Quantitative determination of nonylphenol polyethoxylate surfactants in marine sediment using normal-phase liquid chromatography–electrospray mass spectrometry

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Abstract

A new comprehensive analytical method based on normal-phase liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) has been developed for the quantitative determination of individual nonylphenol ethoxylate (NPEO) surfactants in complex environmental matrices. Clean-up of sample extracts was performed on cyanopropyl silica solid-phase extraction cartridges. Complete NPEO oligomer separation was achieved by using normal-phase LC. Because the non-polar solvents used in normal-phase LC are incompatible with ESI, unique LC–ESI-MS interface conditions were adopted that provided a functional interface and also enhanced the detection response of NPEOs. These provided enhanced ESI signal intensity and stability and facilitated the detection of NPEOs as sodium adducts at parts-per-billion concentration levels. The overall analytical method was validated for accuracy and precision by analyzing sediment samples spiked with known amounts of NPEOs. The method is superior to those currently used for NPEO analysis (LC–UV, LC–fluorescence, LC–thermospray-MS, LC–field desorption-MS, LC–particle beam-MS and GC–MS) in terms of detection limits, specificity and speed of analysis. The validated method was successfully applied to determine levels of NPEOs in sediments from the Strait of Georgia, British Columbia. This work also demonstrates that by proper selection of normal-phase LC–ESI-MS interface conditions this technique is capable of solving separation problems which are not amenable with reversed-phase LC–ESI-MS. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Alkylphenol ethoxylates (APEs) are non-ionic surfactants used widely in industrial and domestic cleaning products, paints, herbicides, pesticides, pulp and paper production, textile manufacturing and in various household products. Much of the production, reported globally to exceed 0.3 metric tons [1], goes ''down the drain'' to enter the environment. APEs and their breakdown products, the alkylphenols (APs), bioaccumulate [2] and are toxic [3–6]. The main environmental concern, however, is not their toxicity but rather the estrogenic potential of APEs which can manifest itself at much lower concen-

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trations [7,8]. The most commonly used APE is nonylphenol ethoxylate (NPnEO), wherein a nonyl group opposes a chain containing n ethoxylate groups ($n=1$ to 20 or more). Upon entering the environment, the APE mixture is subject to breakdown, forming APEs of low ethoxylate numbers and APs [9]. Being surfactants, they attach strongly to particles and tend to accumulate in sediments [10,11].

Separation and quantitation of individual APE oligomers is required to determine the fate of these compounds in the environment. Likewise, the correct assessment of risk requires such information because the aquatic toxicity varies with ethoxy chain length as well as with length and branching of the alkyl chain [12]. The occurrence of APs and APEs at low concentrations together with the complex matrix interference often present in environmental media means that highly sensitive and specific analytical methods are needed for their determination. To date, several analytical schemes have been developed [13,14], usually employing either gas chromatography–mass spectroscopy (GC–MS) or liquid chromatography (LC) with various detectors. Although GC–MS gives excellent sensitivity for low oligomer NPEOs [15] its applicability has been limited to NPEO compounds with less than five EO units [16,17] due to the high polarity, low volatility and thermal instability associated with the higher oligomers. Normal-phase LC with UV detection (LC–UV) [18,19] or with fluorescence detection (LC–FL) [20] is not prone to these limitations. However, these techniques lack the specificity required for the determination of individual NPEO oligomers in environmental samples. Ahel and Giger [18] discussed other drawbacks, such as lower separation efficiencies compared to high-resolution GC techniques and the inability to recognize homologous APEO compounds in environmental samples which may contain a mixture of octyl-, decyl- and nonylphenol polyethoxylates.

Despite a large body of environmental data for APEOs [13,14,23], there remain controversies on the quality of analytical data [11,18], most of which can be attributed to the methodology problems outlined above. What is required, therefore, is a highly specific and sensitive analytical method that provides accurate and precise data for the determination of individual NPEO oligomers. This is achieved by utilizing normal-phase high-performance liquid chromatography (HPLC)–MS instrumentation with an electrospray ionization interface. Normal-phase HPLC provides individual NPEO separation and MS provides the high specificity and sensitivity required for the determination of these compounds in complex environmental samples.

Electrospray ionization (ESI) works best with polar solvents and provides good sensitivity for polar and ionic analytes. Depending on the solution composition and the ionization mode (positive or negative polarity) chosen, protonated, deprotonated and adduct (such as alkali ion attachment) molecular ions are produced. Most LC–ESI-MS applications are focused on the analysis of large biomolecules or pharmaceutical compounds where reversed-phase LC is predominantly used as the separation method primarily due to the inherent compatibility of the mobile phase of the LC system with ESI. Popenoe et al. [21] and Crescenzi et al. [22] have demonstrated that reversed-phase LC–ESI-MS can be used to determine certain non-ionic surfactants in environmental samples. However, when reversed-phase LC–ESI-MS was used to determine NPEOs in water [22], individual NPEO oligomers co-eluted under the chosen conditions, compromising the specificity of the technique. In this case, the non-ionic surfactants examined were separated only as polymer classes and not as individual oligomers. Furthermore, NP, NP1EO and NP2EO, the most important metabolites of NPEO biodegradation process, could not be detected under the given conditions.

Here, we present a new analytical method based on normal-phase LC–ESI-MS for the quantitative determination of trace concentrations of individual oligomers of NP and NPEOs in marine sediments. In this paper we describe: (a) the method of coupling normal-phase LC to ESI-MS; (b) procedures to fully separate NP and all the NPEO oligomers ($n=1$ to 19) using normal-phase LC–ESI-MS; (c) performance characteristics (precision/accuracy) and validation of the overall analytical method and (d) analytical results for NP and NPEO oligomer distributions in Strait of Georgia sediments near a municipal outfall.

2. Experimental

2.1. Preparation of quantitation standards

Commercial products of NPEOs are unsuitable as quantitation standards because they are variable, poorly defined mixtures of NPEO compounds containing decyl-, nonyl- and octylphenol ethoxylates, assorted alkyl-group branching plus many impurities [16,24]. NP standard was, therefore, prepared by purifying technical grade NP using LC. The NPEOs used for both quantitative standards and spiking were blends of Surfonic N-100 from Huntsman (Austin, TX, USA), laboratory-synthesized NP1EO and NP2EO and purified commercial product NP. We chose N-100 because it contains most of the NPEO species of interest ($2 < n_{EO} < 20$). However, N-100 contains only very small amounts of the mono- and diethoxylates of 4-nonylphenol, the two most important breakdown products of NPEOs in the environment, and authentic standards for these were prepared in the laboratory. These were synthesized by reacting NP with 2-chloroethanol and 2-(2-chloroethoxy)ethanol in the presence of KOH and DMSO following classical organic synthesis procedures. The synthesized NP1EO and NP2EO were isolated from the reactant products by utilizing the liquid chromatographic separation method reported by Wahlberg et al. [16]. The purified synthetic and separated commercial products were characterized for molar distributions of n_{EO} by HPLC–UV based on a modified method of Ahel and Giger [18] and each homologue was confirmed by flow injection ESI-MS under the conditions described below. The determined molar distribution of N-100 agreed well with the manufacturer's specifications [30]. The homologue groups were then each dissolved in 10 ml of acetone to be used as reference standards containing known amounts of NP and NPEOs. A blend of N-100, NP, NP1EO and NP2EO, prepared for method and instrument calibration (Table 1), was stored in an airtight container at 4°C in the dark and checked before use by HPLC–UV to confirm the molar distributions. The stock solution was prepared in ethyl acetate at a concentration of 100 mg/ml. The spiking solution was made by dilution of the stock solution in a binary solution of MeOH–acetone

Table 1
Composition of the NPEO stock solution

Compound	% (w/w)[a]	Compound	% (w/w)[a]
NP	8.3	NP10EO	9.2
NP1EO	6.2	NP11EO	9.0
NP2EO	7.5	NP12EO	7.3
NP3EO	1.4	NP13EO	5.5
NP4EO	2.8	NP14EO	3.8
NP5EO	3.2	NP15EO	2.4
NP6EO	4.6	NP16EO	1.6
NP7EO	6.8	NP17EO	0.9
NP8EO	8.8	NP18EO	0.5
NP9EO	9.8	NP19EO	0.4

[a] The weight percents were calculated from the HPLC–UV n_{EO} concentration determinations of N-100, NP, NP1EO and NP2EO. Each fraction was collected and checked by FI-ESI-MS analysis. The composition of the sulfonic N-100 standard matched closely that provided by the manufacturer.

