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Final Report to Hudson River Foundation on

"Alkylphenol Ethoxylate Metabolites in the New York Harbor Complex: An Initial Assessment of their Distribution and Effects"

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Project Abstract

Detailed studies of the distribution and fate of many potentially estrogenic, sewage-derived contaminants in the environment have been hampered by the limited sensitivity, selectivity, or applicability of routinely available analytical techniques. In this work, highly specific analytical approaches were developed, based on HPLC with electrospray mass spectrometry detection, for determining alkylphenol ethoxylate surfactants (APEOs) and their metabolites, in environmental matrixes such as wastewater, receiving waters, and sediments. Two published methods (Chapters 1 and 2 of this report) provide the most sensitive and robust methods available for quantification of APEO metabolites in water, wastewater and sediments.

The developed methods were applied to detailed studies of the environmental fate and transport of APEOs in Jamaica Bay, NY, a heavily sewage-impacted U.S. estuary. Results presented in Chapter 3 of this report indicated that nonylphenol ethoxylate (NPEO) and octylphenol ethoxylate (OPEO) metabolites were present in sediments throughout the bay, although the concentrations varied according to organic carbon content of the sediments and proximity to wastewater treatment plant outfalls. Chlorinated and brominated nonylphenol were identified as wastewater-disinfection byproducts in many sediments throughout the Bay. Biodegradation of APEOs in surface water and sorption to suspended particulate matter were important removal mechanisms for these compounds in estuarine water.

The significance of this project is not limited to development of ultrasensitive analytical methods and a better understanding of APEO fate in urban estuaries. Higher than expected concentrations of APEO metabolites were found in sediments from

Jamaica Bay, and also for limited number of additional sediment samples (another 9, concentrations reported in earlier correspondences to HRF) collected from around the NY Harbor complex. These concentrations are in the same range as total PAHs in sediments and suggest that APEOs should be included in a full assessment of sediment contamination and toxicity in the Harbor. These results led to the current HRF project by Brownawell and Bopp to better assess the sources and fate of APEOs in the NY Harbor basin.

The body of the report is organized as three already published chapters: Chapters 1 and 2 are method development papers and Chapter 3 describes what was learned about APEO distributions and behavior in Jamaica Bay.

CHAPTER 1: ANALYSIS OF ALKYLPHENOL ETHOXYLATE METABOLITES IN THE AQUATIC ENVIRONMENT USING LIQUID CHROMATOGRAPHY-ELECTROSPRAY MASS SPECTROMETRY

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1.1. Abstract

A quantitative method is described for the analysis of the metabolites of alkylphenol ethoxylate (APEO) surfactants in estuarine water and sediment samples using reverse-phase high performance liquid chromatography with electrospray mass spectrometry detection. Nonyl- and octylphenols, nonyl- and octylphenol mono-, di-, and triethoxylates, halogenated nonylphenols, and nonylphenol ethoxycarboxylates were concentrated from water samples using a C_{18} solid-phase extraction procedure. A novel, continuous flow, high temperature, sonicated extraction system was developed to isolate APEO metabolites from sediment samples. Quantitative LC-MS was performed in the negative ion mode for nonylphenols, octylphenols, and halogenated nonylphenols, and in the positive ion mode for nonyl- and octylphenol ethoxylates using selected ion monitoring with isotopically labeled surrogate standards. Recoveries for sediment and water analyses ranged between 78 and 94%, and detection limits for APEO metabolites were between 1 and 20 pg injected on column. This is a significant improvement over previously reported methods. Suppression of analyte response was encountered in the presence of matrix components in sediment samples, but this effect was eliminated by careful selection of surrogate and internal standards. Individual APEO metabolite

concentrations of 1 to 320 ng/L and 5 to 2000 ng/g are reported for water and sediment samples, respectively, from Jamaica Bay, NY (USA).

1.2 Introduction

There is significant interest in the environmental fate of the metabolites of alkylphenol ethoxylate (APEO) surfactants because of the potential effects of these compounds as endocrine disruptors in aquatic organisms. APEO surfactants are manufactured by sequential ethylene oxide addition to a hydrophobic alkylphenol. The most common alkylphenol (AP) used for this application is the technical product known as 4-nonylphenol (NP). This product consists of a mixture of branched nonyl-chain isomers. The end result of APEO synthesis using NP is a complex mixture of polyethoxylated, mixed alkyl-isomer compounds collectively known as nonylphenol ethoxylates (NPEO). A less common, but important AP used in APEO synthesis is 4-(1,1,3,3-tetramethylbutyl)phenol, more commonly known as 4-octylphenol (OP). APEOs manufactured from OP are termed octylphenol ethoxylates (OPEO). Biotransformation occurs when APEO surfactants are exposed to secondary wastewater treatment (Giger, Brunner et al. 1984), and a complex mixture of metabolites is released to the aquatic environment via discharge of the treated sewage. Metabolites include the short (typically 1-3) ethoxy-chain length NPEOs and OPEOs, the APs themselves (NP and OP), as well as carboxylated APEOs (NPEC and OPEC) and ring-brominated or chlorinated APs (such as Br-NP or Cl-NP). There is evidence that many of these substances can cause estrogenic effects in fish and other aquatic organisms(Jobling, Sheahan et al. 1996), and

these compounds may persist in aquatic sediments (Shang, Macdonald et al. 1999) where they may undergo remobilization and bioaccumulate into the food chain.

