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Analysis of nonylphenol and nonylphenol ethoxylates in environmental samples by mixed-mode high-performance liquid chromatography–electrospray mass spectrometry

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Abstract

A new method is described based on mixed-mode high-performance liquid chromatography with electrospray mass spectrometry detection for comprehensive quantitative analysis of nonylphenol (NP) and nonylphenol ethoxylates (NPEOs) in wastewater and sediment. Efficient separation, reduced band broadening, and high sensitivity were achieved by employing a methanol–water gradient on a mixed-solvent gel filtration column designed for MS interfacing. Quantitative accuracy and precision of the method were improved by the use of custom-synthesized [$^{13}C_6$]NPEO analogs as isotope-dilution surrogate standards. Method detection limits for NP and individual NPEOs ranged from 1 to 55 pg injected on column.

Keywords: Environmental analysis; Water analysis; Sediment; Nonylphenol; Nonylphenol ethoxylates; Ethoxylates; Surfactants; Alkylphenol ethoxylates; Alkylphenols

1. Introduction

Nonylphenol ethoxylates (NPEOs) are a class of high volume (1994 USA production $>280{\cdot}10^6$ kg [1]), petrochemical-based nonionic surfactants used in a wide variety of industrial and consumer products including cleaning agents and emulsifiers. NPEO formulations are complex mixtures produced by addition of a large molar excess of ethylene oxide to the 4-nonylphenol hydrophobe, which is itself a mixture of branched nonyl isomers. A typical NPEO surfactant formulation is comprised of nonylphenol with an average of ~10 and a range of 1 to ~20 ethoxy units. Biodegradation of NPEO during wastewater treatment or after discharge to the environment can result in shortening of the ethoxy chain [2,3], leading to more hydrophobic [4] metabolites. Concern over possible toxicity and estrogenicity [5] of several of these NPEO degradation products, including nonylphenol (NP) and the mono-, di-, and triethoxylates (NP1EO, NP2EO, and NP3EO), has led to a ban in the use of NPEO surfactants in household cleaning products in some areas of Europe, most notably Switzerland [6], and initiation of regulatory attention in North America [7,8]. The US Environmental Protection Agency (EPA) has recently added NP1EO, NP2EO, and NP3EO to the Toxic Substances Control Act (TSCA) Section 4(e) Priority Testing List [9]. NP had already been placed on the list in 1996 [10].

The increased attention focused on occurrence of

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NPEO metabolites in the aquatic environment necessitates the development of sensitive and robust analytical methods for determining these compounds in complex environmental matrixes. Methods based on reversed-phase liquid chromatography with electrospray mass spectrometry are particularly suited to the analysis of NPEO metabolites in water and sediment [11,12]. However, these methods are typically not suitable for the determination of the full range of polyethoxylated NPEOs, as well as NP, in environmental samples. NPEO ethoxymers coelute in reversed-phase HPLC, leading under certain conditions (i.e. when analyzing samples containing a large amount of highly ethoxylated NPEOs) to competitive ionization suppression during the electrospray process [11,12] and isobaric interferences between singly- and doubly-charged NPEO ethoxymers [13] (discussed below). Analysis of the full range of NPEO ethoxymers in the environment is desirable in some situations, as their presence may constitute a source for more toxic NP and NP(1–3)EO, via degradation. Although biological wastewater treatment is typically very effective at removing highly ethoxylated NPEOs ([2], discussed below), waters that receive primarily mechanically treated, or untreated wastewater may be contaminated with highly ethoxylated, undegraded NPEOs [14]. In addition, analysis of total NPEO in sediments that were deposited prior to the advent of widespread secondary wastewater treatment requires the use of a method suitable for determination of highly ethoxylated NPEOs.

Electrospray ionization mass spectrometry is ideally suited for the analysis of NPEO surfactants. The high surface activity of most NPEO ethoxymers, coupled with their high affinity for alkali metal cations, leads to efficient ion formation during the electrospray process with resulting high sensitivity. Detection by mass spectrometry also affords a high degree of specificity. However, for analysis of samples containing a disperse mixture of NPEO ethoxymers, it is necessary to separate NPEOs chromatographically prior to mass analysis in order to avoid electrospray competition effects and isobaric interferences. Previously, this separation was achievable only by normal-phase chromatography [13]. While this approach does allow quantitation of a wide range of NPEOs as well as NP, it has several disadvantages. Firstly, the mobile phase is not directly compatible with electrospray ionization, and a post column split and aqueous make-up flow are necessary to obtain a reasonable ion beam, thereby reducing overall sensitivity [13]. Secondly, the separation is such that NP and NP(1–3)EO are eluted first, with relatively short retention time, and without complete chromatographic resolution. As noted by Crescenzi et al. [11], the less ethoxylated NPEOs are more susceptible to ionization suppression by coelution of other ethoxymers. Also, studies have shown that ionization suppression of analytes due to coextracted matrix interferences is most severe at short retention times [15]. Finally, all previous attempts at measuring NPEOs and NP by HPLC–MS have required two separate chromatographic runs in order to provide comprehensive quantitation of all ethoxymers as well as the phenol [12,13,16].

