Simplified determination of lipophilic metabolites of nonylphenol ethoxylates: method development and application in aqueous samples from Buenos Aires, Argentina†‡

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In the present work we have developed an analytical methodology for the determination of nonylphenol (NP) and nonylphenol mono- and di-ethoxylates (NP1EO and NP2EO) in water samples. The applicability of this methodology was proved by means of the analysis of environmentally relevant aqueous samples from Buenos Aires. This constitutes a starting point for a rigorous assessment of the incidence of NP*n*EO surfactants in Argentina, as only very few, qualitative or semi-quantitative data on the occurrence of these compounds in local systems were available up to this time. Enrichment of the analytes was carried out by solid-phase extraction on a C-18 sorbent, followed by elution with ethyl acetate. Normal-phase high performance liquid chromatography on an amino-silica column and fluorescence detection at excitation-emission wavelengths of 230–300 nm were employed for separation and quantification of the analytes. Confirmation of peak assignment in selected real samples was performed by off-line coupling HPLC with GC-MS analysis. A non-polar GC capillary column was used, and a characteristic peak pattern was obtained for the alkyl chain isomers of each ethoxylated homologue and NP. GC-MS analyses yielded in all cases purity levels higher than 80% for the HPLC collected fractions. The elevated concentrations found for the estrogenic metabolites of NP*n*EO are in accordance with an unrestricted use of this class of non-ionic surfactants in the country.

Introduction

Nonylphenol ethoxylates (NP*n*EO) are a well known class of non-ionic surfactants, and constitute around 80–90% of the commercial market of alkylphenol ethoxylates (AP*n*EO).[1,2] They have been intensively applied world-wide in industrial, institutional and household products during the last decades. The main applications include the manufacture of detergents and cleaners, and their use as dispersing or emulsifying agents.[2–4] Nonylphenol ethoxylates are manufactured by sequential addition of ethylene oxide units to technical grade nonylphenol (t-NP). This product consists mostly of a mixture of hydrophobic, branched nonyl chain isomers of 4-nonylphenol. As a result, NP*n*EO surfactants are complex mixtures of polyethoxylated, alkyl isomer compounds.[5] Derived from their use, a large part of NP*n*EO surfactants are disposed of in liquid effluents, hence degrading during wastewater treatment or in the environment when directly discharged into water courses. Bio-transformation occurs, but a complex mixture of persistent metabolites is introduced in the aquatic environment. As has been reported in several field and laboratory investigations, they comprise mainly short chain ethoxymers (NP1EO and NP2EO) and ethoxycarboxylates (NP1EC and NP2EC), and nonylphenol.[2,3,6–9] These bio-refractory compounds are strongly lipophilic and have therefore a great tendency to be adsorbed onto suspended solids, sediments and sewage sludge.[5] Thus, their presence in the aqueous phase could be an important marker of contamination.[4] Due also to their physical–chemical properties, they accumulate in the fatty tissue of aquatic organisms.[1,3,9] Lately, interest in the environmental fate of AP*n*EO surfactants has increased, because of the potential effects of their degradation products as endocrine disrupters. Both *in vitro* and *in vivo* bioassays suggest that the estrogenic activity of short ethoxy chain and non-ethoxylated degradation by-products of AP*n*EO surpasses that of the parent compounds and can affect aquatic organisms.[4,5,9] The short chain nonylphenol ethoxylates and ethoxycarboxylates, and nonylphenol, have been extensively found in water bodies (including sewage, surface, ground and coastal waters) and also in sediments, biota and sludge from diverse regions of Europe, Asia and North America along the last years.[1–3,5,9–14] Due to these findings, regulations in Europe have followed the recommendation of phasing out the use of AP*n*EO surfactants in domestic and industrial cleaning agents.[4,15] Also, Canada has recently adopted NP guidelines for the protection of aquatic life.[2]

In Latin American countries, however, the use of AP*n*EO is still completely unrestricted. Even further, the studies on the

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† This paper is dedicated to the memory of Prof. Dr Daniel A. Batistoni.
‡ The HTML version of this article has been enhanced with additional colour images.

incidence of this class of non-ionic surfactants in the aquatic and terrestrial environments of the region are actually scarce. In 2001 we reported—for the first time in Argentina—the presence of 4-NP, 4-NP1EO and 4-NP2EO in a freshwater body of Buenos Aires Province (Matanza River).[16] However, no quantitative results were presented at that instance. Very recently, and as part of an ecotoxicological study conducted to assess the environmental status of the Luján River (another important freshwater system, situated in the NE of Buenos Aires Province), the presence of bio-accumulatable compounds including NP isomers was checked throughout the river by solid-phase micro-extraction followed by gas chromatography-mass spectrometry (GC-MS) qualitative analysis.[17] In a latest contribution, the concentrations of selected alkylphenols, arising from the application of pesticides, were determined in sediment and surface water samples of four reservoirs in the Atlantic Rainforest south-west of São Paulo, Brazil.[18]

The present work has been designed to meet two main objectives. The first one was to contribute with a simple analytical methodology suitable for the simultaneous, quantitative measurement of 4-NP2EO, 4-NP1EO and 4-NP from water samples. Currently, most of the determinations of NP*n*EO and their biodegradation products are reported to be made by reversed-phase high performance liquid chromatography (HPLC) on octadecyl silica (C-18) columns coupled to mass-spectrometry detection (reversed-phase LC-MS), after sample pre-treatment by solid-phase extraction (SPE) on a hydrophobic sorbent (usually C-18).[2,12,14,19–24] Enhancement of selectivity is provided in this case by the use of on-line MS detection. The instrumentation required for this modern, highly selective technique is, however, still unaffordable for the majority of the environmental laboratories in Argentina and the rest of South America. Based on the coupling of well established enrichment and separation techniques, we propose an alternative, affordable method. It consists of an extraction and pre-concentration step on a C-18 SPE sorbent, with subsequent separation and quantification by normal-phase HPLC on an amino-silica column with fluorimetric detection. This instrumental approach follows basically the one by Ahel *et. al.*[11,25] for determination of estrogenic metabolites of NP*n*EO in waters; however, tedious liquid–liquid extraction of the analytes is in this work replaced by solid-phase extraction on C-18 cartridges. When enrichment on a hydrophobic sorbent is followed by HPLC analysis on a normal-phase column, which interacts through the polar moiety of the analyte molecules, selectivity is expected to be considerably improved. Based upon similar principles (SPE/normal-phase LC/fluorescence detection), Smith *et. al.*[3] determined nonyl-, octyl- and butylphenol in estuarine rivers. Mayer and co-workers[9] reported the determination of NP*n*EO with *n* from 1 to 17 in the bulk water of a coastal marsh, using SPE concentration followed by clean-up on silica gel and normal-phase LC coupled to fluorescence detection; measurement of fully de-ethoxylated NP was made in that case by GC-MS analysis after derivatisation. Here, we also propose that confirmation of peak identities can be readily implemented (when necessary) by off-line coupling with more widespread GC-MS instruments, after evaporation of the highly volatile organic solvents used for normal-phase LC elution and suitable re-dissolution.[1,7] To the authors knowledge, the exact combination of analytical steps set up in the present paper has rarely been exploited for the simultaneous determination of 4-NP2EO, 4-NP1EO and 4-NP.