(70:30, v/v) with NPEOs at concentrations of 1 μg/ml.

2.2. Reagents and chemicals

Acetone, *n*-hexane, toluene, CH_2Cl_2 (dichloromethane; DCM), methanol (MeOH), isopropanol (IPA) and acetonitrile (ACN) were HPLC grade from Mallinckrodt; dimethyl sulfoxide (DMSO, reagent grade) was from Aldrich; 2-chloroethanol and 2-(2-chloroethoxy)ethanol (reagent grade) were from Lancaster. Potassium hydroxide (reagent grade) and sodium acetate (NaOAc, analytical reagent grade) were from Fisher Scientific and BDH, respectively. The internal standard (I.S.), 4-fluoro-4′-hydroxylbenzophenone (97%) from Aldrich, was also used to optimize the ESI-MS daily for maximum signal intensity and stability. All of the above solvents were used without further purification. Sodium sulfate (analytical reagent grade, Mallinckrodt) was baked at 450°C overnight and stored at 110°C before use. The water used was double Milli-Q filtered (Millipore). All chemicals were tested for background levels for the compounds of interest.

Large reservoir capacity (LRC) diol, cyanopropyl (CN), silica and aminopropyl silica (NH_2) solid-phase extraction (SPE) cartridges (500 mg stationary phase, Varian) were used to clean up the extract from the samples. Fine granulated copper (analytical-re-

agent grade, Mallinckrodt) was activated by rinsing it consecutively with 1 *M* HCl, deionized (d.i.) water, acetone and hexane before use. Neutral silica gel (100–200 mesh, 60 A, ICN Biomedicals) was used for product chromatographic purification. Pre-coated thin-layer chromatography (TLC) plates (silica-gel 60, 0.25 mm, E. Merck) were used to monitor the progress of the synthesizing reactions and chromatographic separation. All materials and glassware were repeatedly washed using d.i. water, avoiding detergent, rinsed with HPLC grade acetone and hexane, baked at 450°C overnight and stored at 110°C before use.

2.3. Sample collection

Marine sediment samples were collected from the Strait of Georgia, British Columbia (B.C.), off the Fraser River Delta. Stations were selected using the distribution of Ag concentration in surface sediments to obtain sediments impacted by a municipal wastewater discharge pipe at IONA. Sampling was carried out using a Smith–McIntyre Grab sampler. Sediments were kept frozen at −15°C to −20°C in the dark before being freeze–dried.

2.4. Sample preparation and extraction

Freeze–dried sediment samples were ground to a free-flowing powder before extraction. Soxhlet extraction was applied to 20 to 50 g of sample powder mixed with 50 g of pre-baked sodium sulfate. The sample was placed in a single-walled cellulose thimble which, along with the extractor, had been pre-cleaned by fluxing with hexane–IPA (70:30, v/v) for 2 h. The sample was then extracted for at least 18 h with hexane–IPA (70:30, v/v, 250 ml). The extract was reduced to ca. 5 ml using a rotary evaporator, transferred with hexane–IPA (70:30, v/v) rinses into a 25-ml flask, and evaporated to dryness under N_2 at 40°C. The residue was reconstituted to 6 ml with hexane–DCM (90:10, v/v) before SPE clean-up. A sonication based extraction method was also developed in which three cycles of shaking, sonication and decanting were carried out using 60 ml of hexane–acetone (60:40, v/v). The extracts were treated in the same way as Soxhlet extraction.

To determine extraction efficiency, replicate analyses were performed by spiking a marine sediment sample with 2 ml of a spike solution, which was prepared by adding the appropriate amount of stock solution into acetone. After spiking, the sample powder was homogenized and kept in the dark at 4°C overnight to allow NPEOs to interact with the natural organic material. The next day the spiked sample was air-dried and weighed before being extracted.

2.5. SPE clean-up

Extracted samples were cleaned using 500 mg LRC CN SPE cartridges. To prevent blockage of the 20 μm frits and to remove water, pre-baked sodium sulfate (3 g) was placed on top of the cartridge packing bed. To remove elemental sulfur from the sample extracts a layer of 1.5 g of activated copper powder was sandwiched between the top of SPE packing and sodium sulfate. This multi-layer modified SPE cartridge was found very robust to ''dirty'' samples.

2.5.1. SPE cartridge conditioning, sample loading and extraction

Six SPE cartridges were attached in parallel to a vacuum manifold. Each cartridge was rinsed with 15 ml of DCM to remove any possible background NPEOs arising from manufacture, packaging and handling. The packing bed was dried under vacuum for 1 min and the cartridges were activated by rinsing with 15 ml of hexane. The sample extract (hexane–DCM, 90:10, v/v, 6 ml) was added to the wet cartridge and the flow-rate was adjusted carefully to draw sample through the cartridge dropwise. Without letting the cartridge run dry, two 5-ml hexane rinses of the sample beaker were also run through. The collected extractant and rinse solvent were collected, treated and analyzed the same way as the NPEO desorbing solvent to check for SPE cartridge breakthrough.

Without allowing the SPE cartridge to run dry, three 6-ml aliquots of acetone were used to rinse the beaker containing the sample and then added to SPE cartridge to desorb the retained NPEO compounds. Upon adding acetone, the cartridge was allowed to stand for three min before applying vacuum to draw the desorbing solvent through it.

The extract was carefully reduced to dryness under a mild flow of N_2 at 40°C and the residue was reconstituted with a solution of toluene–DCM–MeOH (60:20:20, v/v/v) containing 0.5 mM NaOAc and the internal standard (I.S.), 4-fluoro-4′-hydroxyl-benzophenone. DCM was required to dissolve high oligomer NPEOs and MeOH to dissolve NaOAc. The reconstituted volume was usually 1.0 ml and 2.0 ml for samples where high concentrations of NPEOs were encountered. The sample vials were shaken for 3 to 5 min and then stored at 4°C in the dark for at least 12 h before analysis. It was determined empirically that the stand-by period significantly improved the reproducibility of the LC–ESI-MS results.

