appears most suited to the AE derivatization aimed at homolog-by-homolog separation.

The separation of the NIC derivatives of AE petrochemical mixtures consisting of C_{11} (A) and C_{12}–C_{15} (B) homologs, respectively, is presented in Fig. 8. Both chromatograms show an unresolved peak overlap in addition to the narrow irregularly shaped peaks. The comparison of the retention times of NIC derivatives of petrochemical AEs, C_{11}–C_{18} primary alcohols as well as oleochemical mixtures, shows clearly that the peak maxima of the petrochemical mixture correspond to the linear AEs, whereas the 2-alkyl substituted AEs are eluted earlier and are responsible for the observed unresolved background (or overlapping).

Therefore, for the trace level determination of AEs in environmental samples, the derivatization by NIC, instead of that by NC, followed by the homolog-by-homolog separation, is far preferable since peak identification and quantitation are easier and more accurate. On the other hand, because of the remarkably different toxicity effects [34] and the possible different biodegradation rate of the individual ethoxymers [35], the ethoxymeric distribution of each homolog is important and the NC appears to be the most suited derivatizing agent to disclose it. Fig. 9 shows the ethoxymer-by-ethoxymer separation of the C_{12} and C_{14} homolog mixture which was obtained by a gradient elution of acetonitrile–methanol. The elution order was inferred by spiking the mixture with individual derivatized ethoxymers. Typically, the shorter ethoxymers and the unethoxylated alcohol of the C_{12} homolog eluted between the most polar ethoxymers of the C_{14} homolog. The

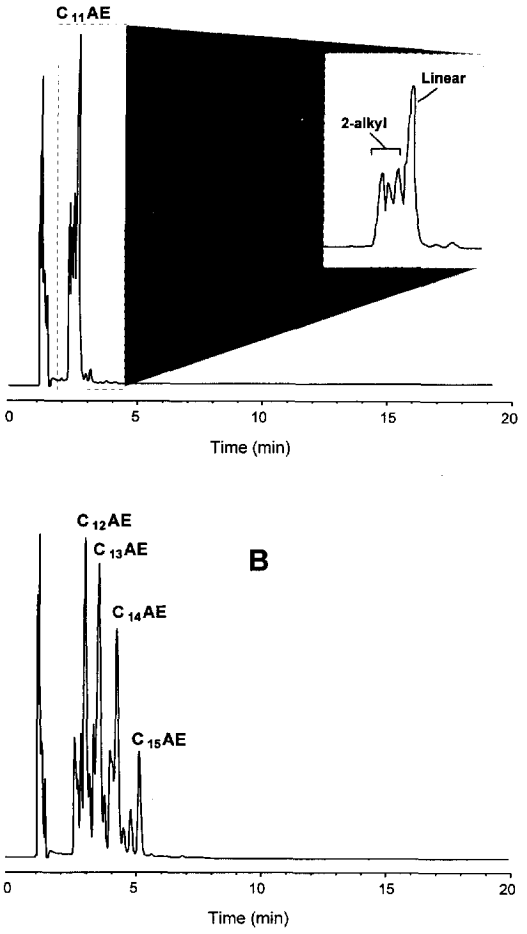


Fig. 8. Chromatograms of the NIC derivatives of a C_{11} homolog and C_{12}–C_{15} mixture of petrochemical linear and branched AEs. Stationary phase: C_{18} column; mobile phase: methanol–acetonitrile.