The second objective of this work was to evaluate the performance of the method proposed in a real case study. The expected quantitative data should also contribute to the assessment of the fate of NP*n*EO and their persistent metabolites, and constitute the first report of its kind for a Latin American country.

Experimental

Materials

4-Nonylphenol (technical grade, Fluka, Buchs, Switzerland), 4-NP1EO and 4-NP2EO (Promochem, Wesel, Germany), were used as analyte standards for the estrogenic metabolites. A commercial NP*n*EO mixture with an average number of ethoxy groups (*n*) of 10 (NP10EO), purchased from Proquimia SA (Chile), was also utilised. *Hexanes* (95% *n*-hexane/5% branched isomers) and cyclohexane were HPLC grade solvents from Sintorgan (Buenos Aires, Argentina). Methanol (MeOH), 2-propanol (2-PrOH) and ethyl acetate (EtAc) were HPLC grade solvents from Merck (Darmstadt, Germany). Purified water (18 MΩ cm) was obtained from a Simplicity model water purification unit (Millipore, SP, Brazil). Sodium chloride and sodium sulfate (anhydrous) were *p.a.* reagents from Merck, and they were heated overnight at 650 °C prior to use. Hydrochloric acid (36.5%) was *p.a.* quality from Mallinckrodt-Baker, México. Glass-fibre pre-filters were APFD 04700 from Millipore, Ireland; they were heated at 450 °C for 5 h prior to use. For solid-phase extraction, glass columns and PTFE frits from Merck were used. Octadecylsilica sorbent was either LiChrolut RP-18 (Merck) or CEC-18 (UCT, PA, USA). An aqueous solution of formaldehyde (37%, *p.a.*, Fluka) was used for preservation of the samples. For the procedures where plastic tubing was needed, fluorocarbon polymer materials were always employed.

Instrumentation

The HPLC system used consisted of a *SpectraSERIES P200* binary pump (Thermo Separation Products, CA, USA). Detection was performed with a *Linear LC-305* fluorescence detector (Linear Instruments, NE, USA). Data were acquired and analysed with the *Konikrom 5.2* software (Konik Instruments, Barcelona, Spain). Column temperature was controlled within 0.1 °C using an Eppendorf CH-30 column heater and an Eppendorf TC-50 controller (Alltech, IL, USA).

Gas chromatography with mass spectrometry detection analyses were carried out with a *Shimadzu GC-17A* split-splitless gas chromatograph coupled to a *MS-QP5050A* mass spectrometer. Data were acquired and analysed with the *Class-5000* software (Shimadzu Corporation, Kyoto, Japan) which includes the WILEY 229 spectral library. Gas chromatography with flame ionisation detection (GC-FID) was performed with an HP 5890-A split-splitless instrument.

Procedures

i. High-performance liquid chromatography with fluorescence detection. Standard solutions of t-NP, 4-NP1EO and 4-NP2EO for HPLC and GC analyses were prepared by dilution of approx.

1000 mg l^{-1} stock solutions of the individual compounds with cyclohexane or *hexanes*. Approx. 10 000 mg l^{-1} stock solutions of the NP10EO surfactant were prepared in 2-PrOH. All solutions were stored at 4 °C.

Separations were performed on a 5 μm particle aminopropyl-silica column (250 × 4.6 mm) with 10 × 4.0 mm guard-column (Pinnacle II, Restek, USA). Column temperature was set at 35 °C. Eluent reservoirs were kept at room temperature. Injection volumes were of 50 μl, unless otherwise stated. Isocratic elution for determination of the lipophilic metabolites was carried out with a mixture of 4.1% 2-PrOH in *hexanes* at a flow rate of 0.65 ml min^{-1}. Simultaneous measurement of both short and longer oligomers was performed by binary gradient elution. Mobile phases were A: *hexanes* and B: 2-propanol. The program started isocratically with 96% A – 4% B at a flow rate of 0.65 ml min^{-1} for 19 min, followed by a linear flow rate gradient up to 1 ml min^{-1} in 2 min; then a linear solvent gradient through 50% A – 50% B was applied in 29 min, and there were finally 6 min of isocratic hold with 100% B. Identification of compounds with less than 3 ethylene oxide units was made with standard spikes of t-NP, 4-NP1EO and 4-NP2EO, while the higher ethoxymers were assigned from their elution sequence.

Excitation and emission spectra of 4-NP, 4-NP1EO and 4-NP2EO were recorded by means of a real time, spectral scan of HPLC eluting peaks. Scans from 200 to 300 nm (excitation) and from 250 to 400 nm (emission) were acquired. The wavelength maxima of each individual compound were identified, allowing selection of the working settings for best sensitivity.