2.6. HPLC–UV and HPLC–FL

Normal phase HPLC–UV analysis was based on the method of Ahel and Giger [18] with minor modifications. Instead of the 277 nm UV absorbance and 1.5 ml/min flow-rate used by Ahel and Giger [18], the UV detector was set at 225 nm for optimum sensitivity and a flow-rate of 1.0 ml/min was used to achieve better separation especially for the early eluting low-mass NPEO oligomers. A Beckman System-Gold, Model 126, HPLC system was used for all analyses (Fullerton, CA, USA). The samples were introduced using a Rheodyne sample loading injector (Model 7225) with a 20-μl loop and analytes were chromatographed on a 25 cm×4.6 mm I.D. column packed with 5 μm d_p NH$_2$ Hypersil APS-1 from Phenomenex. A linear gradient of mobile phase A (hexane–IPA, 98:2, v/v) and mobile phase B (IPA–water, 98:2, v/v) was applied with phase B progressing from 5% B at $t=0$ min to 50% B at $t=30$ min at which time all 19 NPEO oligomers had eluted. To elute any remaining unknown compounds, mobile phase B was linearly increased to 95% between $t=30$ and $t=35$ min and held there for 10 min before returning to 5% from $t=45$ min to $t=50$ min. The system was equilibrated for at least 15 min between injections to achieve constant retention times. Samples were also analyzed using a fluorescence detector (Model 1046A, Hewlett-Packard) with an excitation wavelength of 233 nm, an emission wavelength of 302 nm, slit width of 10 nm and flow cell 15 μl.

2.7. LC–ESI-MS

A VG Quattro tandem mass spectrometer equipped with an electrospray source from Micromass (Manchester, UK) was used. To achieve maximum sensitivity two analyses were performed for each sample, one for the determination of NP (negative ion mode) and one for the determination of all the other NPEOs (positive ion mode). Depending on the type of analysis both full scan (200 to 1100 m/z mass range) and selected-ion monitoring (SIM) modes were used. The ESI probe is a pneumatically-assisted system that uses nitrogen as the nebulizing gas at approximately 80 p.s.i. (1 p.s.i.=6894.76 Pa) and flow-rates between 6.5 and 7 ml/min. Typical ion source and lenses settings used were: source temperature at 70°C; drying gas flow-rate at 0.3 L/min; ESI capillary voltage at 1.92 kV (positive ESI) and 3.67 kV (negative ESI); HV lens at 250 V (positive ESI) and 520 V (negative ESI); cone voltage between 29 and 32 V; skimmer offset at 0 V; lens-3 between 16 and 22 V; and ion energy between 3.4 and 4.1 V. The MS was operated in the unit resolution mode scanning the m/z 200 to 1100 range with a scan time of 5 s.

Mobile phase A was pure toluene and mobile phase B was 0.5 mM NaOAc in toluene–MeOH–water (10:88:2, v/v/v). The linear gradient progressed from 5% B at $t=0$ min to 60% B at $t=25$ and then linearly increased to 95% B from $t=25$ min to $t=30$ min. A cartridge column (250×3.0 mm) packed with Spherisorb CN 5 μm packing materials from Chrompack was used. All 19 NPEO oligomers eluted within this 30 min period. To remove any remaining unknown matrix compounds from the HPLC system, mobile phase B was held constant at 95% for 5 min between $t=30$ and $t=35$ min before returning to 5% B from $t=35$ min to $t=40$ min where it was maintained for 20 min before the next injection.

A performing mobile phase (mobile phase B) was added to the LC effluent after the column and before ESI (Fig. 1) to enhance system sensitivity. Mobile phase B was delivered to the system using a Harvard syringe pump Model 22, PTFE tubing (0.8 mm I.D.), either a zero dead volume (ZDV) Tee or a polyether ether ketone (PEEK) mixing Tee, and ZDV unions all from Upchurch Scientific. The post-column addi-
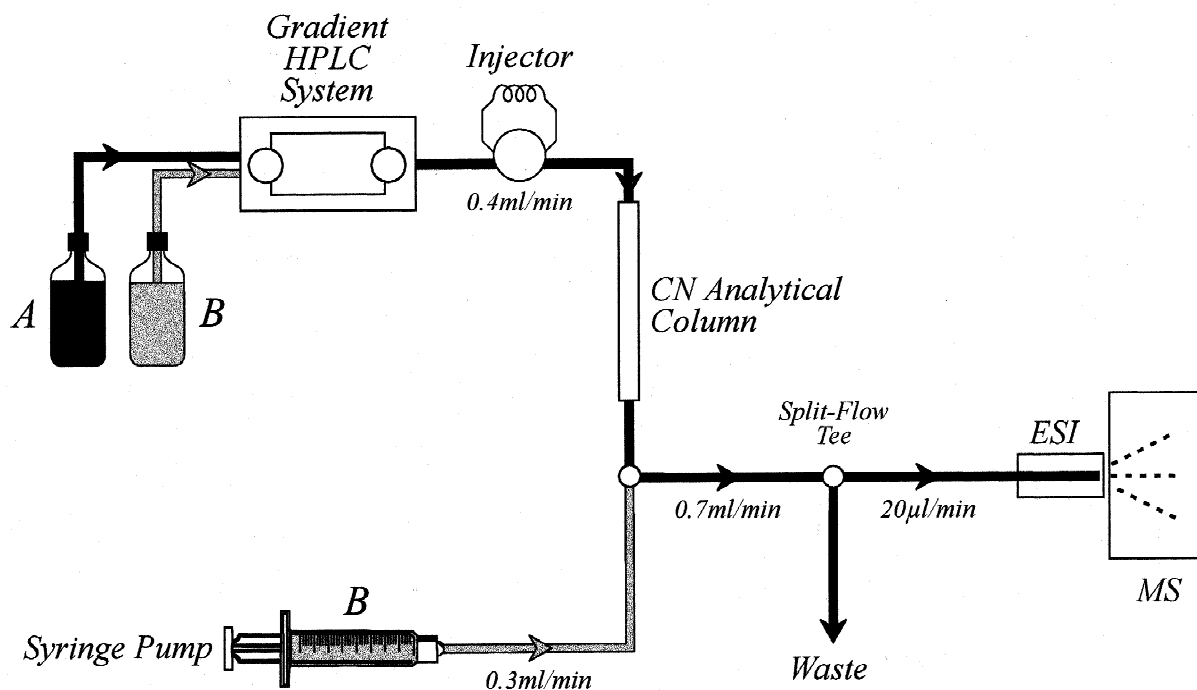
Fig. 1. Schematic diagram of the experimental set-up.

tion Tee was followed by a second Tee after 50 cm of stainless steel tubing (1/16 in. O.D.×0.007 in. I.D.) to achieve complete post-column mixing (1 in.=2.54 cm). The second Tee was needed to reduce the flow (by splitting) to rates that were compatible with ESI. The split ratio was carefully regulated by adjusting the length and I.D. of the restriction small bore stainless steel tube, which is less prone to splitting ratio variation than fused-silica capillary line. With this arrangement about 20 μl/min was diverted to the electrospray interface through a 120 cm×0.10 mm I.D. deactivated, low-polarity fused-silica capillary transfer line (Chrompack). For NP determinations, the mobile phase was operated isocratically with phase 60% A, a flow-rate of 0.4 ml/min, and post-column addition of 0.3 ml/min of mobile phase B. For both NPEO and NP analyses the flow-rate through the analytical column and that of the performing mobile phase were kept constant at 0.4 and 0.3 ml/min, respectively.

2.8. Quantitation

A stock solution was prepared by mixing known amounts of NP (9.1%, w/w), NP1EO (7.9%, w/w), NP2EO (8.2% w/w) and Surfonic N-100 (74.8%, w/w). The % (w/w) distribution of the final solution is shown in Table 1. A series of five standard solutions were prepared by diluting the NPEO blend (Table 1) in toluene–DCM–MeOH (60:20:20, v/v/v) with 0.5 mM NaOAc. The total concentrations (NP plus all the NPEOs) in these solutions were 0.836, 4.18, 8.36, 83.6 and 836 mg/l. The I.S. 4-fluoro-4′-hydroxyl-benzophenone was added to all solutions with a final solution concentration of 20 ppm. Five-point calibration curves for each of the 19 NPEOs and NP were generated by triplicate injections of the standard solutions.

