Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1010 (2003) 25–35
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of alkylphenol and alkylphenolethoxylates in biota by liquid chromatography with detection by tandem mass spectrometry and fluorescence spectroscopy

Isabelle Schmitz-Afonso[a,1], Jorge E. Loyo-Rosales[b], Maria de la Paz Avilés[b], Barnett A. Rattner[c], Clifford P. Rice[a,*]

[a]*Environmental Quality Laboratory, ANRI, ARS/USDA, BARC-West, Building 001, Room 220, 10300 Baltimore Avenue, Beltsville, MD 20705, USA*
[b]*Environmental Engineering Program, Department of Civil and Environmental Engineering, University of Maryland, College Park, MD 20742, USA*
[c]*USGS-Patuxent Wildlife Research Center, 12011 Beech Forest Road, Laurel, MD 20708, USA*

Received 20 December 2002; received in revised form 13 May 2003; accepted 21 May 2003

Abstract

A quantitative method for the simultaneous determination of octylphenol, nonylphenol and the corresponding ethoxylates (1 to 5) in biota is presented. Extraction methods were developed for egg and fish matrices based on accelerated solvent extraction followed by a solid-phase extraction cleanup, using octadecylsilica or aminopropyl cartridges. Identification and quantitation were accomplished by liquid chromatography–electrospray tandem mass spectrometry (LC–MS–MS) and compared to the traditional liquid chromatography with fluorescence spectroscopy detection. LC–MS–MS provides high sensitivity and specificity required for these complex matrices and an accurate quantitation with the use of ^{13}C-labeled internal standards. Quantitation limits by LC–MS–MS ranged from 4 to 12 ng/g in eggs, and from 6 to 22 ng/g in fish samples. These methods were successfully applied to osprey eggs from the Chesapeake Bay and fish from the Great Lakes area. Total levels found in osprey egg samples were up to 18 ng/g wet mass and as high as 8.2 μg/g wet mass in the fish samples.

Keywords: Osprey; Fish; Alkylphenol; Alkylphenylethoxylates; Nonylphenol; Octylphenol; Ethoxylates

1. Introduction

Alkylphenol polyethoxylates (APEs) are effective non-ionic surfactants, widely used in a variety of industrial processes and in cleaning products. About 60% of the total surfactant production enters the aquatic environment [1] by way of industrial and municipal wastewater discharges. Studies conducted in the aquatic environment have shown bioaccumulation by aquatic organisms [2,3], estrogenic activity [4–6], and chronic toxicity [7,8] of this class of compounds. In order to assess the risk of exposure to

*Corresponding author. Tel.: +1-301-504-6398; fax: +1-301-504-7976.
E-mail address: ricec@ba.ars.usda.gov (C.P. Rice).
[1]Present address: ICSN-CNRS Avenue de la Terrasse Bât. 27, 91198 Gif-sur-Yvette Cédex, France.

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00956-7

wildlife and humans, residues of contaminants in all environmental compartments need to be determined. Several studies have found high levels of these compounds in river water and sediments throughout the USA [9–11]. Fewer studies have dealt with the analysis of these compounds in aquatic organisms, and most include only a few of the compounds; especially lacking are data on octylphenol (OP) and the octylphenol ethoxylates (OPEs).

In the USA, nonylphenol (NP) has been found in various fish species at levels up to 0.2 μg/g [12]. The highest levels reported in the USA were a total NP(0 to 3)EO of 4.9 μg/g in one carp sample from Detroit River, MI, USA [13]. Similar high levels were found in UK estuaries [14,15] and Catalonian rivers [16]. These high levels are usually related to the presence of sewage treatment plants (STPs) discharging into the rivers, and to high concentrations in water and sediments. Few data exist for the transfer of these chemicals to avian species. Ahel et al. [2] reported the presence of NP(0 to 2)EO in mallard duck collected from a Swiss river where fish were found to have high levels of APEs.