Several analytical methods have been used to monitor these contaminants in various environmental matrices. Methods include GC-MS with electron ionization (EI) (Giger, Stephanou et al. 1981; Stephanou and Giger 1982; Ding and Tzing 1998) and chemical ionization (CI) (Stephanou 1984; Stephanou 1984; Ding and Tzing 1998), and HPLC with UV absorbance (Ahel and Giger 1985) and fluorescence (Holt, McKerrell et al. 1986; Marcomini and Giger 1987; Chee, Wong et al. 1996; Scullion, Clench et al. 1996; Mackay, Croft et al. 1997) detection. Due to the limited volatility of highly ethoxylated and carboxylated APEOs, GC-MS is restricted to the analysis of APs and short ethoxyl-chain APEOs unless derivatization is incorporated into the protocol. The high resolving power of capillary GC allows separation of many of the alkyl-isomers of NP and NPEOs, resulting in compositional information, but detection at reduced signal-to-noise. In addition, APs and APEOs tend to fragment extensively under EI conditions providing valuable structural information, but limiting the sensitivity of the analysis in selected ion monitoring mode. The application of CI to GC-MS of APEO metabolites provides molecular weight confirmation for each compound, but this approach has not generally been used for quantitative analysis (Stephanou 1984; Stephanou 1984; Ding and Tzing 1998). HPLC with fluorescence detection is very sensitive for the analysis of APEO metabolites; however, no chromatographic separation has been reported which can simultaneously separate APEOs on the basis of alkyl- and ethoxy-chain length. In addition, UV absorbance and fluorescence detection lacks the specificity inherent to the

mass spectrometer, and interferences from complex environmental matrices may complicate the analysis.

HPLC with mass spectrometry detection has been used less frequently to analyze APEOs, their metabolites, and other nonionic surfactants in the environment. Early attempts included LC-MS with particle beam (PB) interface (Clark, Rosen et al. 1992) and thermospray (TS) ionization (Evans, Dubey et al. 1994). The development of robust and sensitive atmospheric pressure ionization (API) sources has greatly simplified coupling of HPLC to MS, while significantly enhancing sensitivity for polar and ionic compounds. Several authors have reported the analysis of ethoxylated nonionic surfactants and their metabolites by atmospheric pressure chemical ionization (APCI) (Castillo, Ventura et al. 1999) and electrospray (ESI) (Crescenzi, Dicorcia et al. 1995; Di Corcia, Costantino et al. 1998; Shang, Ikonomou et al. 1999) LC-MS. HPLC with ESI-MS detection offers excellent sensitivity for ionic and polar molecules, making it an ideal candidate for the quantitative determination of APEO metabolites. Crescenzi et. al (1995) reported excellent sensitivities for alcohol ethoxylate surfactants as well as long ethoxy-chain NPEOs in sewage, drinking water, and river water using HPLC-ESI-MS. Their method, however, was not readily amenable to short-chain ethoxylated analytes, and important metabolites such as APs and APECs were not detected. The HPLC-ESI-MS method of Shang et. al (1999) was able to detect NP as well as NPEOs in marine sediments, but did not detect NPECs or the halogenated NPs. This method utilized normal-phase HPLC, attained only modest sensitivity, and required long run times. Neither method included the OPEO metabolites, which may be important contaminants in some aquatic systems.

The objective of this study was to develop a method which could overcome the limitations of previous analytical approaches to measuring the important APEO metabolites in the aquatic environment. A comprehensive technique is reported which allows for quantitative determination of alkylphenols, halogenated alkylphenols, and APEOs with short (< 5) ethoxyl chains in both water and sediment. Highly ethoxylated APEOs and APECs were also detected in water by this method, although quantitative analysis was not performed. Reverse-phase HPLC-MS with electrospray ionization was optimized for specific and sensitive analysis of the APEO metabolites. In addition, sample preparation methods were improved, and a novel sediment extraction technique was developed for the analysis of APEO metabolites in contaminated sediments. The entire method was validated with environmental samples from a sewage-impacted urban estuary. Important limitations of ESI-MS analysis of environmental samples were considered, including sample matrix-derived interferences, competitive ionization suppression, and interferences from multiple-charged ions. These limitations were examined in the context of quantitative determination of APEO metabolites in sediment and water samples. The method provides the highest currently available sensitivity (1-20 pg per component injected) for APEO biotransformation products and is the first technique to demonstrate applicability to a full range of important metabolites likely to be encountered in the aquatic environment.

1.3 Experimental section

1.3.1 Materials

Stable isotope-labeled surrogate standards ($^{13}C_6$-n-nonylphenol, plus mono,di, and tri- ethoxylates) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Mono, di, and tri- ethoxylated 4-n-nonylphenol internal standards were synthesized from 4-n-nonylphenol (Lancaster Synthesis Inc., Windham, NH) and 2-chloroethanols (Aldrich, Milwaukee, WI) according to the procedure of Mansfield and Locke (1964). Synthetic products were purified by gel permeation chromatography (> 95% by ESI-MS). Monochlorinated NP was prepared by chlorination of technical nonylphenol using sulfuryl chloride according to the method of Stokker et. al (1980), and monobrominated NP was synthesized using elemental bromine (Kammerer, Eberle et al. 1975). Halogenated nonylphenols were purified by reverse-phase HPLC (> 98% by ESI-MS). Authentic standards of technical grade NP, NPEO (characterized for % composition of each ethoxymer), and nonylphenoxyacetic acid (sodium salt) were provided by Dr. Carter Naylor (Huntsman Chemical Co., Austin, TX). OP was purchased from Aldrich, and a mixture of octylphenol mono, di, and tri- ethoxylates was obtained from Chem Service (West Chester, PA). The octylphenol mono, di, and tri-ethoxylate material was characterized for % ethoxymer composition by GC-FID response. All solvents were HPLC grade or equivalent and were purchased from Burdick and Jackson (Muskegon, MI).