Masslynx (the instrument's software) was used to integrate peak areas of standards and samples, and results were normalized against the I.S. to correct for variance from sample injection and instrument response. Over the established concentration range, linear regression of concentration vs. peak area ratios measured (area of analyte divided by the area of the I.S.) gave good fits (typically, R^2 values ≥0.9959) for each of the 19 NPEO species. For real samples the extract concentrations were converted into NPEO

concentrations in the sample by using the amount of sample extracted, the volume of the extract analyzed and recovery rate obtained from a parallel assay of a spiked sample. The sediment sample concentrations were expressed on a freeze–dried weight basis.

3. Results and discussion

3.1. Methodology for the coupling of normal-phase LC to ESI-MS

3.1.1. Optimization of ESI-MS parameters, flow injection experiments

These were conducted by injecting 20-μl aliquots of NPEO standard(s) directly into the flow of the carrier solvent which was maintained at a flow-rate of 20 μl/min. Mass spectra from preliminary experiments using a standard NPEO commercial mixture and a number of different solvent systems exhibited weak protonated molecular ions $[M+H]^+$ for nonylphenol and for all the ethoxylates. In an attempt to maximize analyte signal response a number of solvent systems were tested including: ACN–water (50:50) with and without 0.1% acetic acid; MeOH–water (50:50); IPA–water (50:50); ACN–IPA (50:50) with 0.5 mM NH$_4$OAc; hexane–IPA (50:50); hexane–IPA–water (50:48:2); hexane–acetone–water (45:45:10); toluene–ACN–water (48:50:2); benzene–EtOH–water (48:50:2); and toluene–MeOH–water (48:50:2) with 0.5 mM NaOAc. A typical spectrum is shown in Fig. 2a where the series of peaks represents NPEOs with increasing ethoxylate numbers. Each NPEO, however, comprises $[M+H]^+$ ions plus an indiscriminate number of adduct molecular ions including $[M+NH_4]^+$, $[M+H_3O]^+$, $[M+Na]^+$ and $[M+K]^+$ (Fig. 2b). Furthermore, the relative abundance of the adduct ions varies with the level of ethoxylation (compare for example NP5EO and NP11EO in Fig. 2a). Each of the ion series is separated by 44 Da corresponding to different levels of ethoxylation, but the ionization of the parent molecule(s) is dispersed among many molecular adduct ions reducing analyte sensitivity. Under these circumstances, quantitation reproducibility is poor since the relative abundance of the adduct ions is sensitive to variables which are difficult to control such as Na$^+$, K$^+$ and/or NH$_4^+$ impurities in the solvents used or impurities in the

ionization chamber and the solvent transfer lines. Solvated adduct ions such as $[M+(H_2O)_n]H^+$, $[M+(H_2O)_n+(NH_3)_m]H^+$, $[M+(H_2O)_n+Na]^+$, $[M+(H_2O)_n+K]^+$ ($m=1$ to 3 and $n=1$ to 5) are also observed but can be controlled by optimizing the voltages of the ion sampling lenses (cone, skimmer and lens-3), the source temperature and the composition and flow-rate of the carrying solvent, which will be discussed later in this paper.

In most flow injection experiments, even without adding any solvent modifiers such as NaOAc or NH$_4$OAc to the electrosprayed solution, intense sodium and ammonium adducts were detected with the sodium adducts being usually the most abundant. Na$^+$ is ubiquitous: trace concentrations are present in most solvents, Na$^+$ leaches from the walls of glassware, and marine sediment sample extracts contain small and variable amounts of Na$^+$, K$^+$ and NH$_4^+$. Small and variable amounts of Na$^+$, K$^+$ or NH$_4^+$ in the extracts introduces a large, difficult to control variability on the intensity of the corresponding analyte adduct ions. To address this problem we investigated the effect of additives on analyte signal intensity to establish conditions that would produce abundant and stable molecular adduct ion formation for the entire series of ethoxylates under ESI conditions. Although the relative intensity of NPEO adduct ions may be enhanced by a number of additives (e.g., NaOAc, NaOH, NaCl or NH$_4$OAc), we found that NaOAc produced the most abundant adduct ions ($[M+Na]^+$) for the entire ethoxylate series with good reproducibility. Therefore, in all subsequent experiments defined mM quantities of NaOAc were added to the sample solution and mobile phase solvents. In contrast to the NPEOs, nonylphenol exhibited best detection sensitivity in the negative ion mode and was detected as $[M-H]^-$ with m/z 219.

Contrary to the findings of other studies [22] sodium adducts were detected for both low and high NPEO oligomers provided proper conditions were selected. The sodium adduct intensity of the low NPEO oligomers, NP1EO and NP2EO in particular, was found to depend on reaction time prior to ESI-MS analysis and concentration of NaOAc. The intensity of NP1EONa$^+$ increased five-fold when the NPEOs/NaOAc mixture was allowed to stand for 48 h prior to the flow injection experiments. To ensure maximum sodiation of all the ethoxylates the final
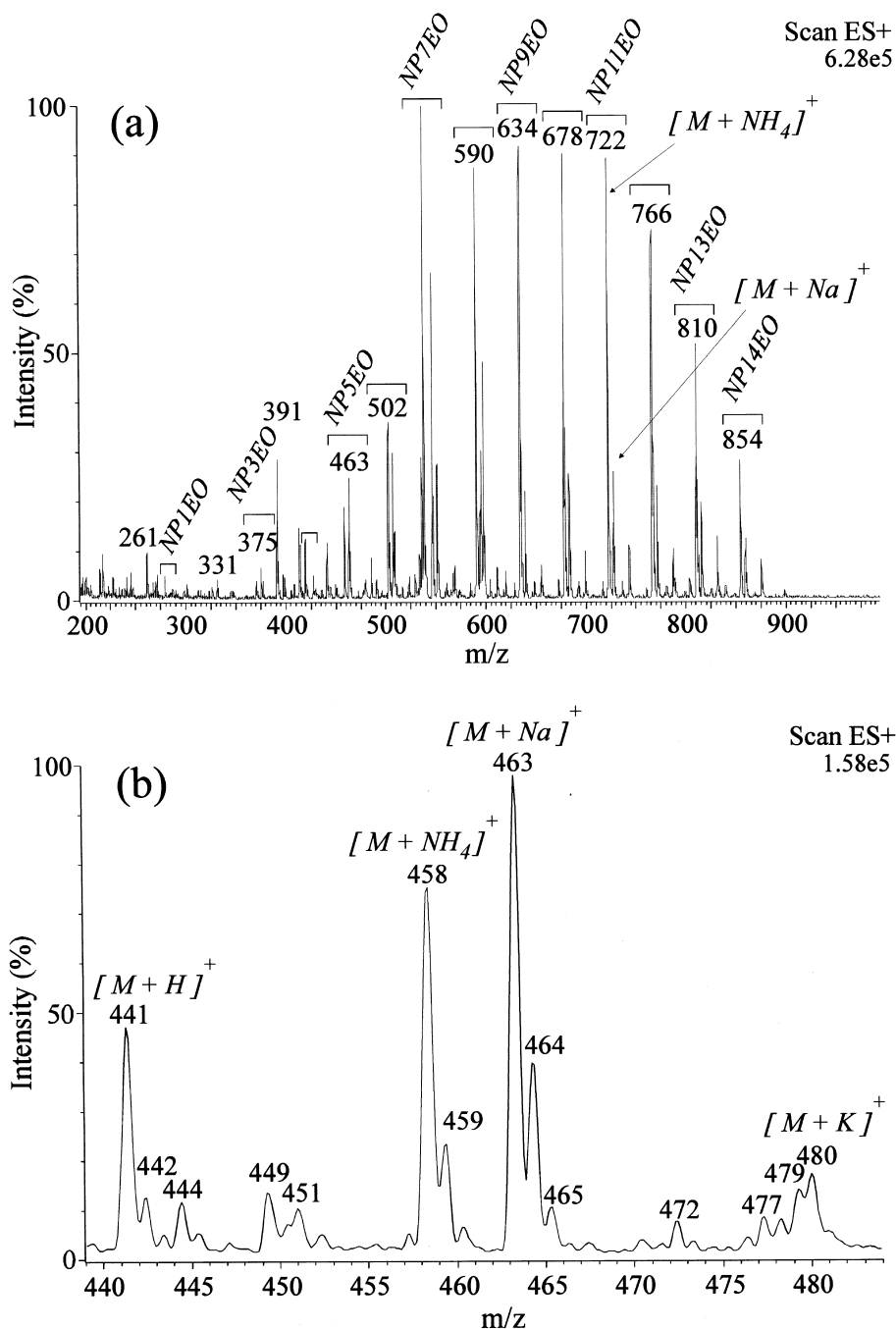
Fig. 2. (a) Positive ion ESI mass spectrum from flow injection of 5 ppm N-100 NPEO commercial mixture. The carrying solvent was Hexane–IPA–water (50:48:2) at 20 μl/min. Bracketed areas designate sections in the spectrum where corresponding adduct ions for each of the ethoxylates are detected: $[M+Na]^+$ (*m/z* 243, 287, 331 etc.); $[M+NH_4]^+$ (*m/z* 238, 282, 326 etc.); $[M+H_3O]^+$ (*m/z* 239, 283, 327 etc.) and $[M+K]^+$ (*m/z* 259, 303, 347 etc). (b) Expanded section of the spectrum covering the range *m/z* 440 to 485 where specific adducts of NP5EO are identified.