In the present work, we report on the application of a mixed-mode HPLC separation, coupled with electrospray mass spectrometry (ESI–MS) detection for the comprehensive analysis of NP and NPEO concentrations and distributions in sediment and sewage samples. The mixed-mode separation, which operates with both size-exclusion and reversed-phase mechanisms, allows the resolution of NPEO ethoxymers prior to introduction to the MS using a solvent system that is readily compatible with electrospray. In this method, elution of NPEOs is reversed relative to normal-phase chromatography, with smaller, less ethoxylated compounds, including NP, eluting last. The separation allows all NPEOs and NP to be quantified in a single chromatographic run, while removing the effects of isobaric interferences and co-analyte electrospray competition. A similar chromatographic approach was recently used by Takino et al. [17] to analyze a narrow range (2–6 ethoxy units) of NPEOs in technical surfactants and in spiked water samples. This method was not applied to the full range of NPEOs present in commercial surfactant products, and did not include the environmentally important NPEO metabolite, NP. Also, the method lacked the sensitivity required to determine NPEOs in realistic environmental samples. In the current work, we describe a fully developed analytical approach based on mixed-mode HPLC–ESI–MS for the comprehensive analysis of NP and NPEOs with high sensitivity in actual environmental

matrixes. We also report on the development and use of stable isotope labeled NP and NPEO analogs as surrogate quantitation standards, in order to increase the reliability of the method when analyzing NPEOs in the presence of a complex, potentially interfering environmental matrix.

2. Experimental

2.1. Materials

Technical NP (lot 4252 TK, 99% nonylphenol) and short ethoxy-chain NPEO (Surfonic N-10, lot 7202-96C, characterized for NP(1–3)EO composition) were provided by Dr. Jennifer Field of Oregon State University. A standard blend of commercial NPEO (Huntsman lot number 7427-20) that had been characterized for percent ethoxymer composition by normal-phase HPLC with UV absorbance detection was provided by Dr. Carter Naylor of Huntsman Corporation. This material had an average EO number of 6.3 and was composed of NP and NP(0–15)EO. This NPEO was used as a standard for quantitation of environmental samples. Internal standards were *n*-NP (Lancaster Synthesis, Windham, NH, USA) and *n*-NP3EO (synthesized from *n*-NP as described previously [12]). Surrogate standards were synthetic [$^{13}C_6$]NP and NPEO materials, prepared as described below. [$^{13}C_6$]Phenol (catalog no. CLM-216, isotopic purity=99%) was purchased from Cambridge Isotopes Labs (Andover, MA, USA). A sample of refinery grade nonene was provided by Imperial Oil of Canada. Proprietary strong acid ion-exchange resin was a gift of Schenectady International (Schenectady, NY, USA). Ethylene oxide (99.5+%), glacial acetic acid, and sodium methoxide (0.5 *M* in dry methanol) were purchased from Aldrich (Milwaukee, WI, USA). Solvents (HPLC grade) were purchased from Burdick & Jackson (Muskegon, MI, USA). Water (>18 MΩ) was purified by a Millipore (Bedford, MA, USA) Synthesis grade system. Because of the ubiquitous occurrence of NPEOs in detergents, special precautions had to be followed to avoid sample contamination. No detergent was allowed to contact glassware used in sample preparation or analysis. Sea sand (obtained from Aldrich) and glassware were baked at 450°C in a muffle furnace overnight prior to use. Extraction columns and associated equipment were thoroughly rinsed with methanol prior to use and between samples.

2.2. Synthesis and characterization of [$^{13}C_6$]NP, [$^{13}C_6$]NPEO

The goal of this synthetic work was to prepare [$^{13}C_6$]NP with an isomer distribution closely matching that of a typical commercial grade product, and then to prepare [$^{13}C_6$]NPEO from it by addition of ethylene oxide. Both of these products would then be used as surrogate standards in the present method. Synthesis of [$^{13}C_6$]NP was performed by acid-catalyzed addition of technical nonene to [$^{13}C_6$]phenol. Nonene was first passed over an alumina column to remove butylated hydroxytoluene that had been added as a polymerization inhibitor. Microscale reaction of nonene with 100 mg [$^{13}C_6$]phenol (2:1 nonene–phenol molar ratio) was performed under anhydrous conditions [18] using a proprietary strong acid ion-exchange resin as the acid catalyst. [$^{13}C_6$]NP was purified from the crude reaction product using normal-phase chromatography (5 ml/min isocratic 5% ethanol in hexane on 250×10 mm, 5 μm Supelcosil LC-Diol column, Supelco, Bellefonte, PA, USA). The purity and isomer distribution of the synthetic [$^{13}C_6$]NP was determined using GC with flame ionization detection. Electron ionization MS spectra of the [$^{13}C_6$]NP were obtained using a Varian Saturn 4D ion trap GC–MS system.

A portion (47.4 mg) of the [$^{13}C_6$]NP synthesized as described above was used to prepare a [$^{13}C_6$]NPEO mixture containing predominately short chain ethoxylates. Base-catalyzed addition of ethylene oxide to [$^{13}C_6$]NP was performed as described by Hannah [19] under anhydrous conditions on a specially designed vacuum line using sodium methoxide as the catalyst. The mole ratio of ethylene oxide to [$^{13}C_6$]NP added to the reaction vial was carefully controlled to 1.5:1 in order to obtain the desired ethoxymer distribution of the product [$^{13}C_6$]NPEO. A second batch of [$^{13}C_6$]NPEO was then prepared using an ethylene oxide to [$^{13}C_6$]NP (44.4 mg) ratio of 10:1 in order to obtain a [$^{13}C_6$]NPEO product containing a wide range of

ethoxy chain lengths. Determination of the ethoxymer distributions of these synthetic [$^{13}C_6$]NPEO materials was performed relative to the characterized NPEO standards obtained from Huntsman Corporation and Oregon State University using mixed-mode HPLC as described below, with diode-array UV detection.

2.3. Sample collection

Sediment samples were collected in 1996 by gravity core from a depositional site within Jamaica Bay, on Long Island in New York (USA). A detailed description of the sampling site, as well as the method used for preparing the sliced sediment core for trace organics analysis is published elsewhere [20].