Collection of HPLC fractions for GC-MS analyses was made by means of a 3-way manual switching valve (Rheodyne 7030RV, USA). One first run was performed in order to accurately determine the time windows of NP1EO, NP2EO and NP. Afterwards, 100 μl of the extracts were injected 2–5 times and the fractions corresponding to each individual analyte were collected together in a glass tube, to make up a compound mass enough for GC-MS determination.

ii. Gas chromatography. HPLC collected eluates were injected in the GC-MS instrument after complete evaporation of HPLC solvents under a stream of N_2 and re-dissolution in an appropriate volume (25–100 μl) of cyclohexane. By use of a capillary column, complex peak patterns were obtained from injection of NP and the ethoxylated homologues, this fact owed to the separation of several nonyl chain isomers.[26–28] The capillary column used in this study was a Zebron ZB-1 of 60 m × 0.32 mm i.d. × 0.50 μm film thickness (Phenomenex, USA). Instrumental conditions selected for the separate measurement of the three compounds of interest were as follows: injector and detector temperature, 300 °C; oven temperature programme, (a) 80 °C (1 min)/6 °C min^{-1}/280 °C (5 min) for NP, and (b) 160 °C (4 min)/3 °C min^{-1}/280 °C (5 min) for NP1EO and NP2EO; column pressure, 90.5 kPa; split ratio, (a) 1 : 10 for NP, and (b) 1 : 5 for NP1EO and NP2EO. Injection volumes ranged from 1.0 to 1.5 μl. The analytes were identified and quantified using the mass-spectrometer in the single ion-monitoring (SIM) mode.[1,18] Every peak was quantified separately and then they were summed up to make the total amount of the target compound.[1,28] Selection of the ion traces to be monitored was made by running the analyte standards in full-scan mode, based upon the premise of enhanced selectivity and sensitivity. These goals were reached by choosing the more intense mass fragments of each compound, provided that the same mass to charge ratios were absent in the spectra of interfering, ubiquitous phthalate esters. Positive identification was made by means of coincident retention times of analytes and standards. In addition, a SIM spectral library was built up with the standards, and every peak in the samples were checked for a matching abundance pattern of the four (one target plus three qualifier) ions monitored. The target ions chosen for quantification are shown in Table 1.

The response with mass-spectrometry detection is proportional to the number of ions formed in the fragmentation of the molecule, and this number is likely to be different for the

Table 1 Mass to charge ratios of the ion traces monitored for the isomer specific GC-MS determination of NP, NP1EO and NP2EO; and proportion of branched isomers in the commercial products used as analyte standards, determined by GC-FID

	GC-MS			GC-FID		
Isomer peak number[a]	NP target ion	NP1EO target ion	NP2EO target ion	% in NP standard	% in NP1EO standard	% in NP2EO standard
1	163	165	251	5.3	4.2	5.2
2	135	179	223	10.6	6.8	5.6
3	107	193	237	12.2	9.5	18.5 (peaks 3 + 4)
4	135	179	223	3.3	16.0	—
5	135	193	223	12.4	6.4	17.8 (peaks 5 + 6 + 7)
6	107	179	237	7.4	6.4	—
7	163	193	223	2.6 (peaks 7 + 8)	7.5	—
8	135	165	237	—	3.1 (peaks 8 + 9)	11.3 (peaks 8 + 9)
9	163	179	251	4.3	—	—
10	107	193	251	4.0	7.2 (peaks 10 + 11)	4.3
11	163	165	223	12.6	—	11.2 (peaks 11 + 12)
12	135	107	237	13.2	7.1	—
13	107	179	251	5.9	4.3	5.0
14	107	107	223	6.3	9.4	16.0
15	—	179	237	—	6.8	5.1
16	—	193	—	—	5.4	—

[a] Isomer peak numbers correlate with those in Fig. 4.

different analyte isomers. In order to know the proportion of every single isomer in the analyte standards, it was necessary to make use of a detection system like flame ionisation, in which the response is equivalent for isomer molecules. Thus, the amount of each isomer present in the technical products were measured by GC-FID and isomer specific determinations were then carried out with the GC-MS instrument.[1] The GC-FID apparatus was equipped with an ULTRA-1 capillary column of 60 m × 0.32 mm i.d. × 0.50 μm film thickness (Hewlett-Packard, USA). Operational parameters were equivalent to those selected for GC-MS analyses, in order to match the isomer peak profiles obtained with both instruments.

iii. Solid-phase extraction of model samples and blanks. Nanopure water spiked with the analytes was employed to make a preliminary evaluation of the enrichment method. A more rigorous assessment of analyte recoveries and method precision was further carried out with the real samples. Spiking mixtures were prepared in MeOH, from stock solutions in this solvent stored at 4 °C. Sample volume assessed was 500 ml, at a concentration level of 2.5 μg l^{-1} of each compound. Blank samples were also included, to check the presence of interfering substances arising at any step of sample treatment and handling.

Formaldehyde at a final concentration of 1% (v/v) was added to the water in the beginning of the procedure. This chemical would act in preserving environmental samples from microbial activity, preventing the analytes from being further bio-degraded after sampling.[5] Afterwards, the samples were passed through glass-fibre filters. The filters were subsequently rinsed with MeOH, because when handling river and effluent samples, it is important to elute the analyte portion adsorbed onto the filter cake to attain a quantitative recovery of the analytes.[29] The methanolic fraction was collected together with the filtered water, resulting in a concentration of 5% of the polar organic solvent, as usually recommended for reverse-phase SPE of large amounts (>100 ml) of aqueous samples, in order to maintain the sorbent surface active. After filtration, sodium chloride was added as a salting-out agent, at a final concentration of 5% (m/v).

The SPE columns were filled with 1 g C-18 sorbent. They were conditioned with 15 ml MeOH as activation solvent and equilibrated with 15 ml nanopure water before sample processing. Samples were then percolated through the cartridges by application of vacuum, at a flow rate of 8–10 ml min^{-1}. After that, the sorbent was washed with 15 ml of a mixture MeOH : water (10 : 90, v/v). A drying step (under vacuum) was incorporated. Details on its duration will be given further along. A total amount of 15 ml of elution solvent (EtAc) was then passed through the column in aliquots of 5 ml, at atmospheric pressure. Anhydrous sodium sulfate was used to remove remaining water traces from the organic phase. The SPE elution solvent was evaporated to dryness under a mild stream of nitrogen (at 50 °C), and the residue was re-dissolved in a known, smaller amount of *hexanes* (typically 0.5–1.5 ml) for HPLC injection. Recovery rates were measured against repeated injections of a standard mixture 0.8 mg l^{-1} of the analytes in cyclohexane.