extracts of samples were spiked with NaOAc and stored in the dark for a minimum of 24 h prior to analysis.

In another set of experiments the effect of increasing [NaOAc] on the intensity of NP1EONa$^+$ was examined. Five test solutions were prepared by keeping [NP1EO] constant at 1.7 ppm and increasing [NaOAc] (0, 0.5, 1, 4 and 10 mM). A 10-fold increase in NP1EONa$^+$ signal intensity was observed by increasing [NaOAc] to 1 mM. Although the higher [NaOAc] (4 and 10 mM) produced higher abundance of NP1EONa$^+$ such conditions are impractical for routine operation because they result in unstable spray accompanied by the formation of a large number of analyte–solvent-sodiated adduct ions which suppress analyte signal intensity and induce signal instability [25,26]. We found 0.5 mM NaOAc to be the best compromise for enhancing the sodium adduct formation without sacrificing system stability. To facilitate cation attachment, both the sample solution and the mobile phase were spiked to produce a [NaOAc] of 0.5 mM. At this concentration, periodic orifice cleaning is mandatory to maintain sensitivity. Once the additive was chosen, the solubility of NaOAc in a particular solvent system became an important factor in the selection of mobile phase to be used in the LC–ESI-MS experiments. Of the mobile phases examined only benzene–EtOH–water and toluene–MeOH–water were suitable. Despite its slightly better performance, benzene–EtOH–water was rejected due to toxicity and odor.

3.1.2. Post-column addition of a performing solvent system

The best chromatographic separation for the NPEOs was achieved under normal-phase LC conditions with gradient elution. However, most normal-phase LC mobile phases are non polar solvents which are not compatible with electrospray ionization. To achieve good chromatographic separation and high sensitivity of MS detection, post column addition of a polar solvent and a modifier is required to facilitate ionization of the target analytes and evaporation of the pre-formed ions into the gas phase via the electrospray process [26]. After a large number of tests, the most suitable post-column addition solvent system was found to be mobile

phase B at a flow-rate ratio of 0.75:1. This system was fully miscible with the LC effluent and gave the best signal enhancement and system stability in both positive and negative ionization modes. High oligomer NPEOs benefit most from the post column addition with over 10-times signal intensity enhancement, whereas low oligomers (NP1EO, NP2EO) show approximately a three times increase in signal intensity compared to results from no post-column addition. For nonylphenol analysis (negative ionization mode), we kept the same post-column addition system so that the MS could be "toggled" seamlessly between the two ionization modes.

The ESI-MS interface was tuned to minimize formation of cluster ions containing single solute species combined with one or more solvent molecules. Sodium ion concentration, solvent polarity and volatility influenced the composition of these clusters and the efficiency of the electrospray process. For the chosen solvent system, conditions that gave minimum solvent cluster formation and full desolvation of analyte ions without dissociation of the covalent bonds were: ion source at 70°C; NaOAc at 0.5 mM concentrations; and sampling cone voltages between 15 and 37 V. Cone voltages higher than 37 V induce fragmentation and thus decrease analyte ion sensitivity, whereas cone voltages lower than 15 V favor cluster ion formation.

3.2. Complete separation of NP and all the NPEO oligomers using normal-phase LC–ESI-MS

Most LC–ESI-MS applications use reversed-phase HPLC mainly because the solvents are polar with high dielectric constant and low surface tension and are therefore compatible with electrospray ionization [25]. In this study we utilized normal-phase HPLC since it provided better separation of NPEO oligomers [27–29] in comparison to reversed-phase. Excellent results were obtained with an NH$_2$ column under normal-phase conditions using hexane–IPA as the mobile phase [18]. However, when this chromatographic method was used in conjunction with ESI-MS, weak signals, long retention times, and in some cases, poor sensitivity was obtained even when post column addition of the performing mobile phase was used. The poor performance was attributed to IPA's relatively low dielectric constant, high viscosi-

ty and low solubility for NaOAc, the modifier used to enhance formation of NPEO sodium adducts.

Although both CN- [11] or NH$_2$-modified [18] silica-packed columns have been used to separate NPEOs, the CN column was found to be particularly suitable because its excellent separation power for NPEOs with methanol–toluene as mobile phase. Methanol is a compatible solvent for electrospray ionization and a good solvent for NaOAc. Amino-propyl packing was too sensitive to methanol concentration in gradient mode, resulting in poor separation of NPEO oligomers. We also found the aminopropyl packing to be prone to fouling, perhaps due to frequent occurrence of carbonyl compounds in marine sediment samples which can form Schiff bases and thus modify the surface properties. On the basis of resolution, reproducibility, solvent saving, column life and cost, we chose the cartridge column (250×3.0 mm) packed with Spherisorb CN 5 μm from Chrompack. The optimum solvent gradient was determined through extensive trials with various solvent combinations, flow-rates, linear and non-linear gradient segment combinations and individual gradient steps.