Grab samples (4 l) of influent and effluent were obtained from the Yonkers (New York, USA) municipal sewage treatment plant (MSTP) in October, 1998, and immediately preserved by addition of 1% methylene chloride. Wastewater samples were stored at 4°C until extraction. The Yonkers MSTP serves 500,000 people, with a flow capacity of $549 \cdot 10^6$ l/day. It operates with full secondary biological treatment (conventional activated sludge method).

2.4. Sample preparation

2.4.1. Sediment

NP and NPEOs were extracted from dried sediment samples (0.2 g) by continuous-flow, high temperature sonication, as described previously [12]. Briefly, sediment was packed into extraction columns assembled from empty HPLC columns, and an aliquot (5 μl) of surrogate standard cocktail, containing ~100 ng of each [$^{13}C_6$]NPEO ethoxymer and [$^{13}C_6$]NP, was spiked onto the sediment. The void space in the column was filled with sea sand that had been baked at 450°C overnight, and the columns were capped. Extraction of NP and NPEOs was performed by pumping methanol through the column at a rate of 0.5 ml/min for 10 min while the column was immersed in an ultrasonic bath held at a constant 65°C. The extract (5 ml) was collected in a baked (450°C) glass test tube. Triplicate blanks were prepared by filling the extraction columns with baked sea sand, spiking with the surrogate cocktail, and carrying out the extraction procedure described above.

Extracts were evaporated to dryness at ~30°C under gentle nitrogen flow, and were then reconstituted in 400 μl of methanol. Particulates were removed from sediment and blank extracts by centrifugal filtration using Millipore Ultrafree-MC 0.45 μm Durapore membrane filters (catalog no. UFC30HV00). Filtered extracts were purified by non-aqueous reverse phase HPLC on two 250×4.6 mm Beckman Ultrasphere C_{18} columns connected in series, with a methanol mobile phase flow of 2 ml/min. Aliquots (200 μl) of extracts were injected onto a Shimadzu HPLC system (LC-6A pumps and SPD-6AV UV absorbance detector). Retention times of NP and NPEOs were verified by injection of standards, and a single time window (3.0 to 4.2 min) was identified for collection of the analytes from sample extracts. Collected fractions from sediment and blank extracts were again evaporated to dryness, and reconstituted with 1 ml of methanol–water (50:50). Internal standard (consisting of 50 ng each of *n*-NP and *n*-NP3EO) was added to the reconstituted extracts at this point.

2.4.2. Wastewater samples

NP and NPEOs were extracted from 4-l influent and effluent samples by liquid–liquid extraction (2×) in separatory funnels, using 2×100 ml methylene chloride. The extraction and analysis of these samples were performed prior to the synthesis of [$^{13}C_6$]NPEOs, during the method development process, and consequently, no surrogate standards were available. Extracts were combined, dried over sodium sulfate, and evaporated to 5 ml using a Kuderna–Danish concentrator. Aliquots of the extracts (10 μl for influent, 100 μl for effluent) were evaporated and reconstituted in 1 ml of methanol–water (50:50). Extracts were spiked with internal standards (50 ng each of *n*-NP and *n*-NP3EO) as described above for sediment extracts.

2.5. Instrumental analysis

2.5.1. Chromatography

NP and NPEOs were separated chromatographically prior to ESI–MS detection. Efficient separation was provided by mixed-mode HPLC, using a

Shodex (Japan) MSpak GF-310 4D gel filtration column. This column had dimensions of 150×4.6 mm, and was packed with a cross-linked poly(vinyl alcohol) phase. The manufacturer describes this phase as suitable for 'mixed-solvent' gel filtration, compatible with both normal-phase and reversed-phase solvents. Application notes provided by the manufacturer indicate that columns packed with this phase are capable of size-exclusion-based separation of poly(ethylene glycol) oligomers in the M_r range between ~200 and ~1500. This molecular mass range is similar to that of the NPEO ethoxymers investigated in the current work. As described in the Results, the poly(vinyl alcohol) column packing also exhibited adsorptive retention of NPEOs at some solvent compositions, facilitating the mixed-mode separation. The HPLC system was a Hewlett-Packard 1100 series, with a G1312A binary pump and a G1313A autosampler. Solvent 'A' was water containing 5 μ*M* sodium acetate, and solvent 'B' was methanol, also with 5 μ*M* sodium acetate. The sodium salt was added in order to enhance and stabilize the formation of sodium adducts of NPEOs during the electrospray process. The operating conditions of the HPLC, including the mobile phase gradient, are shown in Table 1. The run time for the total analysis was 55 min, including a water-wash step (which removed residual salts that otherwise led to ESI baseline instability in subsequent runs) and a column equilibration step between samples. The entire column eluent was directed to the ESI–MS system for analysis.

Table 1
Instrument parameters

HPLC parameters		
Flow rate	0.2 ml/min	
Column temperature	60°C	
Injection volume	15 μl	
Gradient timetable		
Time (min)	% A	% B
0	50	50
22.7	0	100
32.7	0	100
38	100	0
40	100	0
45	50	50
55	50	50
Electrospray MS parameters		
Desolvation gas flow	~550 l/h	
Desolvation gas temperature	250°C	
Source temperature	120°C	
Capillary (positive ion)	2.83 kV	
SIM dwell (positive ion)	0.08 s	
Capillary (negative ion)	−2.48 kV	
SIM dwell (negative ion)	0.2 s	

2.5.2. Electrospray mass spectrometry

The mass spectrometer used in the present study was a Platform LCZ single quadrupole instrument manufactured by Micromass (Manchester, UK). This instrument was fitted with the Z-Spray ion source, which allows high sensitivity and robust operation of the mass spectrometer at relatively high flow-rates (0.2 ml/min) by sampling the electrospray plume in an orthogonal configuration. NPEOs, [$^{13}C_6$]NPEOs, and *n*-NP3EO were detected by selected ion monitoring of the corresponding $(M+Na)^+$ ions in positive ion mode. The polarity of the instrument was switched to negative ion mode after the NPEOs had eluted (at 25.8 min), and the later eluting NP, [$^{13}C_6$]NP, and *n*-NP were detected during the same chromatographic run by selected ion monitoring of the appropriate $(M-H)^-$ ions. Electrospray MS operating conditions are shown in Table 1 for both positive and negative ion detection. The specific ions monitored and the appropriate cone voltages for each ion are discussed in the Results.