Current analytical methods for the detection of APEs in biota are generally based on gas chromatography–mass spectrometry (GC–MS), which is limited to OP, NP and NP(1 to 3)EO [12,14,15,17,18], or normal-phase LC with fluorescence detection (Fl) [2,13,19] for NP(0 to 5)EO. Reversed-phase LC is less commonly used for OP, NP and NP(1,2)EO [20,21]. The method detection limits (MDLs) reported are usually on the order of 1 to 40 ng/g, but can be as high as 100 ng/g [14]. More recent methods involve reversed-phase LC coupled with MS detection for NP, OP and bisphenol A (BPA) in fish with reported method quantification limits (MQLs) in the range 10–50 ng/g [16,22]. To the best of our knowledge, only one method was previously published [23] for the analysis of nonylphenol in egg.

This paper describes a method for the quantitation of NP, OP and their respective ethoxylates (1 to 5) in egg and fish matrices using accelerated solvent extraction (ASE) followed by solid-phase cleanup, and LC–MS–MS analysis, and compares it to LC–Fl. Method development efforts were focused on walleye (*Stizostedion vitreum*) and carp (*Cyprinus carpio*) tissue and osprey (*Pandio haliaetus*) eggs, in order to investigate contaminant exposure and potential reproductive effects in fish from the Great Lakes area and in birds nesting in highly polluted areas of the Chesapeake Bay. Fish tissue methods involved refinement of an extraction method previously developed by our research group [13] and the development of a confirmatory MS method for the APEs in fish samples. The osprey egg method was intended to extend the application of the fish methods to egg samples, particularly to field collected osprey eggs. One challenge encountered was to develop a method able to extract simultaneously all of the compounds of interest from matrices containing high levels of lipid. LC–MS–MS was chosen due to its ability to quantify all compounds with good sensitivity and the high specificity needed for these complex matrices.

2. Experimental

2.1. Standards and reagents

Analytically pure standards were obtained commercially for NP (Schenectady International, Schenectady, NY, USA; purity≥95%), OP (Aldrich, Milwaukee, WI, USA; 97% purity) and NP2EO (Aldrich R&D product; 95% purity). Other ethoxylates were purified in the laboratory by flash chromatography on silica gel as described elsewhere [13,24]: NP1EO from Surfonic N-10 (Huntsman Chemicals, Austin, TX, USA) and NP3EO, NP4EO and NP5EO from POE(4) nonylphenol (Chem Service, West Chester, PA, USA). The OP*n*EOs ($n=1$ to 5) were purified from POE(3) and POE(5) *tert*.-octylphenol; Chem Service. Purity was 99% for all compounds except OP1EO, 94%. Internal standards for LC–MS–MS were *n*-NP (Lancaster Synthesis, Windham, NH, USA), *n*-NP3EO (synthesized by Ferguson et al. [25]), $^{13}C_6$-NP (Cambridge Isotope Labs., Andover, MA, USA) and $^{13}C_6$-NP(1.6)EO (synthesized by Ferguson et al. [26]). All standards were stored at −20 °C. Solvents were high purity, pesticide grade; Burdick & Jackson (Honeywell International, Muskegon, MI, USA). Deionized, carbon-free water (DI water) was purified in a NANOpure water purification system (Barnstead Interna-

tional, Dubuque, IA, USA). Ammonium acetate; Aldrich (purity 99.99%) was stored in a desiccator. To avoid APE contamination in the samples no detergent was allowed to contact glassware used in sample preparation or analysis; plastic was avoided or rinsed thoroughly with solvent prior to use and sodium sulfate (anhydrous Na_2SO_4, granular powder; Mallinckrodt Baker, Paris, KY, USA) and glassware were baked for 4 h at 400 °C prior to use. Finally, procedural blanks were included with each batch of samples to ensure that only minimal contamination occurred, if any at all.