iv. Environmental sampling. As a case study, we selected the facilities of a sewage treatment plant (STP) located in the surroundings of Buenos Aires, 23 km to the NW of the capital city. The STP treats mainly domestic sewage, serving to a population of 30 000 inhabitants. A scheme of the plant is shown in Fig. 1. It operates basically as follows: raw effluent (5800 m^3 d^{-1}) receives a primary treatment consisting of large solids removal in a rotary sieve and further sand removal. The input stream is then mixed with smaller recycling streams in a pumping station, from where a composite effluent is pumped to a partition chamber. Clarification of sewage by settling and sedimentation, along with anaerobic digestion, take place in three Imhoff tanks (I-1 to 3). Nearly 15% of the sewage is treated by I-1, 37% by I-2 and 48 % by I-3. A composite stream from I-1 and I-2 is submitted to secondary treatment on a trickling filter, while the effluent from I-3 is treated by a system of ponds. The effluent from the trickling filter passes to a secondary clarifier; the clarified liquid, together with the water exiting the ponds, make up the whole treated effluent, which is discharged into a natural water stream (Morón creek).

In the first survey carried out, in spring 2005, the two following points were sampled: (a) the brook, at a point 100 m downstream the discharge of the plant (point V in Fig. 1), and (b) the effluent exiting the secondary clarifier (point IV in Fig. 1). In winter 2006, the second survey consisted of sampling at three points: (a) inside the partition chamber (point I in Fig. 1); (b) the composite effluent from I-1 and I-2 (point II in Fig. 1), and (c) the effluent from the trickling filter (point III in Fig. 1).

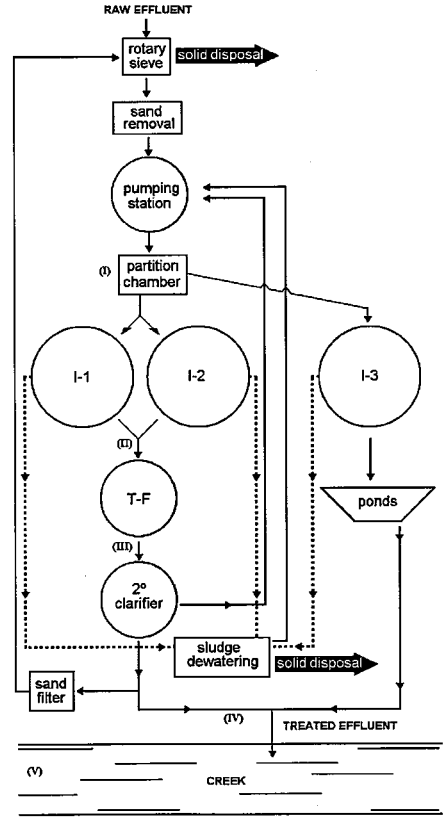

Fig. 1 A scheme of the sewage treatment plant. Liquid flows are drawn as full lines, while solid flows are drawn as doted lines. I-1, I-2 and I-3 indicate Imhoff tanks 1, 2 and 3, respectively. T-F indicates trickling filter. Roman numbers I to V show the sampled points.

Samples were collected with an aluminium can and immediately transferred into various amber-glass bottles (making up replicate samples), where 1% (v/v) of formaldehyde was added. They were stored at 4 °C until analysis.

v. Analysis of real samples

Morón creek and treated effluent (first survey). Sample treatment and conditioning were further studied with Morón creek replicate samples. Firstly, and following the procedure described in *Section iii*, extraction of 500 ml water aliquots was carried out. In view of the high concentration levels found for the analytes, a reduction in sample size from 500 to 100 ml was implemented at that instance. In second place, extraction of 100 ml sample at natural pH (around 9) and at pH = 3 (adjusted with HCl) were assessed. Afterwards, and concomitant with the reduction in sample size, a reduction in C-18 sorbent mass (from 1 to 0.5 g) and in conditioning and elution solvents (from 15 to 8 ml), were carried out. Finally, re-dissolution of the dried extracts in known volumes of HPLC elution mixture, instead of *hexanes*, was also tested.

Chromatographic peak areas were recorded and analyte concentrations in the extracts were calculated. Standard mixtures of the analytes, at concentrations ranging from 0.5 to 3.0 mg l^{-1}, were used for HPLC-fluorescence calibration. Recoveries were calculated after spiking replicate sample aliquots of 100 ml with known amounts of the analytes at two concentration levels: (i) 0.008, 0.006 and 0.005 mg l^{-1} and (ii) 0.036, 0.031 and 0.025 mg l^{-1} for NP1EO, NP2EO and NP, respectively.

Subsequently, aliquots of 50 and 100 ml from replicate bottles of the treated effluent were analysed. All samples were adjusted to pH 3 with HCl, extracted on 500 mg C-18 solid phase and eluted with 8 ml EtAc. Re-dissolution of the evaporation residues was made in 4.1% 2-PrOH in *hexanes*. Analyte concentrations in the extracts were calculated in the same way as for Morón creek. Recovery rates were worked out after spiking replicate sample aliquots at three concentration levels, namely: (i) 0.004, 0.004 and 0.003, (ii) 0.011, 0.009 and 0.008, and (iii) 0.022, 0.019 and 0.016 mg l^{-1} for NP1EO, NP2EO and NP, respectively.

The fractions corresponding to the three analytes in every replicate sample were collected and re-injected in the GC-MS instrument.

Along wastewater treatment (second survey). The sample from the partition chamber, the composite effluent from Imhoff tanks 1 and 2 and the trickling filter supernatant, were processed in the same manner as the secondary effluent. Additional recovery tests were carried out in order to evaluate the homogeneity in the extraction of higher *n* NP*n*EO oligomers. Two NP10EO samples were prepared in nanopure water, one at a concentration below and one at a concentration above the *critical micelle concentration* (CMC) of the surfactant (CMC of NP10EO at 25 °C = 7.5 × 10^{-5} M).[30] The concentrated extracts were analysed by gradient elution HPLC.