2.6. Calibration and quantitation

Five point quantitative calibration series ($r^2 > 0.999$) were prepared in methanol–water (50:50) for the analytes and surrogate standards. Concentrations of individual NP(*n*)EO (where $n=0$–15) ethoxymers in the calibration solutions varied, due to the necessity of preparing these solutions from standardized mixtures of NPEO ethoxymers. The concentrations in the low calibration point ranged from 1.5 ng/ml (NP15EO) to 15 ng/ml (NP1EO), and in the highest concentration calibration solution, the levels ranged from 93 ng/ml (NP15EO) to 910 ng/ml (NP1EO). Internal standards were present at 50 ng/ml in all five calibration solutions.

Surrogate standard compounds (the $^{13}C_6$-labeled NP and NPEOs) were quantified in sediment samples and sediment blanks relative to response of *n*-NP and *n*-NP3EO internal standards, in order to check recoveries and to monitor the effects of matrix-

induced ionization modulation on the analyte signals. Analytes were quantified relative to their respective coeluting $^{13}C_6$-labeled analog surrogate standard, with the exception of NP2EO. This compound's surrogate standard ([$^{13}C_6$]NP2EO) had a severe isobaric interference in sediment samples; therefore, it was quantified relative to [$^{13}C_6$]NP3EO, its nearest chromatographic neighbor. NP and NPEO in sewage samples were quantified relative to the internal standards added just prior to analysis, and therefore were not corrected for recovery or matrix-related effects in the electrospray process. In all cases, samples which contained analyte concentrations above the linear dynamic range of the instrument (which corresponded to the chosen calibration range for most compounds) were diluted sufficiently and then re-analyzed.

3. Results and discussion

3.1. [$^{13}C_6$]NP, [$^{13}C_6$]NPEO synthesis

The chemical purity of the synthetic [$^{13}C_6$]NP material was found to be >98% by GC–flame ionization detection (FID) response after normal-phase HPLC purification. The purified product was free of residual [$^{13}C_6$]phenol and nonene. The nonyl-chain branched isomer distribution of synthetic [$^{13}C_6$]NP closely matched that of the standard NP material, as evidenced by very similar GC–FID peak patterns. The synthetic [$^{13}C_6$]NP was further characterized by electron ionization mass spectrometry. The mass spectrum of the synthetic material was very similar to that obtained from standard NP, with a corresponding shift of peak m/z values by six due to the incorporation of six ^{13}C atoms. The synthetic product therefore provided an ideal standard for mass spectrometric quantitation of NP by stable isotope dilution [21], as it coelutes with the technical NP under all conditions, unlike the commercially available [$^{13}C_6$]*n*-NP. The latter material has been found to separate chromatographically from technical NP by GC and by the mixed-mode separation in the present study, as well as by reversed-phase HPLC in a previous work [12]. The synthetic [$^{13}C_6$]NP also provided the starting material for synthesis of [$^{13}C_6$]NPEOs to be used as surrogate standards for NPEO quantitation.

The ethoxylation of [$^{13}C_6$]NP produced mixtures containing [$^{13}C_6$]NPEOs with a range of ethoxy chain lengths. Characterization of these materials for % molar ethoxymer composition by mixed-mode HPLC with UV detection allowed the raw products to be used as quantitative standards. The two batches of [$^{13}C_6$]NPEO, synthesized with different average numbers of ethoxy groups (average values and molar % compositions shown in Table 2), were blended as needed with [$^{13}C_6$]NP to produce suitable surrogate standard cocktails. The blends were constructed carefully to include individual ethoxymers at similar concentrations. These blends were then used for sediment spiking and in calibration standard solutions. Analysis of the synthesized [$^{13}C_6$]NPEO product materials by ESI–MS showed that the major impurities were polyethylene glycol monomethyl ether and sodium acetate. Neither of these impurities affected the performance of the [$^{13}C_6$]NPEO material as a surrogate standard as they were unretained by the analytical column. Mixed-mode HPLC–ESI–MS experiments confirmed that the synthetic [$^{13}C_6$]NP and [$^{13}C_6$]NPEO material coeluted exactly with technical NP and NPEO analytes.