2.2. Osprey egg extraction

Osprey eggs were collected in the spring of 2000 and 2001. Samples P1 and P2 were collected from the middle Potomac River near Washington, DC, USA; S1, S2 and S3 from the South River and S4 from the West River (Chesapeake Bay, south of Annapolis, MD, USA). The whole egg was homogenized, divided in portions, and kept frozen at −20 °C until analysis. Previous to extraction, the aliquots were defrosted overnight at 5 °C. A 5-g homogenized sample was mixed with 30 g sodium sulfate, dried overnight in a desiccator, and ground with mortar and pestle. This mix was packed into a 33-ml stainless steel ASE cell capped with two filter disks [Soxhlet-extracted for 6 h with dichloromethane (DCM) to remove NP contamination]. The cell was extracted three times with acetonitrile at room temperature in an ASE apparatus (ASE 200; Dionex, Sunnyvale, CA, USA). Conditions were: 10 min static mode, 1000 p.s.i. pressure, 90% flush volume, 200 s N_2 purge. All of the three static extraction volumes were combined, evaporated to 4 ml and transferred to a C_{18} solid-phase extraction (SPE) cartridge (500 mg, 6 ml; Baker) previously conditioned with 6 ml acetonitrile. As the sample was loaded, the clean extract started to be collected; the tube that had contained the samples was rinsed with 3×1 ml acetonitrile, which was also loaded to the cartridge. Elution was completed with 6 ml acetonitrile. This extract was reduced to 2 ml through nitrogen evaporation. A 1-ml portion was used for LC–Fl analysis after evaporating the acetonitrile to near dryness and exchanging the solvent to 1 ml hexane. The other 1-ml portion was used for LC–MS–MS determination: the solvent was exchanged to methanol (MeOH)–water (60:40), the extract was filtered through a hydrophilic polypropylene membrane filter (0.45-μm 13-mm GHP Acrodisc Minispike; Pall Gelman Lab., Ann Arbor, MI, USA), which was chosen as the most efficient after investigation of nylon, poly(vinylidene fluoride) (PVDF) and GHP filters. The filter had to be rinsed with MeOH in order to recover all analytes; the final volume was brought to 1.5 ml. No problem was encountered with solubility of the extract in MeOH–water for all of our samples. In future work, extracts might be kept in 100% MeOH to prevent compound losses during filtration, depending on the egg's origin. Prior to analysis, 20 μl of internal standard mix was added. Final concentrations of the internal standards were: $^{13}C_6$-NP, 133 ng/ml; $^{13}C_6$-NP1EO, 125 ng/ml; $^{13}C_6$-NP2EO, 110 ng/ml; $^{13}C_6$-NP3EO, 26 ng/ml; and $^{13}C_6$-NP4EO, 4 ng/ml. Each APE was quantitated using the corresponding $^{13}C_6$-NP(0 to 4), except for the AP5EOs, which were quantitated using $^{13}C_6$-NP4EO because of the low concentration of $^{13}C_6$-NP5EO.

2.3. Fish tissue extraction

Carp, lake trout and walleye fish samples were collected from various sites throughout the Great Lakes area in 1998 and 1999. They were captured by electro-shocking, held briefly for biological processing (e.g., blood sampling, health examination, etc.), ground and stored at −20 °C until analysis. The extraction was based on a method developed and validated in the laboratory [13]. Briefly, the fish sample, after being thawed, was mixed with Na_2SO_4 in a 1:4 ratio. The mixture was dried overnight in a desiccator before being ground in a mortar. A total of 35 g mixture (7 g fish and 28 g Na_2SO_4) were packed in an ASE cell and accelerated solvent-extracted with DCM using the program described above for the osprey eggs. The extract was evaporated and the solvent exchanged to 7 ml hexane; from which 0.5 ml was transferred to a tared weighing pan. After 2 h under the hood, the difference in mass was measured to calculate lipid content. Lipid determination was performed only when using DCM as extraction solvent. Extract cleanup was accomplished using three aminopropyl cartridges

(500 mg, 3 ml; Supelco, Bellefonte, PA, USA) as follows: (i) conditioning: 3×3 ml acetone, 3 ml DCM and 3×3 ml hexane; (ii) sample loading: extract split into three portions and each loaded to a different cartridge; (iii) rinsing: 4 ml hexane; (iv) elution: 7 ml hexane–isopropanol (90:10, v/v). The collected fractions were pooled together and concentrated to 4 ml hexane. The extract was then ready for LC–Fl analysis. This protocol was modified for carp samples: total amount of sample was 30 g of a fish–Na_2SO_4 (1:5) mixture; acetonitrile was used instead of DCM for ASE. The solvent was evaporated to near dryness and exchanged to 5 ml hexane. Only two aminopropyl cartridges were used for the cleanup, and the final volume of the hexane extract was 2.8 ml for LC–Fl.