Results and discussion

1. High-performance liquid chromatography with fluorescence detection

On the one hand, the chromatographic conditions selected allowed complete resolution of the persistent metabolites 4-NP1EO, 4-NP2EO and 4-NP in less than 20 min isocratic run (see Fig. 3-a). Regarding fluorescence detection, excitation and emission spectra of the lipophilic metabolites, recorded respectively at emission and excitation wavelengths of 300 and 230 nm, are shown in Fig. 2. The three compounds exhibited emission maxima around 300 nm and the best excitation wavelength was around 230 nm. Thus, the method was evaluated for linearity, repeatability and limits of detection at excitation and emission wavelengths of 230 and 300 nm, respectively. For all the compounds good precision was obtained, with %RSD smaller than 7% for peak areas and smaller than 1% for retention times (n = 10, injection of a solution 0.05 mg l^{-1} of each compound). Linear correlation coefficients (R) were always higher than 0.999 for calibration curves constructed for peak

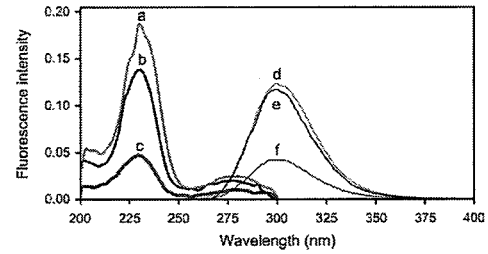

Fig. 2 Fluorescence spectra of the analytes. (a), (b) and (c): excitation spectra of NP2EO, NP1EO and NP, respectively; (d), (e) and (f): emission spectra of NP2EO, NP1EO and NP, respectively. Injected amounts: 100 µl of 0.1 mg l^{-1} solutions of the individual compounds.

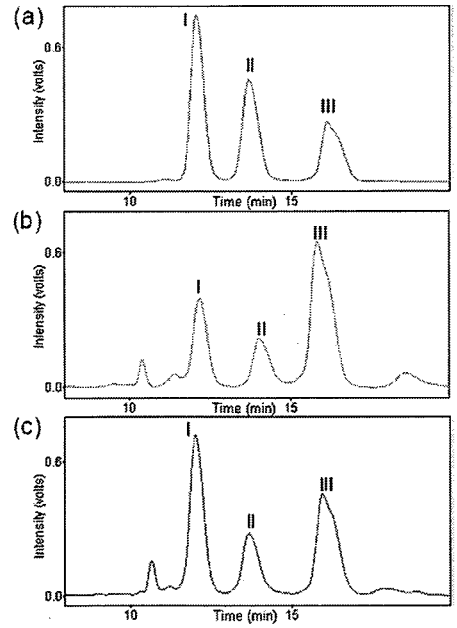

Fig. 3 HPLC chromatograms of: (a) a standard mixture 1.25 mg l^{-1} of t-NP, 4-NP1EO and 4-NP2EO, (b) an extract of the secondary effluent sample (concentration factor = 100) and (c) an extract of the Morón creek sample (concentration factor = 100). Peak labels indicate: (I) 4-NP1EO, (II) 4-NP2EO and (III) 4-NP. Excitation wavelength: 230 nm, emission wavelength: 300 nm; mobile phase: 4.1% 2-PrOH in *hexanes*, flow rate: 0.65 ml min^{-1}; column temperature: 35 °C.

areas, at concentration levels ranging from 0.01 to 1.50 mg l^{-1}. Detection limits, estimated as the concentration for a blank signal of $B + 3\sigma_B$ (where B and σ_B are the blank signal mean value and standard deviation for 10 injections of the solvent), resulted 0.8 μg l^{-1} for NP1EO and NP2EO, and 1.3 μg l^{-1} for NP. The chromatographic method coupled to fluorescence detection attained, in principle, the performance necessary for determination of the analytes at the concentration levels usually found in the environment[5,31] after suitable enrichment of the water samples. Taking into account that enrichment factors up to 1000 (or even higher) are reasonable and practically feasible, we may say that the instrumental limits obtained surpass the requirements for environmental monitoring of moderately contaminated waters (in which the analyte levels are in the order of μg l^{-1}), and fairly meet the requirements for slightly polluted waters (in the ng l^{-1} levels). Even further, injection volumes smaller than 50 μl would, in most cases, be enough for quantification of the analytes in the concentrated extracts.

2. Gas chromatography

Regarding GC-MS measurements, in all cases SIM method allowed to distinguish at least 14 isomer peaks (see Fig. 4), making up accurate finger-prints for confident identification of the analytes. Structure assignment and elucidation of the nonyl chain branching of the separated peaks is beyond the scope of the present work; however, the reader is referred to thorough studies in this field,[27,28,32] which cope specially with NP isomeric peaks identification.

In Table 1 we show the proportions calculated for the peaks separated by capillary GC and using FID detection. On the other hand, limits of detection for GC-MS (as sum of isomers) were found to be around 0.1 μg ml^{-1} for NP, 0.2 μg ml^{-1} for NP1EO and 0.3 μg ml^{-1} for NP2EO.

3. Solid-phase extraction of model samples and blanks

From the most common elution solvents compatible with reverse-phase sorbents, *i.e.* methanol, acetonitrile and ethyl acetate,[33] ethyl acetate was chosen for elution of the strongly lipophilic degradation products under study. Being the less polar among these solvents, cleaner extracts would be expected, as highly polar and ionic organic compounds should not be eluted efficiently. The need for drying the SPE sorbent before elution was tested by comparing analyte recoveries for a set of columns that were submitted to 3 h drying, with that for a second group that was not dried at all. The recoveries resulted almost the same in both cases. This finding could be ascribed to the fact that, even