A typical reconstructed ion chromatogram obtained from the LC–ESI-MS (full scan, positive ionization mode) analysis of a marine sediment extract spiked with 0.4 ppm of the NPEO mixture described in Table 1 is shown in Fig. 3. Although only selected oligomers are shown, good chromatographic separation was achieved for NP4EO to NP19EO, but poor for NP1EO to NP3EO. Partial chromatographic separation of the latter was achieved by using an elaborate gradient method, i.e., addition of a 5-min isocratic elution step (toluene–methanol, 99:1, v/v, as mobile phase C while maintaining the same post-column addition conditions) prior to the gradient program and by decreasing injection volume from 20 μl to 1 μl. Other more complex, nonlinear, or multi-segmented gradients were also tested with limited success in separation of NP1EO, NP2EO and NP3EO at the expense of time and reproducibility. For simplicity, we did not use this approach in our routine measurements since complete separation of all the non-fully resolved components was achieved by the mass spectrometer. The base peak in the mass spectra obtained during elution of a specific NPEO oligomer was the corresponding [M+Na]$^+$ ion (see, for example NP5EO in Fig. 3) where the peak at m/z 463 accounts for more than 90% of the total ion current. The advantages of using normal-phase LC separation and post-column addition of the performing mobile phase in ESI-MS is evident in the difference between Figs. 2b and 4.
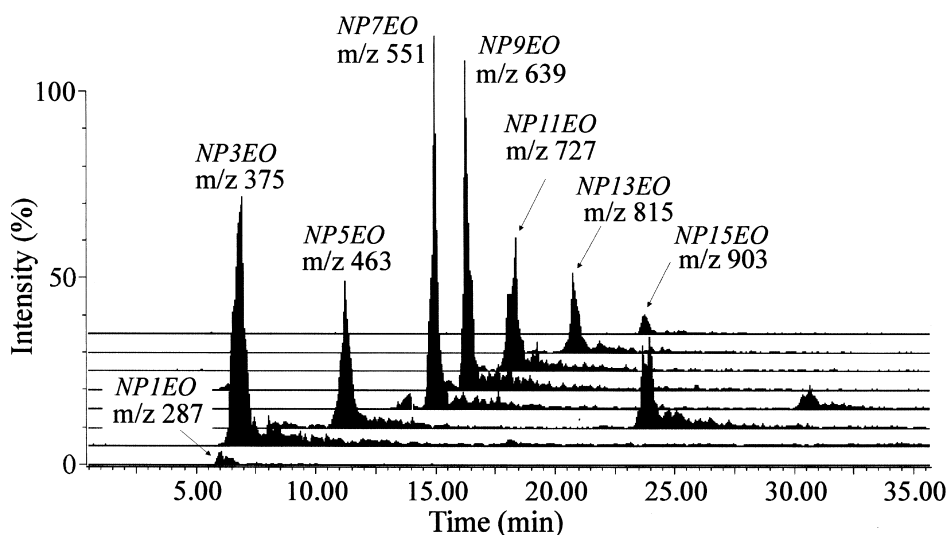

Fig. 3. Reconstructed [M+Na]$^+$ ion chromatograms of specific NPEOs with varying degrees of ethoxylation obtained from the LC–ESI-MS analysis of a marine sediment sample spiked with 0.4 ppm of the NPEO mixture described in Table 1. LC conditions and MS operational parameters are given in Experimental.
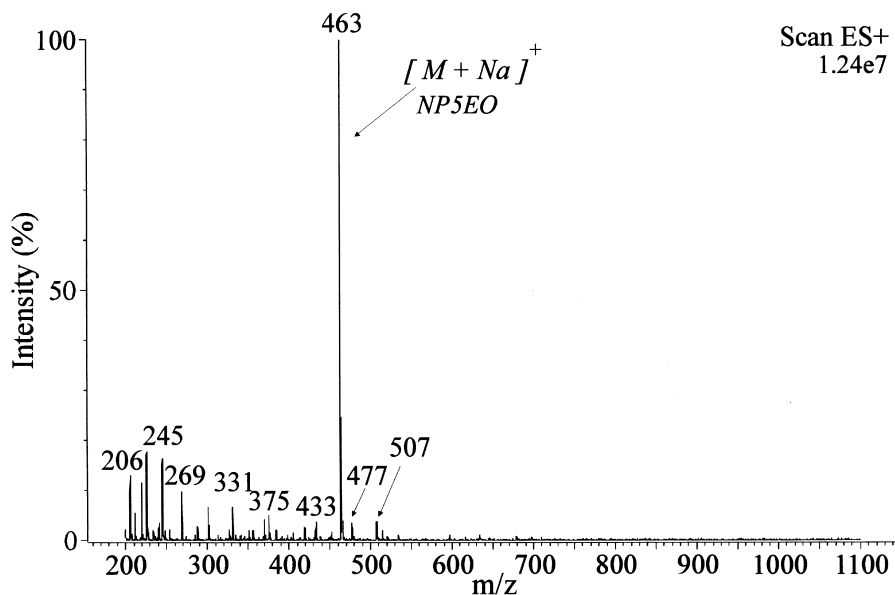
Fig. 4. LC–ESI-MS positive ionization mode mass spectrum obtained during elution of NP5EO, 12.5 min. Experimental conditions as in Fig. 3.

The majority of adduct ions for NP5EO (Fig. 2b) have been converted into the [NP5EO+Na]$^+$ ion (Fig. 4) due to the presence of NaOAc in the performing mobile phase, increasing substantially the detection limits and the specificity of the method.

In the sodium adduct reconstructed ion chromatograms (Fig. 3) two series of peaks are evident in some of the m/z traces: the first eluting peak corresponds to the [M+Na]$^+$ ion of a particular oligomer while the second corresponds to the doubly charged ion of a higher oligomer adduct. For example the m/z 463 trace in Fig. 4 has a peak at 12.5 min ([M+Na]$^+$ of NP5EO) and a peak at 24 min ([NP15EO+2Na]$^{2+}$ of NP15EO) which, of course, co-elutes with NP15EO ([M+Na]$^+$=m/z 903). Without normal-phase LC separation, NP5EO quantitation would be overestimated by 40%. Although singly sodiated ions were detected for all the NPEOs (i.e., NP1EO to NP19EO) doubly sodiated ions were detected only for the NP12EO to NP19EO oligomers. This was an expected observation since formation of multiply charged ions is an intrinsic property of electrospray ionization. As with protein analysis by ESI-MS the probability of multiple charging is expected to increase with the size of the molecule. The presence of doubly charged ions in

the ESI mass spectra of NPEOs underscores the need for complete oligomer chromatographic separation prior to ESI-MS detection.

Since NP exhibited the best detection sensitivity in the negative ionization mode, at the end of the NPEOs analysis of a particular sample the polarity of the MS and the ESI interface was switched and a second aliquot of the sample was analyzed for NP in the negative ionization mode. NP eluted at 3.5 min and was detected as [M−H]$^-$ at m/z 219 with no interference from the NPEOs. The specificity of the LC–ESI-MS technique permitted the detection of NP at low ppb levels in environmental samples without derivatization as required in GC–MS analysis [17].

Although ESI-MS is much more sensitive and specific than other conventional LC detectors it is less tolerant of minor changes in operating parameters and sample matrices. Therefore, an I.S. similar in chemical composition to the analytes is added to compensate for potential variations in instrument response due to variations in volume of sample injected, variations in the composition of the mobile phase and other LC and interface properties known to contribute to system instability. Since labeled NPEO compounds were not commercially available during the method development stage, we selected

4-fluoro-4′-hydroxy-benzophenone to be our internal standard. It exhibits good positive ionization mode sensitivity (detected as a sodium adduct) and even better negative ionization mode sensitivity (detected as $[M-H]^-$ with m/z 215). The I.S. eluted at 4.5 min and is separated chromatographically from all the NPEOs with no detectable concentrations of this compound in marine sediments. The I.S. was added to all the calibration solutions and was used to spike all the samples prior to analysis. As described in the quantitation section the response of NP and all the NPEO oligomers was expressed as a function of the I.S. Prior to analyzing real samples the ESI-MS system was optimized daily in both ionization modes using this I.S.