LC–MS–MS analysis required a supplementary cleanup step. Part of the hexane extract (equivalent to 1.5 g of fish) was evaporated to remove the hexane and exchanged to 2 ml MeOH. The cleanup was similar to that for the osprey egg using a C_{18} cartridge: conditioned with 6 ml MeOH; sample loaded while clean MeOH extract started to be collected; each tube rinsed with 3×1 ml solvent. Sample elution was completed with 6 ml MeOH. The extract was evaporated and filtered for particulate removal through a hydrophilic PVDF membrane (0.2-μm 25-mm Acrodisc LC PVDF; Pall Gelman Lab.) The filter was rinsed twice with MeOH at the end, and the final volume was brought to 1.5 ml in 100% MeOH. Keeping the extract in 100% MeOH produced a completely clear and colorless extract. In MeOH–water, some components would not fully solubilize and stay on the PVDF filter even after rinsing it with MeOH. The use of pure MeOH [20,22] or acetonitrile [16,21] rather than a MeOH–water mixture is consistent with other studies based on reversed-phase analysis of APEs in fish extracts. Internal standards were added as described above.

2.4. LC–fluorescence analysis

NP and NP-ethoxylates were analyzed using normal-phase LC–Fl. The instrument was a Waters (Milford, MA, USA) LC system consisting of a 616 pump, a 600S controller, a 717 Plus autosampler, a 474 scanning fluorescence detector and a Millenium workstation. LC (25 μl injection) was performed on a 5-μm aminopropylsilica column (Hypersil APS; Agilent, Wilmington, DE, USA) 100 mm×4.6 mm I.D., at 23 °C, with a hexane (A)–isopropanol (B) gradient [27] from 98% A (hold, 4 min) to 91% A in 5 min (hold, 5 min). The percentage of A was then taken to 50% in 2 min, held for 1 min, and taken back to the initial conditions in 2 min, where it was allowed to stabilize for 17 min. The flow-rate was 1 ml/min. An aminopropysilica guard column 4 mm× 3.0 mm I.D. (Phenomenex, Torrance, CA, USA) was used. Fluorescence detection wavelengths were 230 nm excitation and 300 nm emission. External standard calibration was performed using five calibration points prepared in matrix; concentrations were 40 to 260 ng/ml for NP and NP1EO; 70 to 280 ng/ml for NP2EO and 95 to 380 ng/ml for NP3EO, NP4EO and NP5EO. Checkpoints were injected every five or six samples.

2.5. LC–MS–MS analysis

The alkylphenols (NP/OP and NP-/OP-ethoxylates) were separated by combined reversed-phase and size-exclusion separation [26] and quantitated using MS–MS detection. The LC instrument was a Waters 2690 Alliance separations module coupled to a triple quadrupole mass spectrometer (Quattro LC; Micromass, Manchester, UK) with an electrospray interface (Z-spray source). LC (10 μl injection) was performed using an MSpak GF-310 4D column, 150 mm×4.6 mm I.D. (Shodex, Shoko, Tokyo, Japan) at 60 °C with an MeOH–buffer (MeOH–10 m*M* ammonium acetate, 50:50) gradient. MeOH was increased from 50 to 95% in 20 min (hold, 8 min), then to 100% for 5 min, and finally brought back to 50%. Rinsing the column in 100% MeOH eliminated components that were insoluble in the MeOH–water phase. A size-exclusion guard column, 4 mm×3.0 mm I.D. (GFC-2000; Phenomenex), was used and was replaced after about 60 sample injections. Samples were maintained at 15 °C. The flow-rate was 0.2 ml/min and was entirely directed into the LC–MS–MS interface.