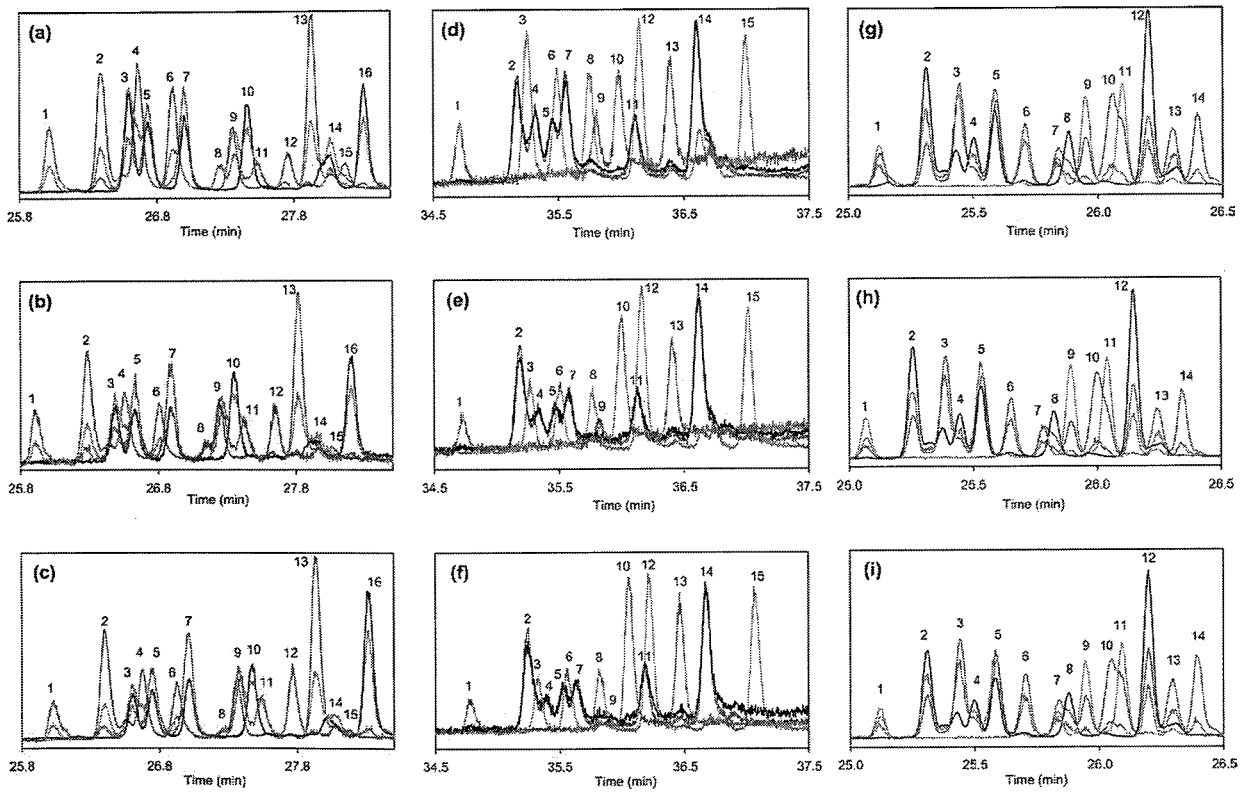

Fig. 4 GC-MS chromatograms corresponding to direct injection of analyte standards and to injection of HPLC collected fractions. (a) standard solution of NP1EO 15 mg l^{-1}; (b) NP1EO in Morón creek sample; (c) NP1EO in secondary effluent sample; (d) standard solution of NP2EO 10 mg l^{-1}; (e) NP2EO in Morón creek sample; (f) NP2EO in secondary effluent sample; (g) standard solution of NP 20 mg l^{-1}; (h) NP in Morón creek sample; and (i) NP in secondary effluent sample. The numbers above the peaks point out the different branched isomers separated, and correlate with those in Table 1. Line colours of the monitored ion traces are as follows: for NP1EO: *m/z* 179 (blue), *m/z* 193 (black), *m/z* 107 (red) and *m/z* 165 (green); for NP2EO: *m/z* 223 (blue), *m/z* 135 (black), *m/z* 237 (red) and *m/z* 251 (green); for NP: *m/z* 107 (blue), *m/z* 135 (black), *m/z* 163 (red) and *m/z* 220 (green). Other instrumental parameters: as indicated in the text.

when water is scarcely soluble in EtAc, this solvent is able of displacing water molecules from the pores and thus interact with all areas of the silica surface.[33] A drying step of 20–30 min was finally included in the protocol, as elution time turned out to be too long when no drying was applied at all. The recovery rates were around 80% for each of the three compounds, with relative standard deviations smaller than 10% (for 4 repeated extractions). The recoveries obtained were considered acceptable for quantification purposes and the reproducibility was also good when percolating the sample at flow rates of 8–10 ml min^{-1}. Therefore, and since lower flow rates would extend the sample preparation time considerably, they were not evaluated in this work. However, the authors think that reducing sample percolation flow rate may help to optimise analytes recovery and this is a variable that could be further explored.

Contribution to blank values was only significant from the nanopure water employed to build up the model samples. Nevertheless, assessment of analyte recoveries was not affected at the concentration level studied. None of the other procedural steps (which are in turn the only steps involved in real samples analysis) introduced interfering substances at detectable levels.

Thus, the whole enrichment procedure was found, in principle, appropriate for quantification purposes.

4. Analysis of real samples

Morón creek and treated effluent (first survey). In Fig. 3 we have gathered HPLC chromatograms recorded from injections of a standard mixture of the analytes, an extract of the secondary effluent sample and an extract of the Morón creek sample (both with a concentration factor of 100). As may be appreciated, baseline resolved, well shaped peaks were apparent for the two samples.

The quantitative results obtained for Morón creek under the varying sample treatment conditions depicted in *Procedures, Section v*, are presented in Table 2. With regards to the method performance, for a total of 14 extractions, around 10% RSD were obtained for the concentration of each analyte after enrichment. No changes in the concentration values, nor in the resolution of the chromatograms, were observed when changing any of the variables, indicating the robustness of the selected methodology. However, acidification of the sample provided a clearer extract. Also re-dissolution of the dry residue in the presence of a more polar solvent (4.1% 2-PrOH) contributed to further clarification of the sample to be injected. For those reasons, the conditions selected involved acidification of the water sample and re-dissolution of the evaporation residue in 4.1% 2-PrOH in *hexanes*. From standard addition of the analytes, calculated average recoveries were 85, 85 and 74% for NP1EO, NP2EO and NP, respectively.

The results for quantification in the treated effluent are listed in Table 3. In this case, for a number of 6 determinations (including sample sizes of 50 and 100 ml) around 5% RSD were achieved for the concentration of each analyte after enrichment. Average recoveries, calculated from standard addition of the analytes, resulted 91, 85 and 73% for NP1EO, NP2EO and NP, respectively.