3.3. Development of optimal extraction protocols and a time-saving sample work-up method

Frequently, incomplete recovery of target compounds is a problem with environmental matrices as in the case of irreversible sorption of linear alkyl-benzenesulfonate on marine sediment [31]. Therefore, we evaluated a variety of protocols to extract and clean up NPEOs from marine sediments. The aim was primarily to achieve high and consistent recoveries of the widest possible range of NPEOs while eliminating interfering compounds and, secondarily, to produce a rapid and convenient sample preparation scheme.

Using consistency of spike recovery as a criterion, we examined Soxhlet and sonication extraction techniques with single solvent and a number of solvent mixtures of increasing polarity. The variety of adsorption sites in sediments and chemical characteristics of the NPEOs (polarity, reactivity, molecular size) requires an exhaustive extraction technique to recover all the analytes from the sediments. Lipophilic oligomers like NP1EO and NP2EO are easily extracted with pure hexane. On the other hand, the high oligomers with poor solubility in hexane exhibited low recoveries. We found that the addition of 30% IPA to hexane greatly improved the extraction of high oligomer NPEOs using Soxhlet. This can be attributed to the higher solubility of the high oligomer NPEOs in the polar solvent, IPA. The solvent system that gave best results with the sonication technique was hexane–acetone (60:40, v/v). NPEO

recoveries almost equal to those of an 18 h Soxhlet extraction were obtained. Using more acetone in the sonication extraction did not improve recovery. Actually, it hindered settling after sonication and made the decantation difficult. Table 2 shows the average recovery of the two methods used. Since the sonication method offered the similar extraction efficiency with considerable time saving, we decided to use the sonication method in this work to extract NPEOs from the sediment samples. We found the removal of water from the sediment before extraction to be crucial for good recovery. Freeze–drying was preferable to air drying because it gave more consistent recoveries.

To optimize SPE sample clean-up, we tested four SPE sorbents; aminopropyl, cyanopropyl, diol and unbonded silica. All of these normal-phase SPE sorbents exhibited similar breakthrough volumes when used under the same clean-up protocol. Although slightly better recoveries were achieved with diol SPE cartridges for the whole range of NPEOs, CN cartridges are a better choice because of the added benefit of HPLC column protection as an off-line guard column. The retention solvent for the SPE extraction process had to be sufficiently polar to dissolve all NPEOs, which have a wide range of polarity and solubility, but still deliver a low breakthrough volume. TLC experiments led us to a combination of hexane–DCM (90:10, v/v) as the retention solvent, and pure acetone to ensure complete desorption of NPEOs from the SPE cartridge. Without the small amount of DCM, we found higher MS background noise for the low oligomers, NP1EO and NP2EO. However, high DCM concentrations led to loss of NP, NP1EO and NP2EO in the SPE process. SPE breakthrough can present a problem especially with sediment samples containing high concentrations of NPEOs. To prevent this we always checked the concentration of NP that eluted from the SPE cartridge with the loading solvent, hexane–DCM. If a significant amount of nonylphenol was found in this fraction, 5 to 10 g instead of 20 g of the original sample were re-processed and analyzed.

Because surfactants readily adsorb to surfaces, particular attention was paid to the potential loss of NPEOs onto the glassware. At first, all glassware was silanized but it was later found that the loss of NPEOs could be prevented by rinsing the glassware

Table 2
Results of spiked sample experiments with Soxhlet and sonication protocols

Compound	Soxhlet extraction		Sonication extraction	
	Mean recovery (%)[a]	RSD (%)[b]	Mean recovery (%)[a]	RSD (%)[b]
NP	84.2	3.2	88.1	4.1
NP1EO	75.4	7.7	79.3	6.0
NP2EO	79.9	6.5	76.0	4.6
NP3EO	88.4	4.1	91.5	5.2
NP4EO	90.0	5.6	82.9	3.4
NP5EO	93.2	6.9	92.3	7.1
NP6EO	103.6	4.9	87.4	6.5
NP7EO	98.9	4.7	81.2	4.3
NP8EO	89.3	7.9	91.3	7.2
NP9EO	88.3	8.1	79.6	7.3
NP10EO	79.4	7.9	81.2	5.2
NP11EO	83.0	6.3	89.4	4.1
NP12EO	81.1	11.3	76.0	6.8
NP13EO	91.3	9.0	88.3	12.2
NP14EO	82.0	14.0	78.9	13.4
NP15EO	72.5	11.3	55.3	14.3
NP16EO	62.7	19.4	61.3	19.3
NP17EO	52.3	18.5	78.0	16.9
NP18EO	61.4	23.2	45.2	21.0
NP19EO	52.9	26.9	51.3	23.5

[a] The sediment samples (20 g) were spiked with a total of 20 ppm of the stock solution (Table 1) prepared in MeOH–acetone (70:30, v/v), homogenized thoroughly and left overnight (18 h) at 4°C in the dark before extraction. Triplicate spiked sediment samples, two blank (unspiked sediment) and two procedure blank samples were prepared for each extraction process. The sample used was a freeze–dried gravity core (Bal-2). Details on extraction procedures are given in Experimental.

[b] RSD=Relative standard deviation. Standard deviation of analyses expressed as % of average compound recovery.

with polar solvents such as methanol or acetone during sample pretreatment.

3.4. Method validation

The overall analytical method was tested for detection limits, linear dynamic range, precision, selectivity and ruggedness. The detection limit, defined as the minimum amount of a compound present in a sample that produces a signal-to-noise ratio of 3 upon final analysis, was typically 2–10 ng/g, positive ionization mode SIM, depending on the individual NPEO oligomer. Under negative ionization mode SIM conditions the detection limit for NP was 4 ng/g. In all cases, the limit of detection was based on an injection of a 20-μl aliquot from the final 1 ml extract of a 20 g freeze–dried sediment sample. The sensitivity obtained from the SIM experiments was approximately five-times higher when compared against the corresponding extracted ion chromatograms obtained from full scan experi-

ments. All real samples were analyzed under SIM conditions.

The linear dynamic range of the LC–ESI-MS instrumentation was evaluated by analyzing in triplicate five NPEO standard blend solutions at the following concentrations: 0.836, 4.18, 8.36, 83.6 and 836 mg/l. Since only about 3% of the LC effluent was diverted into the ESI source, the actual amount of total NPEOs delivered to the MS system was between 0.5 and 500 ng. Within this range, the system was found to be linear for most of the NPEO oligomers and the correlation coefficients of the 19 calibration curves obtained were between 0.9959 and 0.9999.

Over the concentration range tested the within-day precision was excellent for NP and most of the NPEOs with a relative standard deviation (RSD) of about 5%; the RSDs for NP17EO, NP18EO and NP19EO were slightly higher (8.9%, 12.4% and 15.0%, respectively). Between days the precision was somewhat lower, 12% RSD for most of the

NPEOs and 8% for NP. It should be noted that the use of concentrations obtained from LC–UV analysis of standard blend incorporates the error (standard deviation) of the LC–UV method into the LC–ESI-MS analysis. This error associated with the LC–UV analysis is between 3 to 8% (RSD) depending on the NPEO oligomers.

Reproducibility of the complete method was determined from triplicate analysis of spiked sediment samples (Table 2). For NPEOs with n_{EO} between 0 and 11 the RSD was 3.2–8.1%, and slightly higher (11.3–26.9%) for the higher oligomers, n_{EO} 12 to 19. The poor reproducibility observed with the higher NPEOs, i.e., RSDs >20%, was due to lower sensitivity obtained for these oligomers (see Fig. 2) and thus working close to the detection limits. The lower recoveries obtained with the higher NPEOs was not attributed to degradation as that would had

resulted higher than 100% recoveries for the lower NPEOs. High NPEO oligomers are known [11] to degrade to lower oligomers when exposed to air, however that was not observed with our spike recovery experiments.