Both quadrupoles were tuned using a polypropylene glycol standard in MeOH. Operating parameters of the electrospray interface were optimized in real conditions (standards introduced by

infusion pump coupled with an LC pump delivering 0.2 ml/min of MeOH–buffer, 50:50). All ethoxylates were detected in the positive ionization mode whereas both OP and NP were detected in the negative ionization mode. The method and fragmentation patterns are described elsewhere [24]. Briefly, the main parameters were: source temperature, 140 °C; desolvation temperature, 400 °C; nebulisation gas (N_2), 80 l/h; desolvation gas (N_2), 600 l/h; capillary voltage, 3.5 kV in the positive mode, −2.9 kV in the negative mode. Higher capillary voltages (4.5 kV) in positive electrospray ionization (ESI+) enhanced sensitivity for NP1EO and OP1EO but also created an electric arc between the tip of the capillary and the sampling cone, causing ionization problems. Analyses were performed using MS–MS in the multiple reaction monitoring mode. Specific parent ions were $[M+NH_4]^+$ for APEs and $[M-H]^-$ for APs. Analyte concentrations were calculated by the internal standard method using $[^{13}C_6]$NP*n*EOs (n=0 to 4) as internal standards. Six calibration points were prepared in MeOH–water (50:50): 20 to 700 ng/ml for NP, OP, NP1EO and OP1EO and 6 to 200 ng/ml for the rest. Peak integration and quantitation were performed automatically using MassLynx 3.5 (Micromass).

3. Results and discussion

3.1. Method development and validation

3.1.1. Osprey egg extraction

Due to the limited availability of osprey eggs, the method development presented in this section was done both with osprey and chicken eggs as matrix. The only method for NP in egg found in the literature [23] involves steam distillation extraction, which requires large amounts of sample and solvents, and long extraction times. For this work, ASE was chosen for extraction because of its rapidity, its convenience and low consumption of solvents. DCM and acetonitrile were tested as extraction solvents, the latter producing much cleaner extracts with good recoveries for all APEs. Two cleanup procedures were initially tested [28,29]: phase partitioning with water at pH 3 or 8 in presence of NaCl, which resulted in low cleanup efficiency and was time consuming; and normal-phase cleanup with alumina-neutral and aminopropyl cartridges, but yolk components coeluted with the compounds of interest. Finally, octadecyl cleanup [30,31] using acetonitrile yielded good results; the solid-phase adsorbent retained the interferences (oils and pigment) but not the analytes, which eluted slowly through the cartridge with the acetonitrile. It was found that reduction of the extracts to 4 ml prior to introduction to the cartridge was the optimum volume for this stage. Lower volumes led to precipitation of the extracts resulting in lower recoveries and higher volumes caused interferences to break through the cartridges. A 6-ml elution volume proved to be optimal for achieving good recoveries but caused breakthrough of the egg interferences in 3-ml cartridges; therefore 6-ml cartridges were used.

The entire procedure was validated with both LC–Fl for NP and NPEOs and LC–MS–MS for all APEs (Table 1). Recoveries were validated under reproducibility conditions, performing the analysis on different days, and at two different concentrations for injections by LC–MS–MS. Both chicken and osprey egg spikes were tested. Recoveries were above 80% for most compounds, and even the lowest recovery (74%, OP1EO) was within US Environmental Protection Agency (EPA) criteria, where recoveries are considered acceptable between 70 and 130% [32]. The method was reproducible for all compounds with RSDs<18%, except 29% for OP2EO with LC–MS–MS detection. LC–Fl calibration curves were linear over the calibration range with all correlation coefficient (r) values greater than 0.996. All checkpoint injections showed that the quantitation was accurate along the run. Procedural blanks analyzed by fluorescence showed no interferences except for NP at a level of 6 ng/g, which is below the MQL. LC–MS–MS quantitation was linear over the calibration range (r>0.998). Procedural blanks showed residual peaks for most compounds, especially for NP with a concentration of 8 ng/g. These residual peaks originated from the column itself and also from the analytical procedure for NP, despite all the precautions taken to avoid it. None of the sample results were blank corrected. The LC–MS–MS method was more sensitive for all compounds, as indicated by lower MQL values. LC–MS–MS qual-