Due to the ubiquitous occurrence of alkylphenols and alkylphenol ethoxylate materials in plastics and detergents, glassware and sampling apparatus required special treatment prior to use. No detergents were allowed to contact any glassware. All

glassware was solvent rinsed (methanol, acetone and dichloromethane) and baked at 450° C prior to use. No plastics, other than Teflon and PEEK, were used in any part of the procedure. All adsorbents used in chromatographic cleanup or extraction were soxhlet extracted with methanol for 24 hours.

1.3.2 Water samples

Estuarine water samples (1 L) were collected from the surface of Jamaica Bay, NY (USA) using a stainless steel and Teflon Kemerrer bottle (Wildco Instruments, Saginaw, MI). Particulate material was immediately filtered onto precombusted GF/F (0.7 μm nominal) glass fiber filters (Whatman, Maidstone, England) under vacuum. The filtered water samples were spiked with $^{13}C_6$-labeled surrogate standards and acidified to pH < 2 by dropwise addition of concentrated sulfuric acid. Five mL of methanol was added to aid extraction, and the water samples were stored in glass-stoppered Erlenmeyer flasks (1 L) until extraction (within 8 h).

APs and APEOs were isolated from water samples using solid-phase extraction. Previous workers have used C_{18}-bonded silica (Marcomini, Dicorcia et al. 1993), hydrophobic polymers (Castillo, Alpendurada et al. 1997), and graphitized carbon black (Crescenzi, Dicorcia et al. 1995) as sorbents for extraction of APEOs and their metabolites from aqueous solution. We found that the use of 1.0 g of octadecylsilane (Bondesil 40 μm, Varian, Sugarland, TX) provided efficient recovery of the analytes in the current work. SPE cartridges were custom packed using 6 mL glass columns and PTFE frits (Supelco, Bellefonte, PA). Cartridges were pre-rinsed with 10 mL acetone, 10 mL methanol, and 10 mL of acidified Milli-Q water, prior to addition of sample. Water

samples were applied via suction through custom-modified Teflon siphon tubes. Field blanks, consisting of Milli-Q water which had been carried through the sampling procedure described above, were extracted along with water samples. Sample vessels were rinsed three times with acidified Milli-Q water, and the rinses were passed through the SPE cartridges. Cartridges were air-dried under vacuum, and elution was performed with 2 × 3 mL acetone. Extracts were taken gently to dryness under a nitrogen stream, reconstituted in 500 μL 60% methanol/water, and spiked with internal standards. No further treatment was necessary for APEO analysis of water extracts; however, it was necessary to add 50 mg of mixed bed strong ion-exchange resin (AG 11 A8, Bio-Rad, Melville, NY) to the extracts prior to AP analysis to remove interferences from chromatographically unresolved APECs. It should be noted that qualitative determination of APECs in water extracts by LC-MS was performed prior to the addition of ion-exchange resin to the sample.

1.3.3 Sediment Samples

Sediment samples had been collected previously from the New York Harbor Complex (USA) and freeze-dried to remove water. Subsamples were thoroughly ground and homogenized using a mortar and pestle. Previous workers have reported quantitative extraction of APs and APEOs from sediments using soxhlet extraction with nonpolar solvents (Marcomini, Pavoni et al. 1990) or exhaustive steam distillation (Ahel and Giger 1985; Naylor, Mieure et al. 1992). We attempted to adapt both of these methods to our sediment analysis, but found that they provided insufficient recovery. In the case of soxhlet extraction with pentane, up to 30 % of NPEO that had been spiked onto wet

sediment remained adsorbed to the sediment after extraction (18 h), as determined by exhaustive extraction of the sediment residue by high power sonic probe extraction (600 watts, Cole Palmer Instrument Co., Vernon Hills, IL) in methanol and dichloromethane. Even using acetone or acetone/hexane (50/50) as a soxhlet extraction solvent, up to 20 % of NPEO remained on the sediment after the extraction. However, in a recent study (Shang, Ikonomou et al. 1999), high recoveries of NPEOs were reported using soxhlet extraction with 70:30 hexane:isopropanol. The success of this approach may have been due to the use of the protic solvent, isopropanol, in the extraction.