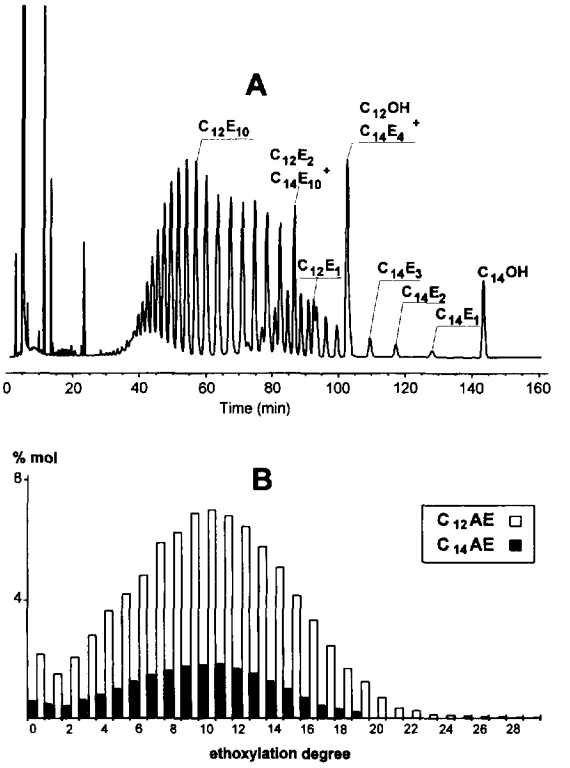


Fig. 9. Chromatogram of the ethoxymer-by-ethoxymer separation of the NC derivatives of the C_{12} and C_{14} AE homologs. Stationary phase: C_{18} column; mobile phase: acetonitrile–water.

quantitative estimation of the contribution of the individual ethoxymers can be evaluated by assuming that the ethoxymers of each homolog obey a Poisson distribution. If so, as is the case with detergent AEs, the signal intensity of each ethoxymer can be approximated to the average of the area counts of the ethoxymers preceding and following that under consideration.

A satisfactory resolution of the individual ethoxymers was obtained only in the case of oleochemical mixtures consisting of even homologs. Taking into account that about 50% of the marketed AEs are of petrochemical origin, the reversed-phase ethoxymer-by-ethoxymer method can be applied to biodegradation screening tests or product quality assurance rather than environmental analysis.

3.4. Separation of PEG and NPE derivatives

In the case of PEGs, which are far more polar than AEs, the ethoxymer-by-ethoxymer separation was also obtained by derivatization with NC (Fig. 10A). In the chromatogram of PEG 600 (i.e. the PEG with an average molecular mass of 600) (Fig. 10A) the penta(ethylene glycol) (E_5) was used as the internal standard and the lower components (i.e. E_n with $n<4$) appear only at trace level. The elution, under the same chromatographic conditions, of the NIC derivatives of PEG 600 resulted in the formation of a broad unresolved peak, and a further increase of

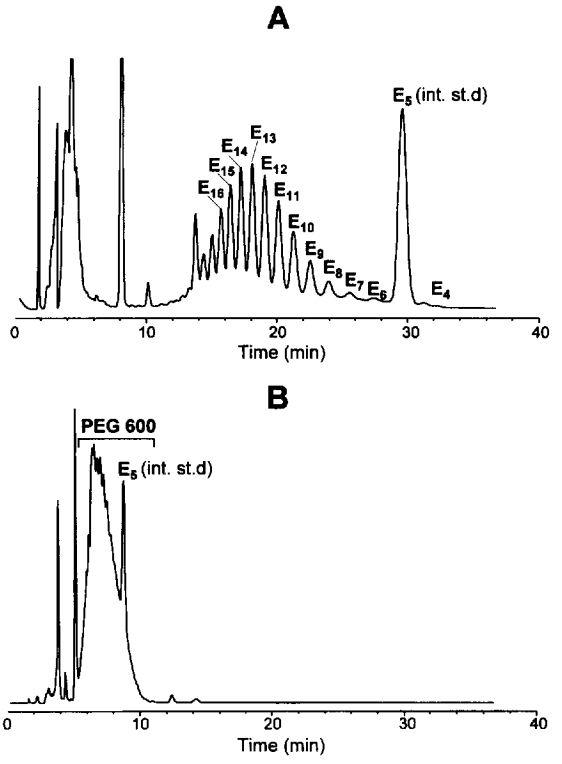


Fig. 10. Chromatograms of PEG 600 after derivatization by NIC (A) and NC (B). Stationary phase: C_{18} column; mobile phase: acetonitrile–water.

water content in the mobile phase did not provide a satisfactory ethoxymer-by-ethoxymer separation (Fig. 10B). Although the simultaneous separation of the NC derivatives of PEGs and AEs is feasible using a gradient program of aqueous acetonitrile, the long elution time required and the resulting partial overlap of AE homologs suggest that we should run the PEG and AE separations separately.

The possible presence in environmental samples of both NPEs and AEs justified the searching of conditions for the simultaneous separation of NPEs and AEs. This was possible after derivatization by NIC and under the chromatographic conditions previously found suitable for the separation of AE derivatives. Fig. 11 shows the simultaneous separation of NPE and AE derivatized by NIC. The NPE ethoxymers eluted under the same peak and coeluted with the C_{11} AE homolog.