Table 1
Method validation for egg samples, analyzed by LC–MS–MS and LC–Fl

Component	LC–MS–MS			LC–Fl			
	Spike* (ng/g)	Recovery (%) (RSD, %)	MQL (ng/g)	Spike** (ng/g)	Recovery (%) (RSD, %)	MQL (ng/g)	Check point (%)
NP	40–62	97 (15)	12	40	92 (13)	16	85
NP1EO	40–64	75 (11)	12	40	86 (14)	16	96
NP2EO	19–70	84 (15)	4	70	83 (11)	28	102
NP3EO	19–95	82 (14)	4	95	88 (14)	38	98
NP4EO	19–95	80 (18)	4	95	80 (10)	38	103
NP5EO	19–95	91 (17)	4	95	77 (17)	38	76
OP	63	78 (11)	12				
OP1EO	62	74 (10)	12				
OP2EO	19	83 (29)	4				
OP3EO	20	82 (16)	4				
OP4EO	21	94 (9)	4				
OP5EO	19	95 (9)	4				

* $n=13$ different samples (seven in common with the LC–fluorescence validation).
** $n=11$ different samples.

ity control parameters were similar or better than LC–Fl. Tandem mass spectrometry provides high specificity for identification, which makes it a preferable method to determine APEs in osprey eggs, especially at low levels.

3.1.2. Supplementary cleanup for fish extracts

The cleanup method originally used in our laboratory for fish samples [13] was validated and applied routinely to several batches of fish. This aminopropyl-based cleanup method eliminated approximately 80% of the lipids and other interferences from the extracts, which could be then analyzed by LC–Fl. Although this procedure was adequate for LC–Fl, the remaining coextractants interfered with the LC–MS–MS determination, and an additional cleanup had to be developed to perform routine analysis.

A normal-phase cleanup, using Florisil SPE coupled to hexane–acetonitrile partitioning, based on published methods [16,20,33], was tested to eliminate the remaining lipids but failed to produce a cleaner extract, apparently because the lipids have similar physical–chemical properties to the higher ethoxylates and coeluted with them. A reversed-phase cleanup based on the C_{18} procedure for eggs was implemented to further purify the extracts. This step provided adequate results, helping to remove material insoluble in the LC–MS–MS mobile phase, and improving performance of the LC column which was important to maintain good peak resolution (chromatogram in Fig. 1). This supplementary cleanup was validated by spiking fish extracts after the aminopropyl cleanup step. Recoveries were evaluated under reproducibility conditions, performing the tests on different days, with carp and walleye extracts, and at a concentration in the middle of the calibration range. Results were satisfactory with most recoveries above 80% and RSDs$<$19% (Table 2).

The original procedure that was validated with lake trout had to be adapted for carp samples due to low NP recovery results (44%) and poor reproducibility (RSD=57%, $n=22$). In contrast, lake trout processed simultaneously showed satisfactory results (96% recovery, RSD=26%, $n=11$); while recoveries for all the ethoxylates were acceptable for both types of fish. Several tests performed to identify the cause of this problem suggested that they were due to the nature of the fish and not to the analytical method. An optimized protocol using acetonitrile as extraction solvent and a longer (overnight instead of 2 h) drying step prior to the ASE was found to yield acceptable recoveries (above 74%) for carp samples. The method was reproducible with RSDs$<$14%, except for NP1EO that was 29%. Standard curves were linear over the range studied ($r>0.996$). Procedural blanks contained low residual contamina-