An efficient sediment extraction method was developed which provided quantitative recovery of APEOs with minimal time and solvent requirements. The extraction of dried sediments was performed under solvent flow, with elevated temperatures and ultrasonic irradiation. A diagram of the experimental apparatus is shown in Figure 1.1. Dried sediment (1-2 g) was packed into a 150 mm stainless steel column (4.6 mm i.d.) fitted with 0.2 μm stainless steel frits (Alltech Chromatography, Deerfield, IL) and spiked with $^{13}C_6$-labeled surrogate standards. The column void volume was filled with sea sand which had been baked at 450° C overnight. The sediment column was immersed in a heated ultrasonic bath (65 °C), and methanol was pumped through the extraction cell at 0.5 mL/min. A backpressure regulator (Alltech Chromatography) was used to maintain the solvent as a liquid under the elevated temperatures. This approach, which uses low power ultrasonic energy to aid extraction, is different from the high energy ultrasonic probe extraction method (i.e. USEPA method 3550) mentioned above for determining extraction efficiency. Sediment extraction using the apparatus described in Figure 1.1 was complete (further extraction yielded no more

APEO) after seven minutes with a total solvent consumption of 3.5 mL per sample. Blank sediment extractions, consisting of baked sea sand spiked with surrogate standards and packed into extraction columns, were performed alongside actual samples.

A two-step cleanup approach was used to isolate APEOs from the sediment extracts. First, a normal-phase SPE approach, using NH_2-modified silica, was utilized (modified from Marcomini, Pavoni et al. (1990)). Aminopropyl silica (Supelco,) (750 mg) SPE cartridges were constructed from glass columns and Teflon frits. Columns were pre-rinsed with 2 × 5 mL acetone, then conditioned with 2 × 5 mL pentane. Sediment extracts (in 5 mL hexane) were passed through the columns. Columns were rinsed with 2 mL pentane, and APs and APEOs were eluted with 5 mL of 25% acetone/pentane. This normal-phase cleanup step served to remove APEOs > 5 EO as well as coextracted pigments and other polar material.

A second, reverse-phase cleanup technique was used to further purify the extracts. This step served to remove material which proved to be insoluble in the more aqueous HPLC-MS mobile phase. It also removed salts and some nonpolar material which would otherwise foul the analytical column. Eluents from the aminopropyl SPE cartridges were taken to dryness under nitrogen flow and resuspended in methanol. An automated, semi-preparative HPLC fractionation scheme was utilized in the cleanup. Two analytical (4.6 mm i.d. × 25 cm) C_{18} columns were placed in series and connected to a UV absorbance detector and a Gilson 203 fraction collector. Extracts (200 μL) were injected using a WISP 210B (Waters Associates, Milford, MA) autosampler. Chromatographic run conditions were isocratic 100 % methanol at 2 mL/min. The fraction containing APs and APEOs was collected between two and three minutes. The run was continued until the

UV detector response returned to baseline, typically 25 minutes. Collected fractions were again taken to dryness, resuspended in 60 % methanol/water, and spiked with internal standards.

1.3.4. Chromatographic Conditions

A Hewlett Packard 1100 series HPLC, consisting of a G1312A binary pump and a G1313A autosampler, with a 15 cm narrowbore (2.1 mm i.d.) C_8 column (5 μm particle size, Keystone Scientific, Inc., Bellefonte, PA) was used for LC-MS analyses. The separation was performed under isocratic conditions with 80 % methanol/water at ambient temperature (200 μL/min) and an injection volume of 20 μL. These conditions resulted in a run time of 13 minutes with no column equilibration step required between injections.

1.3.5. Mass Spectrometry

LC-MS analysis was performed using a Platform LCZ single quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Z-Spray ion source fitted with a pneumatically-assisted electrospray probe. The use of the orthogonal Z-Spray interface allowed the entire column effluent (200 μL/min) from the HPLC to be directed into the source without flow splitting and contributed to the greatly enhanced sensitivity. As in an earlier application reported by Shang et. al (1999), it was necessary to perform two separate analyses for each sample in order to quantify both APs and APEOs. APs and halogenated APs were detected in negative ion mode as $(M-H)^-$. In the negative ion mode, the capillary voltage was 2.52 kV, and the cone was set at 45 V. APEOs were

analyzed in positive ion mode as sodium adducts with capillary and cone voltages of 2.69 kV and 31 V, respectively. In both the positive and negative ion mode, source gas flow (250° C) was 600 L/h and the source block temperature was 150° C.

1.3.6. Quantitation

Quantitative analysis was performed using selected ion monitoring (SIM) in order to maximize sensitivity. Analyte concentrations were calculated relative to $^{13}C_6$-n-NP(0-3)EO surrogate standards, which were added to the samples prior to extraction, at a level that would give a final extract concentration of 100 ng/mL. Recovery of the surrogate standards was calculated relative to n-NP(0-3)EO internal standards, which were added to the final extracts at a concentration of 100 ng/mL. Five-point quantitation calibrations were performed daily for all analytes and the surrogate standards over the linear range of the instrument, which was typically three orders of magnitude above the analyte-dependent instrument detection limit (approximately 0.1-5 pg injected, based on the amount giving a peak with S/N = 3). Concentrations of analytes in calibration standards ranged from < 1 ng/mL to 900 ng/mL. Precision of the method was determined from extraction and analysis of triplicate samples. Method detection limits were calculated as 3 × standard deviation of analyte concentrations determined in field blank samples (n = 3). Qualitative spectra were acquired under full-scan conditions.