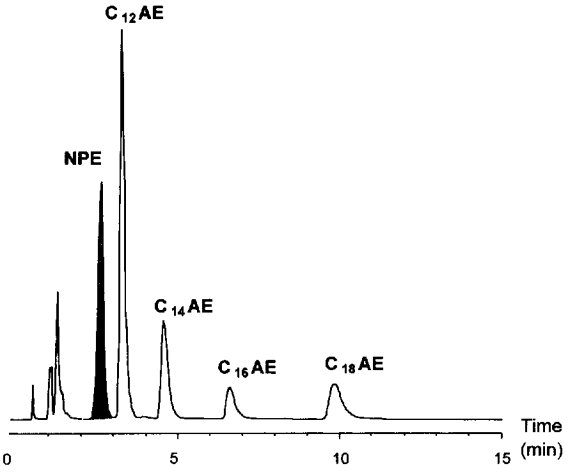


Fig. 11. Simultaneous separation of NPEs and AEs derivatized by NIC under the same elution conditions reported in Fig. 6.

3.5. Chromatographic reproducibility and sensitivity

The retention times of all tested derivatives were remarkably constant over the time, provided that the mobile phase was prepared accurately, thermostating, weighing and mixing together the eluent solvents, particularly in the case of the acetonitrile–water mixture [20]. The chromatographic reproducibility, obtained by multiple injection of the same derivatized mixture, was found for the various derivatives to be systematically $<1\%$ of the total area of each peak. The sensitivity, expressed as the injected amount of each derivative leading to a signal-to-noise (S/N) ratio of 10, was 5 ng for the C_{12}AE homolog with an average ethoxylation number of 9, derivatized by NIC and eluted using methanol–acetonitrile; we found a value of 10 ng for the $C_{14}E_8$ derivatized with NC and eluted using methanol–acetonitrile; we also found a value of 6 ng for the E_6 PEG ethoxymer derivatized by NC and eluted using acetonitrile–water.

3.6. Extraction from aqueous samples

In view of the application of the proposed separations to the analysis of treated and untreated wastewaters, and biodegradation test liquors, AEs

and PEGs were isolated from aqueous samples by SPE using GCB as the sorbent. Previous work [3,4] demonstrated GCB's capability to extract efficiently the nonionic surfactants of the NPE and AE types. The performance of the GCB extraction had been, however, evaluated by determining unaltered AEs as well as NPEs. In this work, the extraction conditions were similar to those previously reported [4] – in particular, the compound of interest was eluted from the cartridge by passing methylene chloride–methanol (80:20) – but PEGs were also included in the set of tested analytes and the performance evaluation was carried out after derivatization of eluted AEs and PEGs by NIC and NC, respectively.

The extraction efficiency of both AEs and PEGs at the concentration levels expected for treated and untreated wastewaters and surface waters was evaluated by quadruplicate recovery of the same amount (about 20 μg) of C_{12}–C_{18} AEs and PEG 400 from 10, 100, 1000 ml of tap water. Moreover, the possible matrix effects on the extraction efficiency were checked by extracting comparable amounts of the same compounds from 10 ml and 100 ml of influent and final effluent, respectively, of a sewage treatment plant. No statistically significant differences were found between the recovery values of AEs and PEGs from tap water and environmental samples. The recovery was significantly dependent on the length of the alkyl chain. The C_{12}, C_{14}, C_{16} and C_{18} homologs were recovered to an average extent of 97%, 94%, 90% and 85%, respectively; for the tested commercial C_{12}–C_{18} AE mixture, where the lower homologs accounted for approximately 70% of the total AEs, the overall recovery was, however, systematically higher than 94%, with relative standard deviations of individual homologs between 2.2 and 6.5%.

The recovery of the PEG 400 ethoxymers from the environmental samples was systematically >94% (average R.S.D.: ±4.5%), and no change of the distribution of the different ethoxymers was observed.

The dependence of the recovery on the degree of ethoxylation was investigated, with respect to STP matrices and to AEs, by triplicate spiking 10 ml influent and 100 ml final effluent with ca. 10 μg of $C_{12}OH$, $C_{12}E_2$, $C_{12}E_4$ and $C_{12}E_6$ pure ethoxymers.