3.5. Method application – analysis of marine sediments

Six marine sediment samples were analyzed to evaluate the method's performance in handling real samples. NPEOs, found at all sites, were composed predominantly of NP and lower oligomers (NP1EO, NP2EO) as shown in Fig. 5. The results were procedural blank corrected, i.e., levels measured in the blanks, which in most cases were below the systems detection limits, of the corresponding batch of samples were subtracted from the real samples.
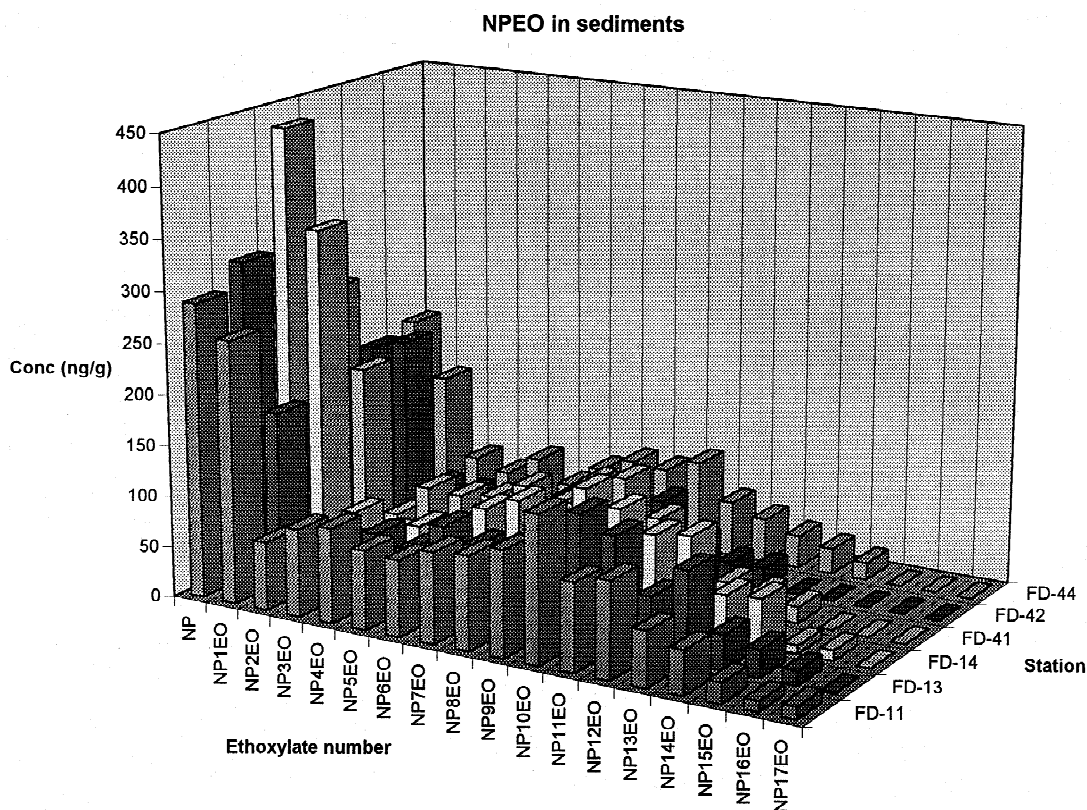

Fig. 5. NPEO distribution in six grab core sediment samples collected in the Strait of Georgia, B.C. Concentration determinations by LC–ESI-MS.

These results are in line with the findings of Giger et al. [9] where it is reported that under aerobic conditions, hydrolytic shortening of the polyethoxy chain of NPEO is favored, leading initially to the formation of lower oligomers (NP2EO, NP1EO), and ultimately to the completely-unethoxylated product, nonylphenol. However, in contrast to other methods, the LC–ESI-MS results revealed that appreciable amounts of high oligomer NPEOs were also present in the sediment samples as seen in Fig. 5. The small hump centered at NP8EO suggests the presence of small amounts of undegraded commercial product in the sediments.

Furthermore, LC–UV and LC–FL analyses gave systematically higher NPEOs concentrations when compared against the LC–ESI-MS data, see Table 3. This is due to the limited specificity and detection limits (DLs) obtainable from these techniques; LC–UV (low μg/g DL) and LC–FL (low ng/g DL). The higher concentrations of NP, NP1EO and NP2EO measured with LC–UV and –FL could be caused by co-eluting compounds present in the final extracts some of which could be alkylphenols other than nonylphenols and/or other alkyl ethoxylates. In LC–ESI-MS analysis co-eluting compounds are discriminated against by monitoring the m/z of the target analytes. For the higher oligomer congeners further confirmation is obtained from the doubly charged species as previously discussed. The comparative data of Table 3 clearly show that chromatographic

separation of the NPEO compounds, in real samples does not prevent peak overlapping with interfering compounds and leads to uncertainty when the analysis is carried out with non specific detectors.

We found a very limited number of reports where concentrations of nonionic surfactants (all oligomers) in marine sediments were measured. Using LC–FL the concentrations of NP, NP1EO and NP2EO in surface sediments from the Venice lagoon were measured between 0.1–6.6 μg/g. In another report Lee and Peart [17] using supercritical extraction and GC–MS analysis measured much higher NP concentrations in sludge samples (137 to 470 μg/g) and river sediments near pulp mills (0.29–1.28 μg/g). In the most comprehensive investigation to date, Naylor et al. [32] used LC–FL to measure river water and sediment samples. Approximately 95% of the sediment samples contained less than 0.635 μg/g of NP and less than 0.1 μg/g of NP1EO. However, none of these studies report values for oligomers higher than NP2EO in environmental samples. The present LC–ESI-MS technique provides a very sensitive and interference free approach for the determination of NP and all the NPEOs (n_{EO} 1–19) in complex environmental matrices.

4. Conclusions

The newly developed normal-phase LC–ESI-MS

Table 3
Comparison of the NPEO concentrations measured in marine sediments by LC–ESI-MS, LC–FL and LC–UV

	Average concentration (ng/g, dry wt.)					
	NP	NP1EO	NP2EO	NP3EO	NP4EO	NP5EO
Spiked[a]						
LC–ESI-MS	821	1201	1389	942	582	610
LC–FL	902	1428	1190	981	611	562
LC–UV	981	1569	1311	1010	710	712
Unspiked						
LC–ESI-MS	18	21	8	12	11	6
LC–FL	29	41	21	18	16	12
LC–UV	ND[b]	ND	ND	ND	ND	ND

[a] The sediment samples examined were from a gravity core (Bal-2) that was freeze–dried and homogenized thoroughly prior to analysis. The data shown are average values from triplicate analysis of the final extract. LC–FL and LC–UV operation conditions are given in Experimental.
[b] ND=Not detected, concentration below the detection limit.

overcomes some of the limitations of currently used GC–MS, LC–UV, LC–FL and reversed-phase LC–ESI-MS methods. Addition of 0.5 m*M* NaOAc into the mobile phase(s) and sample solvent as well as post-column addition of a performing mobile phase enhanced the method sensitivity and method ruggedness. For optimum detection limits both positive and negative electrospray ionization modes have to be used in the determination of NP and NPEOs in the environment. Using the separation power of normal-phase LC and specificity of a MS detector, doubly charged ions and impurity interference can be effectively avoided, thus increasing the detection accuracy and precision. Simple sonication extraction with a binary solvent of hexane and acetone and SPE clean-up protocols were developed and validated. Preliminary data obtained with this analytical method from Strait of Georgia sediments suggest that the NPEOs survived primary treatment processes and are preserved in marine sediments.

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