1.4. Results and discussion

1.4.1. Chromatography

With isocratic elution on a C_8 reverse-phase column, alkylphenolic compounds were separated on the basis of alkyl-chain length (Fig. 1.2a-d). Distinct peaks are evident in the TIC (Fig. 1.2a) for OP, NP, and n-NP. The separation of the 4-n-nonylphenol internal standard from the technical 4-nonylphenol analyte (a mixture of branched nonyl-isomers, containing no detectable n-nonylphenol) provides an advantage in SIM analysis (Fig. 1.2c). These compounds have the same molecular weight, therefore, they can be monitored using a single *m/z* channel, increasing the relative instrument dwell time and enhancing sensitivity. The $^{13}C_6$-4-n-nonylphenol surrogate standard co-elutes with the unlabeled 4-n-nonylphenol internal standard (Fig. 1.2b,c), however these signals are easily resolved by the mass spectrometer. Octylphenol is the least hydrophobic AP, so it is the first to elute (Fig. 1.2d). Like the APs, the APEOs separate on the basis of alkyl-chain length under the specified chromatographic conditions; however, the ethoxymers of a given alkyl-chain length co-elute (Fig. 1.2e-g). This presents a problem for quantitation of the individual ethoxymers when using reverse-phase HPLC with spectroscopic detection, however, as is evident in Figure 1.2e-g, the co eluting APEOs are resolved by the mass spectrometer in reverse-phase LC-ESI-MS due to their characteristic mass shift of 44 Da (mass of one ethoxylate moiety).

1.4.2. Mass Spectrometry

Under negative ion conditions, APs gave exclusively $(M-H)^-$ ions , consistent with previous reports in the literature for this class of compounds (Shang, Ikonomou et al. 1999). These ions are formed by loss of a proton from the phenolic moiety.

Polyethoxylated molecules show a remarkable affinity for alkali metal ions (Okada 1990), which is reflected in the positive ion electrospray spectra of these analytes (Figure 1.3). Even in the absence of added electrolyte, APEOs were detected as Na^+ adducts, presumably due to the ubiquity of this metal in the solvents and surfaces employed. As reported previously by Crescenzi et. al (1995) for alcohol ethoxylates, an increase in absolute response in the ESI-MS signal with increasing degree of alkylphenol ethoxylation was observed. In Figure 1.3, NP1EO was present at higher concentration than NP2EO, yet the intensity of the $(NP2EO+Na)^+$ ion is much greater than that of the $(NP1EO+Na)^+$ ion. The ratio of the NP2EO response factor to that of NP1EO was 5.4. Crescenzi et. al (1995) reported that this increase in response continued to about 6 EO, after which the response appeared to level off.

Because of a possible reduction in APEO ionization due to insufficient metal ion availability, it was necessary to fortify the samples extracts with 10 μM sodium acetate prior to injection. This provided sufficient sodium for analyte-sodium adduct formation. In the absence of this spike, response decreased for minor APEO components due to competition between the ethoxymers for the limited sodium pool. A related effect was noticed by Crescenzi et. al (1995) in their analysis of alcohol ethoxylate surfactants. Injection of multiple ethoxymers simultaneously resulted in overall suppression of the analyte signals relative to those obtained on injection of the ethoxymers individually. This effect was attributed to competition between ethoxymers for sodium adduction. The suppression was much more pronounced for lower-numbered ethoxylates in the presence of equal amounts of higher-numbered ethoxylates (Crescenzi, Dicorcia et al. 1995). The addition of sodium to the mobile phase was reportedly not sufficient to eliminate the

effect. It should be noted that these results were based on coinjection of up to 60 ng of alcohol ethoxylates, and that each ethoxylate was present at the same concentration. This situation may be markedly different from that in the present technique, in which injections of total APEO usually did not exceed low ng quantities, and, due to the normal-phase SPE cleanup technique, highly ethoxylated alkylphenols were not present in the standards or the sediment extracts. Competition for sodium metal adduct ions by APEOs may not completely account for the reported analyte signal suppression. Other mechanisms, such as saturation of the electrospray ion current or modification of ion evaporation processes from small droplets may produce similar results. Further consequences of these processes will be discussed later with regard to matrix effects.

To examine the competitive ionization suppression of less ethoxylated APEO oligomers by highly ethoxylated APEO, experiments were conducted in which increasing concentrations of a "polydisperse" NPEO mixture (avg. 9 EO, range 0-17 EO) were added to a constant (50 ng/L) level of pure NP2EO. The mixtures were analyzed by LC-ESI-MS in positive ion mode. No significant suppression of the NP2EO signal was evident until the concentration of the polydisperse NPEO mixture reached ten times that of the NP2EO. Even when the concentration of polydisperse NPEO was a factor of 40 higher than NP2EO, the signal of the latter was only suppressed by 30% relative to the response of pure NP2EO alone, and in this case, the linear response of the instrument for total NPEO had been exceeded. In this study, the concentration of highly ethoxylated APEOs never exceeded that of the mono-, di-, and tri-ethoxylated oligomers by more than a factor of two in any of the water samples analyzed (as evidenced by the paucity of higher mass peaks in the spectra of analyzed APEO samples, not shown). In fact, due to

the nature of the samples analyzed in this work, the mono- and di- ethoxylates tended to be, by far, the most concentrated neutral APEO metabolites present (due to biodegradation processes during and after sewage treatment). This is consistent with previous work which showed that NP1-2EO were the dominant neutral NPEO metabolites in a sewage-impacted river (Ahel, Giger et al. 1994). As a result, it appears that competitive ionization suppression from highly ethoxylated APEOs did not compromise quantitative results derived from the present method.