In the determination of organic contaminants, the problem of interfering substances is a matter of concern. Ahel and co-workers[25] presented a thorough discussion on the particular case of measuring NP*n*EO and their lipophilic derivatives in sewage effluents, using normal-phase HPLC with fluorescence detection. Briefly, they state that this might be a site-specific problem and then each place intended to routine analysis should be examined independently; however, some general considerations are done by the authors. They suggest that the interference of normally occurring phthalate esters and polycyclic aromatic hydrocarbons is mainly overcome through the use of fluorescence detection. Thus, the most important compounds potentially interfering with the determination of NP, NP1EO and

Table 2 Concentrations measured after enrichment of Morón creek sample (concentration factor = 100)

Replicate sample	Sample volume/ml	Sample pH	SPE sorbent mass/mg	SPE eluent volume/ml	Re-dissolution solvent		NP1EO concentration/mg l^{-1}	NP2EO concentration/mg l^{-1}	NP concentration/mg l^{-1}
I	500	9	1000	15	*Hexanes*		1.42	0.55	1.89
I	100	9	1000	15	*Hexanes*		1.33	0.60	2.04
II	100	9	1000	15	*Hexanes*		1.37	0.58	2.39
I	100	3	1000	15	*Hexanes*		1.12	0.47	1.77
II	100	3	1000	15	*Hexanes*		1.28	0.53	2.05
I	100	3	500	8	*Hexanes*		1.40	0.52	2.17
II	100	3	500	8	*Hexanes*		1.29	0.58	2.02
I	100	3	500	8	*Hexanes*		1.15	0.49	1.84
I	100	3	500	8	4.1% 2-PrOH in *hexanes*		1.05	0.46	1.82
II	100	3	500	8	*Hexanes*		1.08	0.41	1.73
II	100	3	500	8	4.1% 2-PrOH in *hexanes*		1.33	0.58	2.08
I	100	3	500	8	*Hexanes*		1.20	0.51	1.85
II	100	3	500	8	4.1% 2-PrOH in *hexanes*		1.47	0.57	2.31
II	100	3	500	8	4.1% 2-PrOH in *hexanes*		1.35	0.54	2.02
						Average	1.27	0.53	2.00
						Standard deviation	0.13	0.05	0.20
						%RSD	10.4	10.4	9.9

Table 3 Concentrations measured after enrichment of the secondary effluent sample (concentration factor = 100)

Replicate sample	Sample volume/ml		NP1EO concentration/ mg l^{-1}	NP2EO concentration/ mg l^{-1}	NP concentration/mg l^{-1}
I	50		0.74	0.63	2.64
II	50		0.70	0.60	2.51
I	100		0.78	0.66	2.60
II	100		0.80	0.67	2.68
III	100		0.74	0.60	2.42
IV	100		0.73	0.59	2.39
		Average	0.75	0.62	2.54
		Standard deviation	0.04	0.04	0.12
		%RSD	5.0	5.6	4.7

NP2EO remain other alkylphenols, which could have significant fluorescence responses at the same detection settings of the analytes. Because of this, appropriate sample pre-treatment and good chromatographic resolution become key steps. In the present work we have not made any study on interfering compounds present in the samples after enrichment by the C-18 SPE procedure developed. Instead, we have submitted the HPLC peaks attributed to the analytes to subsequent analysis by GC-MS. The HPLC fractions collected from injection of the samples yielded purity values above 80% when analysed by this second technique. The GC-MS chromatograms corresponding to the HPLC collected peaks are shown in Fig. 4, together with runs of standard solutions of the analytes. Altogether, both the neat HPLC chromatograms and the high purity found by GC-MS analysis for each individual HPLC peak, suggest that the whole analytical procedure offers a degree of selectivity suitable for simplified monitoring of these persistent pollutants in fresh and treated water samples.

On the other hand, the detection limits of the entire method calculated for a sample size of 100 ml, an enrichment factor (defined as the rate of sample volume to extract re-dissolution volume) of 100 and rounded recoveries of 80%, are about 0.013 μg l^{-1} for each of the analytes (considering an average instrumental detection limit of 1 μg l^{-1}). As the amount of water treated can be increased and the final volume of the extract minimised according to the analytes level, enrichment factors 10–50 folds higher than the one above can be obtained and, therefore, the present method should allow determination of the analytes even at extremely low concentrations that may occur in environmental compartments of interest, like for example drinking water, for which NP values as low as 0.001 μg l^{-1} have been reported.[5] Regarding environmental protection residue limits established in different countries, they can also be met by the proposed procedure. For instance, NP guidelines for protection of aquatic life in Canada are 6 μg l^{-1} (Québec regulation) and 1 μg l^{-1} (national regulation),[2] while Dutch surface and groundwater maximum permissible concentrations (MPCs) are 0.33 and 0.11 μg l^{-1} for NP and NP1EO + NP2EO, respectively,[34] all these values measurable with the proposed methodology.

Speaking of the sample levels found for the analytes (calculated from Tables 2 and 3, taking into account the actual recovery rates) they were, for NP1EO, NP2EO and NP, respectively, 8, 7 and 35 μg l^{-1} in the effluent, and 15, 6 and 27 μg l^{-1} in the creek. In general terms, it might be said that these values are consistent with those reported for other countries along the last decades.[5,31] They are all relevant if considering bio-accumulation and, in the case of NP, the levels measured could be placed in the upper extreme of concentrations reported in the literature; these values are also well above the threshold for vitellogenin induction (a common marker for estrogenic activity) in a number of aquatic organisms.[3–5] On the other hand, the concentrations in the two samples were similar, speaking of a minute dilution effect from the sewage output throughout the 100 m of the receiving water stream, and evidencing the high impact that the discharge of bio-refractory compounds may have in the environment. To get a better understanding on the significance of the concentrations measured, they can be contrasted with the aforementioned Canadian and Dutch limits for protection of the aquatic environment.