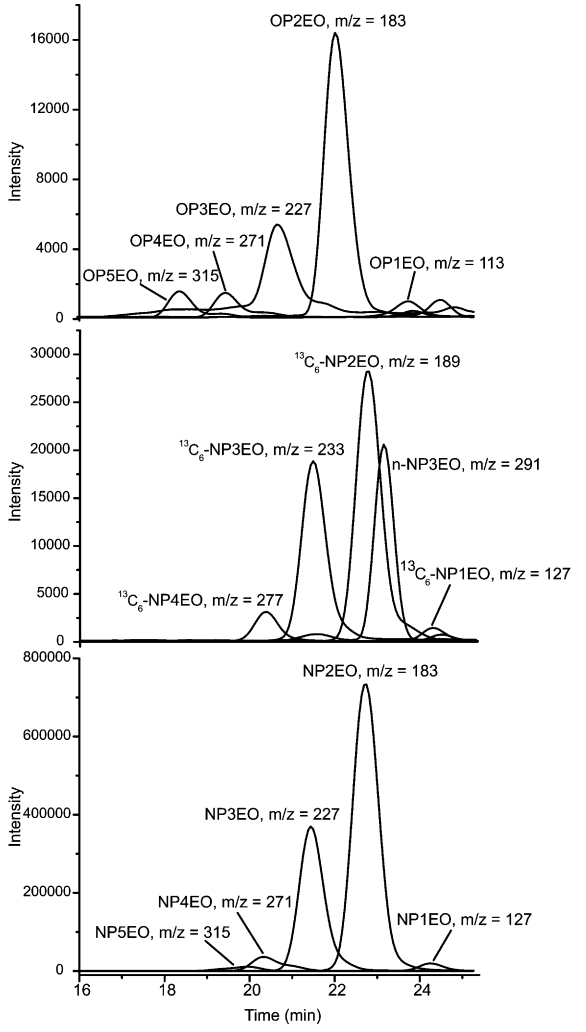

Fig. 1. LC–MS–MS chromatogram in the positive ionization mode for one walleye fish.

tion with NP at an average level of 19 ng/g. The method had similar MQLs for NP and NP1EO to LC–Fl and was more sensitive for the higher ethoxylates.

3.1.3. LC–MS–MS

One difficulty for the analysis of biota samples by LC–MS–MS arises from the nature of the extract and its compatibility with the MS interface, in comparison to water and sediment matrices. The extracts were discovered to contain impurities, as lipids, that caused varying amounts of matrix interference in the MS interface. To attempt to correct for these interferences, both ESI and APCI sources were investigated for the LC–MS–MS analysis [34,35]. From the two, ESI was the most sensitive to the compounds of interest, especially for OP and NP (100 times). Sensitivity was similar for OP1EO and NP1EO (two times) and higher for NP2EO (15 times). Because sensitivity was one of the most important criteria for this study, ESI was used despite the fact that it is more susceptible to matrix-induced ionization suppression effects [36]. Two types of compounds were tested as internal standards: NPs with linear nonyl chains (*n*-NP and *n*-NP3EO) and $^{13}C_6$-labeled branched-chain nonylphenols ($^{13}C_6$-NP in negative mode and $^{13}C_6$-NP(1.4)EO in the positive mode) [26,37]. By spiking samples prior to injection, it was observed that matrix suppression was more important for *n*-NP and *n*-NP3EO than for the targeted analytes, resulting in overestimated results if these are used as internal standards. The main reason might be that *n*-NP and *n*-NP3EO elute at different times than the corresponding NP and NP3EO, and therefore are subjected to different interferences coeluting from the column. For example, *n*-NP elutes 1.5 min after NP at a higher MeOH concentration (95 vs. 91%), where non-soluble interferences might also be eluting from the column, inducing more important matrix effects. In contrast, the $^{13}C_6$ internal standards have the same structure and fragmentation patterns (see for example $^{13}C_6$-NP's mass spectrum in Fig. 2) as the NPs [24]; they coelute with the respective NPs and allow compensation for the matrix suppression effect, which results in more accurate quantitation of samples.

Table 3 illustrates the different matrix suppression effects on all the internal standards. It shows the important differences between *n*-NP and $^{13}C_6$-NP; *n*-NP's signal intensity in matrix extracts was only 20% of the intensity in mobile phase solvent, compared to 42% for $^{13}C_6$-NP. Apparently, egg extracts cause less ionization suppression (maximum 17%, $^{13}C_6$-NP3EO) than fish samples, which produce signal suppression up to 36% in ESI+ ($^{13}C_6$-NP1EO) and 58% in ESI− ($^{13}C_6$-NP). Variation among fish samples (37%) is usually more important than among egg (15%). Egg extracts can be injected without affecting the sensitivity of the instrument

Table 2
Validation of supplementary cleanup for fish samples (analyzed by LC–MS–MS)

Component	With cleanup only		With whole carp protocol		
	Spike* (ng/ml)	Recovery (%) (RSD, %)	Spike** (ng/g)	Recovery (%) (RSD, %)	MQL (ng/g)
NP	205	76 (19)	150	77 (5)	22
NP1EO	214	83 (14)	157	96 (29)	20
NP2EO	64	83 (26)	47	78 (10)	6
NP3EO	64	84 (21)	47	74 (12)	6
NP4EO	63	82 (10)	46	74 (11)	6
NP5EO	67	90 (7)	49	88 (13)	6
OP	210	83 (8)	154	83 (2)	20
OP1EO	208	84 (9)	153	76 (6)	20
OP2EO	65	86 (13)	48	114 (22)	6
OP3EO	67	100 (18)	49	86 (14)	6
OP4EO	69	88 (9)	51	88 (7)	6
OP5EO	64	93 (15)	47	112 (5)	6

* $n=8$ different carp and walleye samples.
** $n=5$ different carp samples.