Because the samples studied in this work contained primarily mono-, di-, and tri-ethoxylated APEOs, and these compounds co-elute from the HPLC column, LC-ESI-MS experiments were performed in order to address the possible effects of co-elution of these analytes on signal response. Pure n-NP3EO was added at increasing concentrations to solutions containing n-NP1EO at a steady concentration (50 ng/mL). In this case, no positive ion ESI signal suppression was noted for n-NP1EO until the concentration of n-NP3EO had exceeded the linear range of the instrument (a factor of 20 higher than n-NP1EO). The scenario tested here can be considered to be the worst possible case, since, as noted by Crescenzi et al. (1995), signal suppression was most severe for less ethoxylated analytes in the presence of more highly ethoxylated compounds. Concentrations of mono-, di, and tri-ethoxylated APEOs in the present study were always within the linear range of the instrument, and, in most cases, the AP1,2EOs were more concentrated than the AP3EOs (see Table 1.1). Consequently, it is unlikely that the co-occurrence of AP1-3EO in the samples analyzed compromised analyte quantitation in the positive ion mode.

1.4.3. Method Validation

APEO metabolites were quantified in triplicate sediment and water samples from a sewage-impacted urban estuary (Jamaica Bay, NY, USA) using the present method. Results from these analyses are shown in Table 1.1. Concentrations of NP and NPEO were consistently higher than those of OP and OPEO in both sediment and water (Table 1.1). This trend reflects usage patterns of these surfactants, where 80 % of the APEO market is comprised of NPEOs and 15-20 % is made up of OPEOs and other ethoxylated alkylphenols (SDA 1999). In sediment, APs and AP(1-2)EO made up the bulk of the APEOs measured, with minor contribution of AP3EO. NPEOs in water showed a different pattern, where NP3EO was present at approximately the same concentration as NP, and NP2EO was the most concentrated APEO metabolite measured. Overall precision of the data was excellent, with relative standard deviations of the measured AP and APEO concentrations falling generally between 3 and 5 %, with some higher values, especially in the case of OPEOs in water. These higher RSDs may have been related to the generally low concentrations of these analytes.

Recoveries of spiked $^{13}C_6$-labeled n-nonylphenol and n-NPEO surrogates were consistently between 80 and 90 % for both water and sediment (Table 1.1). These recoveries were calculated relative to the n-nonylphenol and n-NPEO internal standards, which were added immediately prior to injection. No trend in recovery was evident with regard to degree of ethoxylation for either water or sediment.

The high sensitivity of this analytical technique is illustrated by the method detection limits (MDL) for water samples reported in Table 1.1. These MDLs represent injections of 1-4 pg for APEOs and 1.5-30 pg for APs. MDLs for sediments were

dependent on the amount of dry sediment extracted, but were typically 0.2 ng/g for 1 gram of sediment. Instrument detection limits were typically a factor of 5-10 lower than the MDLs for each analyte. These detection limits are an improvement over previously reported mass sensitivities for normal-phase LC-ESI-MS analyses of AP and APEO (1 – 4 ng injected) (Shang, Ikonomou et al. 1999), and are nearly an order of magnitude lower than results reported for reverse-phase LC-ESI-MS analysis of APEOs (20 pg injected) (Crescenzi, Dicorcia et al. 1995). The significant increases in sensitivities reported in this work are due to (1) the use of second generation pneumatically-assisted electrospray source technology, enabling high flow rates and orthogonal ion sampling; (2) the employment of aqueous mobile phases that increase electrospray efficiency; and (3), the ability of this method to analyze the entire 0.2 mL/min. HPLC eluent without the necessity for post column splitting or make-up flow.

1.4.4. Other APEO metabolites identified

Short-chain APEOs and APs are not the only metabolites that have been reported to arise from exposure of APEOs to wastewater treatment processes. Many authors have reported the presence of carboxylated alkylphenols (such as the nonylphenol ethoxycarboxylates (NPEC)) in sewage effluent and receiving waters (Di Corcia, Samperi et al. 1994; Field and Reed 1996; Di Corcia, Costantino et al. 1998). NPECs were detected in estuarine surface waters using the current technique (Figure 1.4). These analytes were detected in both negative (Figure 1.4a) and positive (Figure 1.4b) electrospray mode. No standards were available at the time of analysis, therefore, quantitative analysis was not possible. As with APEOs, the NPECs gave regularly

spaced peaks in the mass spectra, due to the 1 EO difference in the various structures. Sodiated ions were dominant in the positive ion spectrum, and the corresponding $(M-H)^-$ ions were present in negative ion spectra, reflecting the loss of an acidic proton. The profile of pseudomolecular ions in both positive and negative ion spectra reveal that the carboxylated NPEOs are dominated by short ethoxyl-chain oligomers.