3.2. Chromatographic separation

As shown in Fig. 1, NP and NPEOs are efficiently

Table 2
Ethoxymer distribution of synthetic [$^{13}C_6$]NPEO standards

Compound	Mol.% composition	
	Avg. EO = 1.6	Avg. EO = 9.5
[$^{13}C_6$]NP	0.15	0.13
[$^{13}C_6$]NP1EO	51.28	0.00
[$^{13}C_6$]NP2EO	38.91	0.09
[$^{13}C_6$]NP3EO	8.10	0.89
[$^{13}C_6$]NP4EO	1.21	2.70
[$^{13}C_6$]NP5EO	0.24	5.06
[$^{13}C_6$]NP6EO	0.12	7.47
[$^{13}C_6$]NP7EO	–	10.04
[$^{13}C_6$]NP8EO	–	11.79
[$^{13}C_6$]NP9EO	–	12.68
[$^{13}C_6$]NP10EO	–	12.16
[$^{13}C_6$]NP11EO	–	11.07
[$^{13}C_6$]NP12EO	–	8.64
[$^{13}C_6$]NP13EO	–	6.62
[$^{13}C_6$]NP14EO	–	4.73
[$^{13}C_6$]NP15EO	–	3.04

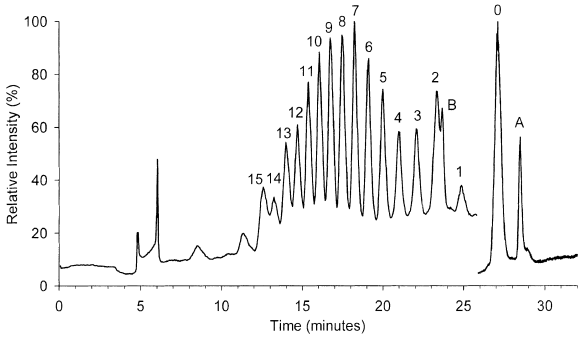

Fig. 1. Mixed-mode HPLC–ESI–MS summed ion chromatogram of a sediment extract (32–36 cm depth core slice) showing resolution of NPEOs and NPs. Numbered peaks correspond to NPEOs and [$^{13}C_6$]NPEOs with the indicated number of ethoxy groups (0 = NP, [$^{13}C_6$]NP; 1 = NP1EO, [$^{13}C_6$]NP1EO; etc.). Peaks 'A' and 'B' are the internal standards, *n*-NP and *n*-NP3EO, respectively. Note the discontinuity at retention time 25.8 min, corresponding to the shift in MS polarity from positive to negative ion mode.

separated and analyzed in a single chromatographic run by the gradient conditions employed in the mixed-mode HPLC–MS analysis. Highly ethoxylated, larger NPEOs elute first, closest to the void volume, while NP is the last analyte to elute. The internal standards, *n*-NP3EO and *n*-NP, are retained longer than their branched-isomer counterparts. Retention times for all analytes were very reproducible between runs (retention times generally varied by $<1\%$). This was likely due to careful temperature control of the column and sufficient equilibration of the polymeric phase between injections. It should be noted that the NPEO and NP peaks shown in the chromatogram from a sediment extract (Fig. 1) represent contribution from both the analyte species (unlabeled), and the isotopically labeled synthetic surrogate species used for isotope-dilution quantification.

The improvement in peak shape and resolution obtained for NPEOs through the use of gradient elution conditions on a mixed-solvent gel filtration column in the present work indicates that the mode of separation was likely mixed, with retention based on both size-exclusion and adsorption. We found significant adsorptive retention of NP and the less highly ethoxylated NPEOs by the polyvinyl alcohol phase in the column under isocratic conditions with a mobile phase consisting of less than 60% methanol. This retention was reduced by increasing the organic strength of the mobile phase. A separation based solely on the size-exclusion mechanism should not be affected greatly by changing the elution solvent. Therefore, the mode of separation reported here, under methanol–water gradient conditions, is probably best described as mixed-mode with both size-exclusion and reversed-phase adsorption mechanisms operating.

As mentioned previously, when analyzing highly ethoxylated mixtures of NPEOs in the environment it is necessary to separate the ethoxymers chromatographically prior to ESI–MS analysis for several reasons. The competitive ionization suppression effect caused by coelution of numerous highly ethoxylated NPEOs in reversed-phase HPLC–ESI–MS has been discussed by Crescenzi et al. [11]. Another important reason to separate NPEO ethoxymers prior to ESI–MS analysis is to avoid the isobaric interference which exists between doubly charged ions of highly ethoxylated NPEOs with odd numbers of ethoxy groups and singly charged ions of less ethoxylated NPEOs [12,13]. An example of this effect is the pair: NP15EO and NP5EO. NP15EO forms a singly charged sodium adduct ion at $m/z = 903$, and a doubly charged, disodiated ion at nominal $m/z = 463$. The singly charged $(\mathrm{NP5EO} + \mathrm{Na})^+$ ion also has a nominal m/z of 463. Obviously, if NP5EO and NP15EO coeluted, as in reversed-phase HPLC, the quantitative determination of NP5EO by ESI–MS would be compromised in the presence of NP15EO, due to the contribution from the latter compound's doubly charged ion. It should be noted that this situation is not alleviated by the choice of an alternate adduct ion, such as lithium or ammonium; the use of these ions produces the same interferences, with the mass scale correspondingly shifted. It may be possible to alleviate this isobaric interference by the use of high resolution MS ($R > 20{,}000$ would be required), but in that case, the competitive ionization effect would remain. Therefore, the separation provided by the current method provides a good choice for alleviating artifacts in the ESI–MS analysis of NP and NPEOs.

3.3. Mass spectrometry

Sodium adducts were found to be a good choice

for quantitative analysis of NPEO [11–13]. Addition of a low concentration of Na^+ ions to the mobile phase served to stabilize the adduct ion signals in positive ion mode, and did not adversely affect sensitivity for detection of $(M-H)^-$ phenolate ions in negative ion mode. Significant enhancements in both sensitivity and chromatographic peak shape were obtained by using selected ion monitoring (SIM) of analyte, surrogate, and internal standard ions in both positive and negative ion mode. The analyte ions monitored by SIM are shown in Table 3. The choice of *n*-NP and *n*-NP3EO as internal standards helped to reduce the number of SIM channels monitored, as these compounds gave ions with the same m/z as their branched nonyl-chain analyte counterparts.