Among the tested compounds, only $C_{12}OH$ showed a significantly lower recovery (92±2.1% from the influent, 89±10% from the effluent). All the remaining C_{12}AE ethoxymers exhibited recoveries higher than 98% in both influent and effluent samples.

3.7. Applications

The proposed extraction, derivatization and separation procedures were applied to the analysis of biodegradation test liquors and influents and effluents of wastewater-treatment plants.

The biodegradation screening tests are very useful in the prediction of the ready biodegradability of water-soluble non-volatile chemicals and in the identification of the possible formation of persistent biotransformation products. We tested a series of AE compounds of increasingly complex composition [2,33]. Fig. 12A shows the chromatograms of a standard solution, before the start of the test, containing the NIC derivatives of NPEs with an average ethoxylation number of 10, the homolog C_{12}AE with an average number of 9 ethoxy units, and the individual ethoxymer $C_{14}E_8$ which was used as the internal standard. A standard solution of the NC derivatives of PEG 600 containing the internal standard pentaethylene glycol (E_5) is presented in Fig. 12B. In Fig. 12C the chromatograms obtained 2 days after the start of the biodegradation test are shown. The C_{12}AE had disappeared completely already and NPE was found unaltered (Fig. 12C); meanwhile, a significant amount of PEGs was found (Fig. 12D) with an ethoxymeric distribution reaching into the lower oligomers, compared to the PEG 600 standard (Fig. 12B). The implication of this finding on the aerobic biodegradation mechanism of AEs is discussed elsewhere [2,33].

The chromatograms of the NIC derivatives of AEs and NPEs, and the NC derivatives of PEGs in the influent and final effluent of a sewage-treatment plant are presented in Fig. 13. The chromatogram of the influent (Fig. 13B) reflects the domestic usage pattern of oleochemical and petrochemical AEs. NPEs are present to an extent of about 20% (on a molar basis) of the overall AEs, but a possible coelution of NPEs with C_{11}AE should be taken into account. In the effluent (Fig. 13C), the expected enrichment of NPEs and an unexpected enrichment

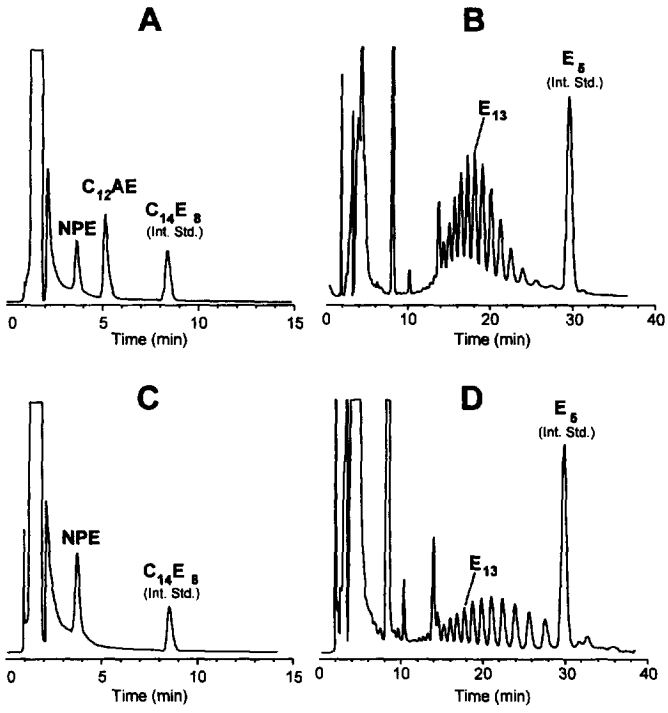


Fig. 12. Chromatograms of the NC derivatives of AEs, NPEs and PEGs in standard solutions (A,B) and extracts of screening test liquors (C,D) taken 2 days after the start of the biodegradation test. Stationary phase: C_{18} (A,C) and C_{18} endcapped (B,D) columns; mobile phase: acetonitrile–water.

of the C_{18} homolog were found. Whether $C_{11}AE$ is present significantly in both influent and effluents (thus coeluting with NPEs) and the structural confirmation of the $C_{18}AE$ homolog in the effluent are issues currently under investigation using HPLC–MS. A similar distribution of PEGs was found in the influent and final effluent (Fig. 13E, Fig. 13F). The concentrations of AEs, NPEs and PEGs in the influent were 1.3 mg/l, 0.26 mg/l and 0.15 mg/l; in the effluent they were 28 μg/l, 22 μg/l and 25 μg/l, respectively.