Several authors have reported the formation of halogenated residues of APEO metabolites during wastewater and drinking water chlorination and their occurrence in the aquatic environment (Sheldon and Hites 1978; Reinhard, Goodman et al. 1982; Ball, Reinhard et al. 1989). In order to examine the possible persistence of these compounds in the environment, sediment extracts were analyzed for the presence of monochlorinated and monobrominated NP. An example of a reconstructed-ion chromatogram for Br-NP in a standard (Figure 1.5a) and a sediment sample (Figure 1.5b) is shown. The doublet signal in the mass spectrum of Br-NP due to the bromine isotope contribution (^{79}Br:^{81}Br – 50.5:49.5) provides additional confirmation of the presence of this compound in sediment samples, in addition to that given by the retention time match with the standard. It is clear from the low concentrations of the halogenated NPs in sediments that these compounds constitute a relatively minor component of the persistent pool of APEO biotransformation products in the marine environment (as little as 0.2 % of NPEO metabolites in sediment). However, this class of APEO metabolites may prove useful as a tracer of input of chlorinated sewage or pulp and paper mill effluent to nearshore environments.

1.4.5. Potential limitations of the present method

The quantitative analysis of APEOs by reverse-phase HPLC-ESI-MS presents several important problems which must be addressed before reliable measurements can be made. The effect of co-eluting APEO analytes on signal intensity has been discussed above. Another potential problem was highlighted by Shang et. al (1999) during their work on normal phase HPLC-MS analysis of NPEOs. In that study, it was noted that isobaric interferences from doubly-charged ions of highly ethoxylated NPEOs could impact quantitation of less ethoxylated NPEOs under conditions in which all ethoxymers co-elute. It was also discovered during the course of the present work that analyte signal suppression due to the presence of an environmental extract matrix must be corrected in order to prevent artifacts in the quantitative analysis of APEO metabolites by electrospray HPLC-MS.

The interference of doubly charged, highly ethoxylated APEO ions with singly charged ions of less ethoxylated APEOs is unique to the NPEO series. The doubly-sodiated ion of NP15EO gives a nominal *m/z* of 463. This also happens to be the nominal mass of NP5EO+Na. In reverse-phase LC-MS this creates a potential problem, since all APEOs of a given alkyl-chain length co-elute under a single peak, and hence, the NP15EO doubly charged ion can contribute to the peak area of NP5EO in the extracted ion chromatogram of *m/z* 463. Shang et. al (1999) reported that the error caused by this effect can be up to 40 %, and it is not limited to the NP15EO/NP5EO pair. All odd-numbered highly ethoxylated NPEOs caused interferences in the spectrum of less ethoxylated NPEO to some degree, depending on the relative concentrations of the NPEO pairs. The previous authors (Shang, Ikonomou et al. 1999) solved the problem by

separating all ethoxymers chromatographically with normal phase HPLC. In the present technique, the problem was eliminated by the removal of highly ethoxylated APEOs by normal-phase SPE from all sediment extracts. Water extracts were not cleaned up by normal-phase SPE, but examination of full scan ESI spectra revealed that interferences from double-charged APEO ions were not an issue. Even-numbered highly ethoxylated APEOs gave double-charged ions which were spaced 22 Da between the peaks for single-charged ions of less ethoxylated APEOs. The absence of peaks corresponding to these even-numbered double-charged ions in all water sample spectra indicated that the interfering peaks, 22 Da on either side, were also absent. This result is consistent with the typically low abundance of APEO ions with EO > 5 found in the spectra of water extracts analyzed in this study.

The intensity of a given analyte ion signal in ESI-MS is a function of both the concentration of the analyte and of the total ions in solution. As the concentration of co-occurring compounds increases beyond a certain threshold, the relative contribution of the analyte to the total ion current decreases, resulting in suppression of the analyte signal (Kebarle and Tang 1993). This "matrix effect" has been noticed in the positive ion ESI-MS analysis of alkyl ethoxysulfates (Jewett, Ramaley et al. 1999).

Matrix-induced analyte signal suppression is likely to be of major concern in the analysis of complex environmental samples by HPLC-ESI-MS. Removal of inorganic ions and other extraneous material by sample cleanup can help to reduce, but not eliminate the potential for co-eluting species to cause matrix effects; however, it is usually impractical or impossible to remove all possible interferences. In order to examine the impact of matrix effects on the present technique, we prepared two spike

series containing the surrogate standard $^{13}C_6$-n-NP and the internal standard n-NP; one in clean solvent, and one in sediment extract. The solutions were spiked with increasing concentrations of $^{13}C_6$-n-NP (treated as the analyte) and constant concentrations of n-NP. When these standards were run under negative ion HPLC-ESI-MS conditions, the response for $^{13}C_6$-n-NP was considerably lower in the presence of sediment extract (Figure 1.6a). It should be noted that the results shown are for alkylphenolic compounds in the negative ion ESI mode; therefore, the signal intensity does not reflect the contribution of adduct ion availability, as might the APEOs in positive ion mode. Because of this, the decreased response in the sediment extract can be attributed to a genuine matrix effect, and not to competition for adduct ions. It is clear from the results shown that calculation of analyte concentrations in actual environmental samples based on external standard response relative to "clean" calibration standards would result in significant errors. Separate experiments indicated that without the reverse-phase HPLC cleanup step, the signal intensity of $^{13}C_6$-n-NP was reduced by 70 % in a particular sediment extract by the matrix effect. This signal reduction was limited to 50 % in that same sediment extract after the reverse-phase HPLC cleanup.

When the $^{13}C_6$-n-NP response was normalized to the internal standard (n-NP in this case), the relative response was nearly identical for clean solvent and sediment extract (Figure 1.6b). This result indicates that the use of an appropriate internal standard reduces the net effect of matrix-induced signal suppression on calculated analyte concentration. It is clear, therefore, that internal and surrogate standards should be chosen which closely mimic the ionization behaviour of the analytes of interest in order to increase the quantitative reliability of ESI-MS in environmental analysis.