In general, NPEO ESI–MS response in positive ion mode was greatly dependent on both the length of the ethoxy chain and the applied cone voltage in the ESI source. Longer ethoxy chains have a higher native affinity for alkali metal ions [22], and this explains the enhanced sensitivity observed at a given cone voltage in the present method for NPEOs with ethoxy chain lengths >3 (Fig. 2). At relatively low source cone voltage settings, the maximum ESI–MS response was obtained for NP6EO. The decrease in response with greater ethoxy chain length was likely due to a combination of the greater formation of doubly sodiated ions by these ethoxymers (due to their higher capacity for binding), as well as the decrease in transmission of higher mass ions by the

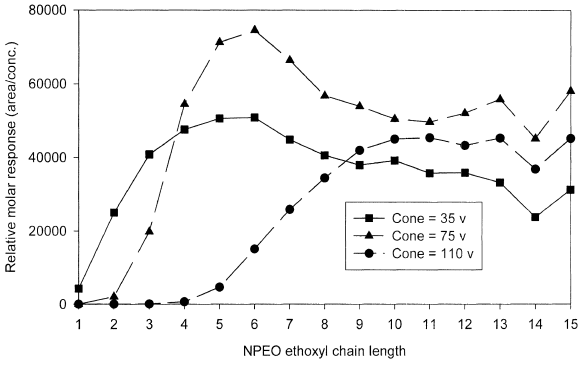

Fig. 2. Relative molar responses of individual NPEO ethoxymers at three different static ESI source cone potential settings. In mixed-mode HPLC–ESI–MS analysis of NPEOs, cone potentials were programmed to the optimum value for each SIM mass monitored (see Table 3 for values).

Table 3
Method validation parameters

Compound	Cone (V)	SIM mass	Apparent % recovery (SD)[b]	Sediment concentration (ng/g) (RSD, %)[c]	MDL (ng/g)[a]
NP	−42	219	95 (14)	4120 (1.4)	21.5
NP1EO	35	287	64 (7)	7290 (0.8)	37.3
NP2EO	35	331	n.d.[d]	1200 (4.4)	2.73
NP3EO	39	375	123 (13)	567 (5.0)	2.90
NP4EO	50	419	121 (7)	372 (9.1)	1.60
NP5EO	62	463	123 (9)	265 (8.4)	0.78
NP6EO	60	507	124 (13)	276 (4.2)	0.96
NP7EO	78	551	124 (18)	279 (6.0)	0.86
NP8EO	83	595	123 (19)	283 (3.8)	1.16
NP9EO	94	639	125 (23)	270 (1.8)	1.24
NP10EO	94	683	127 (25)	264 (2.9)	1.67
NP11EO	95	727	123 (25)	266 (0.9)	1.99
NP12EO	100	771	118 (23)	265 (2.7)	2.17
NP13EO	100	815	115 (23)	264 (3.9)	4.96
NP14EO	105	859	110 (30)	261 (1.6)	3.70
NP15EO	105	903	107 (24)	248 (2.4)	3.40

[a] Calculated as the concentration giving a peak with $S/N = 3$.
[b] Based on recovery of $^{13}C_6$-labeled surrogate standards, $n = 18$.
[c] Triplicate measurement.
[d] Not determined.

quadrupole analyzer. At much higher cone voltage (110 V), the response of NPEOs with ethoxy chain lengths less than nine was greatly reduced (Fig. 2). This was likely the result of collision-induced dissociation of the noncovalent sodium-NPEO adduct species, leading to formation of sodium ions. The fact that this effect was most pronounced for the less ethoxylated NPEOs is likely related to their lower binding affinity for alkali metal cations, mentioned previously. At intermediate cone potential (75 V), the response of NPEOs with ethoxy chain lengths greater than three was enhanced. This increase in ESI–MS response of NPEOs at intermediate (non-fragmenting) potentials was very likely due to the focusing effect which occurs after the free-jet expansion region behind the sampling cone and before the extractor cone in the first stage of ESI source pumping [23]. This mechanism has been shown to have a dramatic effect on the observed distributions of sodiated polyethylene glycol ions in ESI mass spectra, with higher mass ions requiring higher potentials for optimum transmission [23]. In any case, in the present study it was possible to perform infusion of the analytes in solution into the ESI–MS system, and by modulating the cone voltage iteratively, to choose the specific cone voltage which gave optimum sensitivity for individual NPEO ethoxymers. These values are given in Table 3. Optimized cone voltage values increase with increasing NPEO ethoxy chain length, consistent with the observation that higher potentials are necessary to focus higher mass, singly charged ions through the CID region of an ESI source [23].

3.4. Analytical figures of merit

The current method was evaluated for recovery, precision, and method detection limits using actual sediment samples. Recovery was calculated for the [$^{13}C_6$]NP and [$^{13}C_6$]NPEO synthetic surrogate standards, relative to the internal standards *n*-NP and *n*-NP3EO. A total of 18 sediment samples were extracted, and the % recovery data presented in Table 3 represent the mean and standard deviation of individual surrogate recoveries from these separate determinations. Note that no recovery data are available for [$^{13}C_6$]NP2EO, since this compound was obstructed by a severe isobaric interference in the analysis of all sediment extracts.