4. Conclusion

The proposed chromatographic procedures are the first to include the simultaneous extraction of the aliphatic and aromatic nonionic surfactants of the AE and NPE types, respectively, and the neutral biodegradation intermediates, PEGs. The routine determination of AEs and PEGs in biodegradation test liquors and environmental aqueous samples can now be undertaken more profitably than in the past, by using the commercially available derivatizing agents NIC and NC, since the resulting derivatives are amenable to fluorescence, thus greatly increasing the selectivity; they can be separated by reversed-phase to obtain detailed information on the individual homolog and ethoxymer compositions.

Acknowledgments

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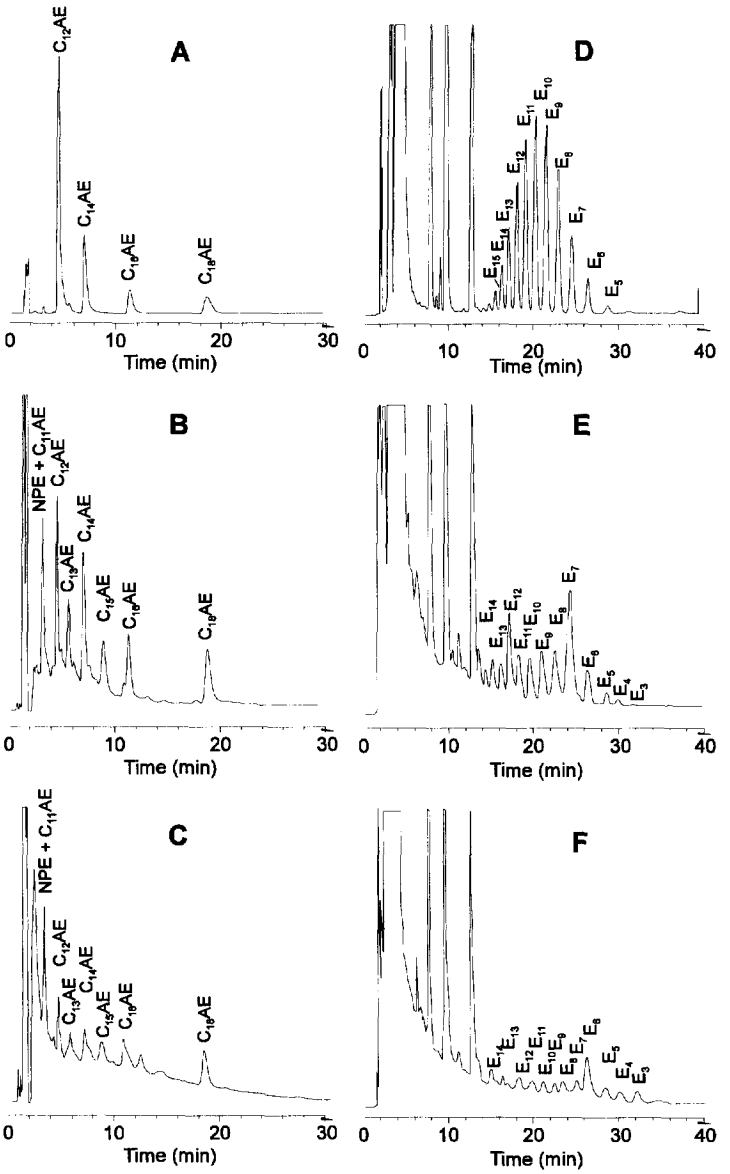

Fig. 13. Chromatograms of the NIC derivatives of oleochemical AEs in standard solution (A), AEs and NPEs in extracts of the influent (B) and final effluent (C) of a sewage-treatment plant. Chromatograms of the NC derivatives of PEGs in a standard solution (D) and extracts of the influent (E) and final effluent (F). Stationary phase: C_{18} column; mobile phase: methanol–acetonitrile (A,B,C) and acetonitrile–water (D,E,F).

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