Since in the present study the surrogate and internal standards did not co-elute with the analytes of interest, it was necessary to confirm that the matrix-induced ionization suppression observed at the retention time of the standards was predictive of that observed for the analytes of interest. Sequential dilution of a sediment extract containing NP, and spiked with surrogate and internal standards, showed that the decrease in response with increasing sediment extract matrix concentration did not differ significantly among the internal and surrogate standards (n-NP and $^{13}C_6$-n-NP) and analytes (NP and OP) tested (data not shown). This suggested that, over a limited retention time window, there was little change in matrix-induced suppression of analyte ionization, and that the use of the chosen internal and surrogate standards was appropriate.

1.5. Conclusions

Reverse-phase HPLC-ESI-MS has been shown to be a highly sensitive, rapid, and robust method for the analysis of the full suite of APEO metabolites in environmental samples. The high sensitivity of the technique allows the use of very small sample sizes, making it possible to determine the concentrations of APEO biotransformation products in suspended particulates trapped on filters and in water samples far removed from contaminant sources. The technique could also be adapted to the analysis of small biological tissue samples and low-concentration matrices, such as air. This flexibility will enable a more complete study of the aquatic fate of the APEO metabolites. The ability to simultaneously monitor APEOs and APs of varying alkyl chain length may prove useful for contaminant source tracing, as variations in the ratios of OP to NP in the

environment may be reflective of specialized use patterns in industrial or municipal applications. It appears that the present method will be most useful for analyzing APEO metabolites in waters and sediments where there has been much degradation of highly ethoxylated APEOs. The complementary normal-phase HPLC-MS method recently developed by Shang et. al (1999) may be more appropriate for analyzing less degraded APEO mixtures in the environment. Finally, this and other papers have shown the effect of matrix-associated effects on analyte signal response in environmental samples. It is clear that the application of electrospray LC-MS techniques to quantitative problems in environmental analytical chemistry must take these effects into account in order to reduce artifacts introduced by the sample matrix.

Table 1.1. Method validation parameters

	% Recovery (SD)[a]		Environmental conc. (RSD)[b]		
Compound	Water	sediment	water (ng/L)	sediment (ng/g)	MDL (ng/L) in water
Nonylphenol	78.2(4.0)	82.8 (5.4)	201 (3%)	846 (12%)	0.92
NP1EO	90.2 (5.6)	93.5 (6.7)	157 (4%)	2120 (5%)	0.20
NP2EO	87.4 (5.5)	84.3 (5.9)	320 (3%)	1020 (2%)	0.24
NP3EO	90.5 (4.6)	80.4 (4.7)	202 (4%)	110 (4%)	0.36
Octylphenol			3.27 (3%)	8.11 (5%)	0.08
OP1EO			26.0 (12%)	60.7 (5%)	0.20
OP2EO			4.89 (8%)	13.0 (3%)	0.21
OP3EO			1.04 (30%)	5.96 (8%)	0.04
Cl-NP			ND[c]	4.38 (10%)	0.21[d]
Br-NP			ND	9.38 (3%)	0.27[d]

[a]Recovery of ^{13}C-labeled surrogate standards (n = 12)
[b]Based on triplicate sample measurements
[c]ND = not detected
[d]Extrapolated from sediment MDLs

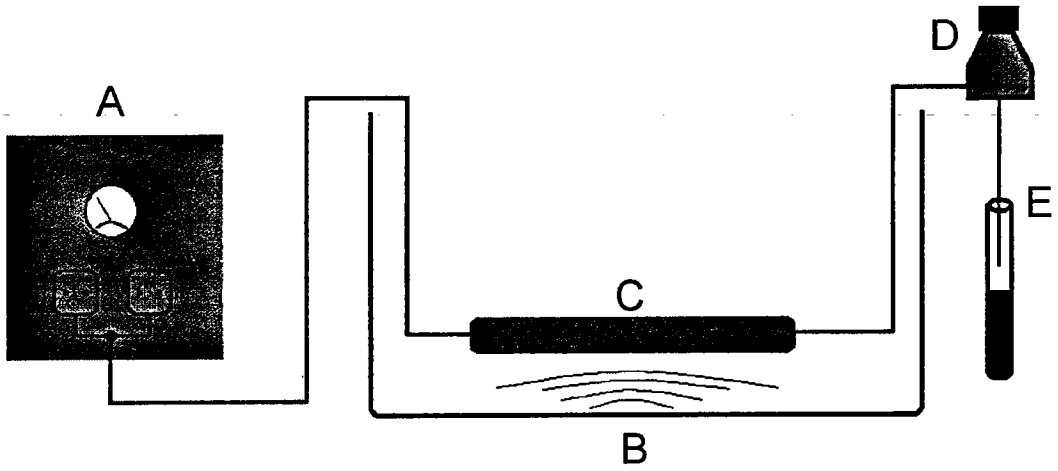

Figure 1.1. Diagram of the high-temperature continuous-flow sonication extraction apparatus, showing the solvent pump (A), heated ultrasonic bath (B), packed sediment extraction column (C), backpressure regulator (D), and extract collection tube (E).