The percent recovery data reported in Table 3 represent both actual recovery of the surrogates from the 18 individual samples and the effects of coextracted matrix interferences on the ESI–MS response of the individual surrogate compounds. In this case, it is likely that matrix-induced ionization suppression [12,15] and enhancement [24] of surrogate compounds did occur during analysis. The enhancement effect is evident from the greater than 100% recoveries obtained for NPEOs with ethoxy chain lengths greater than three. This effect has been observed by Furlong et al. [24] where, in the analysis of herbicides in environmental samples by ESI–MS, apparent recoveries were systematically greater than 100% for several analytes. It is not possible to definitively conclude what the actual surrogate recoveries were in the present case, but the apparent recovery for [$^{13}C_6$]NP3EO (123%) can be compared with that obtained for the [$^{13}C_6$]*n*-NP3EO during previous work in our laboratory using a similar extraction and purification method, with reversed-phase HPLC–ESI–MS detection [12]. Using that method, which removed the effect of matrix-induced ionization suppression of the surrogate by normalization to a coeluting internal standard, a [$^{13}C_6$]*n*-NP3EO recovery of 80.4% from sediment was obtained [12]. Assuming that the actual recovery of the [$^{13}C_6$]NP3EO in the present study was similar to that obtained previously for [$^{13}C_6$]*n*-NP3EO, and that the degree of ionization enhancement in the present work was similar for all of the [$^{13}C_6$]NPEOs with ethoxy chains longer than three, a range of actual recoveries of 70.0–83.0% can be calculated for [$^{13}C_6$]NP(3–15)EO. The low recovery of [$^{13}C_6$]NP1EO obtained in the present work (64%, Table 3) was likely due to ionization suppression, as the recovery of [$^{13}C_6$]*n*-NP1EO was previously reported as 93.5% from sediment in our laboratory using similar sample preparation methods [12]. [$^{13}C_6$]NP shows no clear evidence of either matrix-induced suppression or enhancement in the current study (recovery = 95%, Table 3).

Interestingly, the variability in surrogate recoveries from sediments ($n = 18$), shown by the standard deviations in Table 3, is rather high (greater than 10% in most cases) and seems to increase with

increased retention distance from the internal standards (*n*-NP3EO, which eluted closest to [$^{13}C_6$]NP2EO, and *n*-NP, which eluted after [$^{13}C_6$]NP, see Fig. 1). This supports the conclusion that the recovery data reported in the present work were impacted by effects on analyte ESI–MS response due to coextracted matrix interferences in the samples. These effects were not identical from sample to sample, and would thus be expected to vary according to retention time and sample composition. Hence, effects on individual surrogate compounds would best be accounted for by quantitation relative to a closely eluting internal standard. The variability in surrogate recoveries and the evidence for ionization suppression and enhancement also illustrate the value of using the isotope dilution technique for quantitation of the NP and NPEO analytes in the present work. Since the analytes are quantified relative to their respective coeluting $^{13}C_6$-labeled surrogate analog, any effects such as reduced sample recovery during sample preparation or matrix-induced signal instability during the electrospray process are accounted for in the quantitation calculations, since these effects should impact the surrogate and analyte equally.

Overall precision of the analytical technique was evaluated by extraction and analysis of triplicate samples of a single sediment core depth horizon (28–32 cm). The resulting concentrations and associated relative standard deviations (RSDs) are shown in Table 3. In general, quantitative precision for NP and NPEO determination was excellent, with RSDs below 5% for most analytes, and below 10% in all cases. As stated above, this high precision was likely due to the use of isotopically labeled surrogate standards for NP and NPEO quantitation. Sediment NP and NPEO concentrations reported in Table 3 are reflective of a relatively degraded NPEO mixture, with high levels of the metabolites NP, NP1EO, and NP2EO [2]. This degradation may have occurred during wastewater treatment before the NPEOs were discharged to the environment, or by diagenetic processes after sediment deposition.

Method detection limits (MDLs, Table 3) for NPEOs in sediment samples were calculated as the concentration of analyte in a sediment that would give a signal-to-noise ratio of three in the mixed-mode HPLC–ESI–MS analysis. MDLs were consistent with the instrumental response of individual NPEOs shown in Fig. 2. Greatest sensitivity was obtained for NP(5–7)EO, with detection limits below 1 ng/g. NP and NP1EO were detected with somewhat reduced sensitivity (MDLs$>$20 ng/g). These MDLs represent mass sensitivities for NP and NPEOs between 1 and 55 pg injected into the ESI–MS system. The current method therefore provides the highest available sensitivity for comprehensive NP and NPEO analysis in environmental samples. This sensitivity was more than sufficient for the determination of NP and NPEOs in sewage impacted sediments (concentrations in Table 3). The high mass sensitivity obtained for the analytes in the current study was likely due to a combination of the use of orthogonal ESI ion source geometry in the instrumentation and the utilization of an ESI-friendly mobile phase (methanol and water) in the separation. Levels of NP and NPEO in sediment blanks were in all cases below the MDLs ($n=3$).

3.5. Concentrations and ethoxymer distributions of NP and NPEOs in sediment and wastewater samples

As noted previously, a total of 18 discrete samples from the collected sediment core were extracted and analyzed for NP and NPEOs using the current method. Presentation and discussion of the complete data set is beyond the scope of this work; therefore, we will focus on a comparison of the concentrations of NP and NPEOs found in surface (0–2 cm) sediments with those in a typical wastewater influent and effluent. It is useful to examine the changes in NPEO ethoxymer distributions and concentrations between sewage influent, which has not been subjected to mechanical and biological wastewater treatment, and plant effluent, discharged after treatment. Changes in the concentrations and distributions of NPEOs should reflect the removal efficiency of these compounds by the wastewater treatment process, as well as the degradation processes that may have occurred during the treatment. Also, a comparison of the concentrations and distributions of NP and NPEOs in wastewater effluent (discharged to receiving waters) and sediments, which are important repositories for these relatively particle reactive contaminants in the aquatic environment, should

provide insight into the processes (such as sorption or further biodegradation) impacting NP and NPEOs after discharge.