It is also worth mentioning that differences between the GC-MS isomeric patterns were noted for the samples when compared with the standard materials (see Fig. 4). Relative intensities significantly changed inside the peak groups *3–5* and *8–11* in the case of NP1EO, inside the peak group *2–10* in the case of NP2EO and, in a lesser extent, inside the peak group *2–5* in the case of NP. With the collected information, we are not able to identify the origin of the differential profiles found for the standards and the samples. It could be ascribed to differences in the composition of the raw material used in the synthesis of parent NP*n*EO and NP, but also to the varying recalcitrance of the different isomeric structures. Even so, this observation may be of interest, as it is lately becoming clearer that an isomer-specific point of view is required to evaluate properly the biological and environmental effects of nonylphenol and the ethoxylates, since their estrogenic potential may differ among isomeric forms, as also do their degradation processes.[35,36,8]

Along wastewater treatment (second survey). Based upon the encouraging results presented above, the developed methodology was assessed for its application in the tracking of the NP*n*EO degradation process inside the wastewater treatment plant. The most commonly used surfactants of the nonylphenol polyethoxylate type have chains containing five to twenty oxyethylene groups.[26] Among them, the most popular commercial product is the one containing an average of 10 ethoxy units (NP10EO),[13,37] and this was therefore the parent product assessed. The ethoxymer profiles recovered after SPE percolation of NP10EO synthetic solutions were, at the two concentration levels analysed, similar to the pattern obtained from direct injection of a standard solution, as may be observed in Fig. 5-a

and 5-b. The total recovery, calculated from the sum of areas of NP*n*EO (*n* from 0 to 10), resulted around 75%. Chromatograms obtained for the samples of partition chamber, composite effluent from Imhoff tanks and trickling filter supernatant, with a concentration factor of 100, are also shown in Fig. 5. Although in some cases unidentified peaks partially overlap with the analytes, the chromatograms of the samples offer enough resolution as to reliably identify the compounds of interest by the addition of standard spikes.

Mixtures of NP, NP1EO and NP2EO are not widely utilised in detergent formulations due to their low water solubility, and they are therefore not expected to occur significantly in aqueous effluents. For those reasons, the presence of important amounts of NP, NP1EO and NP2EO in the selected samples would be explained by the recalcitrance of these by-products.[26] This fact is apparent in the three samples collected. Assuming that the starting material is mostly NP10EO, degradation of this detergent can be appreciated already in the primary effluent. Results for the sample from the partition chamber (Fig. 5-c) suggest that long chain ethoxymers ($n > 7$) have undergone a rapid primary degradation that resulted in a transient increase of medium chain molecules ($3 < n < 6$). This sample also contains substantially higher proportions of low n NP*n*EO oligomers ($0 < n < 2$) than the starting product; this fact has been observed before in raw effluents,[5] but in our case there are re-circulation flows that could contribute to these recalcitrant metabolites as well. Concentrations measured in the extract correspond to sample levels of 13 μg l^{-1} of NP1EO and NP2EO, and 16 μg l^{-1} of NP. After digestion in the Imhoff tanks, ethoxymers with $n > 3$ have almost vanished in favour of their persistent metabolic products NP2EO, NP1EO and NP (see Fig. 5-d). The amounts measured in this extract correspond to sample concentrations of 23 μg l^{-1} for NP1EO, 10 μg l^{-1} for NP2EO and 36 μg l^{-1} for NP. During the subsequent aerobic process in the trickling filter, oligomer profile did not change significantly (see Fig. 5-e), as neither did the accumulated amount of estrogenic metabolites, which yielded values of 24, 10 and 41 μg l^{-1} of NP1EO, NP2EO and NP, respectively.

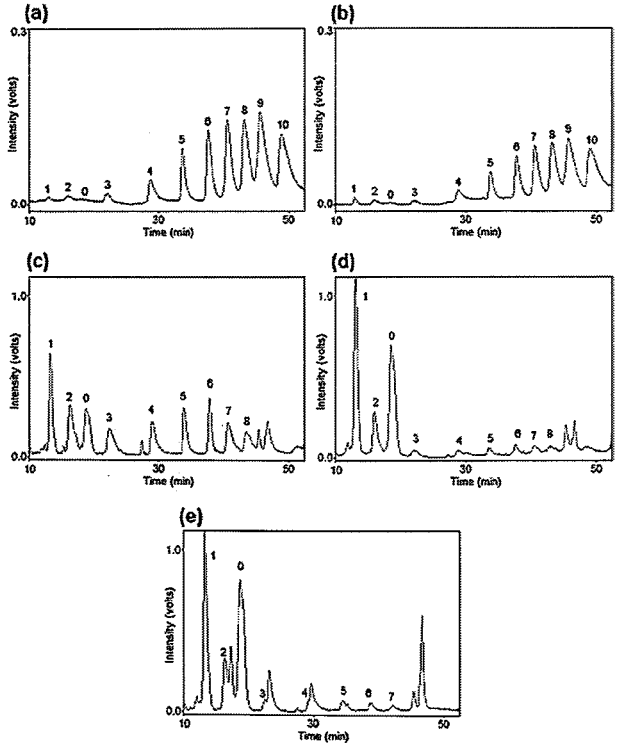

Fig. 5 HPLC chromatograms of: (a) direct injection of NP10EO 10 mg l^{-1} standard solution; (b) standard solution of NP10EO 10 mg l^{-1} after solid-phase extraction; (c) sample from the partition chamber (concentration factor = 100); (d) Imhoff tanks composite effluent (concentration factor = 100); and (e) trickling filter supernatant (concentration factor = 100). Peak labels indicate the number of ethoxy units (n) in the corresponding NP*n*EO oligomer. Mobile phase gradient and flow rate program: as indicated in the text. Other instrumental parameters: same as Fig. 3.

Conclusions

Clearly, the site studied represented just one point in space. However, it was chosen because it can be considered representative of the dynamics of the target compounds in sewage systems of the region. In this way, the utility of the proposed analytical methodology was demonstrated and, at the same time, results relevant from the point of view of the fate and incidence of NP*n*EO were obtained. The levels found for the bio-refractory, lipophilic metabolites of these surfactants (in the tens of μg l^{-1}) are undoubtedly of environmental concern. In order to get a better description of the occurrence and distribution of nonylphenolic pollutants in local systems, a vast sampling campaign has already been started by the group; this program will also extend to the study of river sediments and sewage sludge.

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