The concentrations of summed NP and NPEO ethoxymers decreased markedly from sewage influent to effluent (Table 4). A percent removal of 93% can be calculated for NPEOs based on these values, indicating that the combined mechanical treatment and activated sludge biological treatment process used in the wastewater treatment plant (WWTP) from which the samples were taken was relatively effective in removing bulk NPEO loading from the influent stream. These results are similar to those reported by Ahel et al. [2] for removal of NPEOs by biological sewage treatment in Switzerland. Also, as reported by Ahel et al. [2], there was a marked shift in the distribution of NPEO ethoxymers between influent and effluent. NPEO distributions in influent were similar to those in typical commercial NPEO surfactant formulations [2,14], with a Poisson distribution of ethoxymers centered around ~9–10 ethoxy units. In effluent, NPEO distributions were dominated by NP, NP1EO, NP2EO, and NP3EO, with NP2EO contributing nearly 50% of the total mass concentration of NPEOs (Table 4). NPEOs with ethoxy chain lengths greater than 5 are nearly absent from the biologically-treated effluent. This result is also consistent with previous reports in the literature [2], and reflects the mechanism of NPEO biodegradation by ethoxy chain shortening during biological wastewater treatment. It should be noted that other NPEO biodegradation products such as the carboxylated [2,25] and dicarboxylated [26] NPEOs may be formed during aerobic biological wastewater treatment [27], but these compounds were not quantified in the present study.

A comparison of the relative distribution of NP and NPEO ethoxymers between sewage effluent and sediment (Table 4) reveals that sediment is greatly enriched in NP and NP1EO. The site from which the sediment sample was taken for the current work does not receive wastewater effluent from the specific WWTP for which influent and effluent NPEO concentrations were determined; however, it does receive substantial input of sewage from a WWTP of similar flow capacity ($378 \cdot 10^6$ l/day) and treatment regime (activated sludge process) as the one studied here. The increase in NP and NP1EO contribution to the summed NPEO concentration in sediment, relative to effluent, is likely due to a combination of increased sorption of these hydrophobic NPEO metabolites onto suspended particulates after waste-

Table 4
Relative distributions and summed concentrations of NP and NPEO ethoxymers in wastewater influent, effluent, and surface (0–2 cm) sediment extracts

Compound	Fraction of total NPEO (%)		
	Sediment	Effluent	Influent
NP	53.42	7.21	4.64
NP1EO	36.39	18.89	3.75
NP2EO	4.63	50.22	2.95
NP3EO	0.84	10.43	1.76
NP4EO	0.79	6.05	2.97
NP5EO	0.48	4.94	4.94
NP6EO	0.41	0.77	6.91
NP7EO	0.36	0.46	8.25
NP8EO	0.40	0.26	10.52
NP9EO	0.36	0.21	10.83
NP10EO	0.34	0.23	11.41
NP11EO	0.36	0.17	9.89
NP12EO	0.35	0.15	7.95
NP13EO	0.32	0.00	6.01
NP14EO	0.29	0.00	4.14
NP15EO	0.25	0.00	3.07
Total NPEO concentration	41.4 μg/g	100.9 μg/l	1434 μg/l

water discharge, and further degradation of the NPEO mixture by bacteria in the water column and within the bedded sediment [28]. This degradation may have occurred during transport of the NPEO material through the water column, while sorbed to suspended particles, or after the suspended particles bearing the NPEO became incorporated into the sediment bed. In any case, it is clear from the data presented in Table 4 that, although biological wastewater treatment is generally effective at removing bulk NPEO contamination from sewage, the effect of the treatment is to increase the proportion of the more toxic [5,29] and bioaccumulative [30] NP and NP(0–3)EO in the effluent. Post-discharge degradation of NPEOs and subsequent sorption of the hydrophobic metabolites onto suspended particulates leads to a build-up of these contaminants in organic-rich sediments. In this case, surface sediment was highly contaminated with NPEO metabolites, consistent with previous reports for this estuarine environment [28]. The measured level of NP (>20 $\mu g/g$) in this sediment approaches levels that could cause acute toxicity to benthic organisms [29].

4. Conclusion

Mixed-mode HPLC–ESI–MS as described in the present work provides high sensitivity, selectivity, and precision for the analysis of NP and NPEOs in complex environmental samples such as sediment and wastewaters. The method is most useful for analysis of environmental samples having large amounts of highly ethoxylated NPEOs. The use of specially synthesized, isotopically labeled NP and NPEOs as surrogate standards for quantitation increases the reliability of the method and helps to eliminate impacts of matrix-induced ionization suppression and enhancement on analyte quantitation. The method is the first, based on mass spectrometric detection, to allow simultaneous determination of NP as well as the full range of NPEO ethoxymers in a single chromatographic run. This is possible because of the highly efficient separation provided by the mixed-solvent gel filtration column operated under gradient elution conditions at elevated temperature, coupled with the ability to switch the detection polarity of the mass spectrometer during the chromatographic analysis. The separation of NPEOs by ethoxy chain length also eliminates analytical artifacts caused by isobaric interferences and co-analyte electrospray competition among the various ethoxymers. Individual NPEO ethoxymer ESI–MS response is highly dependent on the ethoxy chain length and the cone voltage applied to the ESI interface of the mass spectrometer. The utility of the method for evaluating the concentrations and distributions of NP and NPEOs in sediments and wastewater has been demonstrated. With appropriate modification of sample preparation techniques, the current method will find additional application in the analysis of NPEOs and their neutral metabolites in dilute surface and groundwaters that have been impacted by discharge of treated or untreated municipal wastewater, as well in biological tissues of organisms exposed to NPEO-contaminated media.

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