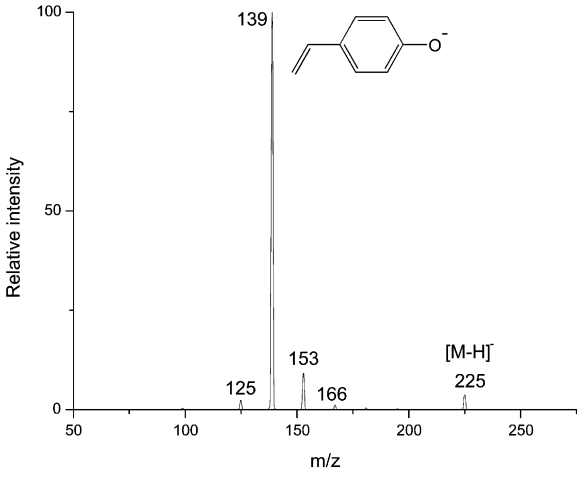

Fig. 2. MS–MS spectrum of $^{13}C_6$-NP's $[M-H]^-$ ion, m/z 225.

over the run, whereas fish samples result in a gradual loss of sensitivity; in spite of this, quantitation remains accurate thanks to the $^{13}C_6$ internal standards. Up to 80 samples, including 50 fish and 30 eggs, have been successfully analyzed in the LC–MS–MS maintaining good performance (linearity and sensitivity). Therefore, this method can be used routinely to analyze large numbers of samples.

3.2. Application

3.2.1. Application to osprey eggs from the Chesapeake Bay area

All the eggs (P1, P2, and S1 to S4) were analyzed by both the LC–Fl and the LC–MS–MS method. Lipid concentrations in these samples [38] ranged from 2.8% for S3 to 8.3% for S1 with an average of

Table 3
Evaluation of ionization suppression for fish and egg matrices

Matrix		*n*-NP	$^{13}C_6$-NP	$^{13}C_6$-NP1EO	$^{13}C_6$-NP2EO	*n*-NP3EO	$^{13}C_6$-NP3EO	$^{13}C_6$-NP4EO
Fish	Signal intensity[a] (%)	20	42	64	66	60	84	100
	RSD[b] (%)	39	37	31	29	20	22	19
Egg	Signal intensity[a] (%)	89	93	89	88	80	83	88
	RSD[b] (%)	27	9	13	12	17	15	11

[a] 100%=intensity of the standard prepared in mobile phase.
[b] Variation of ion current in the sample matrix along the sequence.

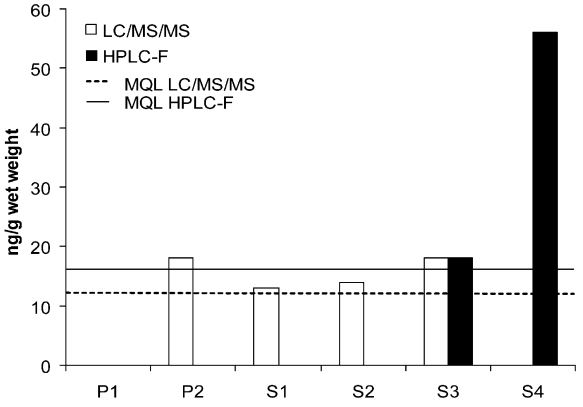

Fig. 3. Nonylphenol in osprey eggs from the Chesapeake Bay area. Samples P (1&2) were collected from the middle Potomac River Area and S (1–4) from the reference site near Annapolis, MD (South and West River).

4.9%. From all the compounds analyzed, only NP was detected at concentrations above the MQL (Fig. 3). Results were similar for samples P1 and S3 by both methods. In samples P2, S1 and S2, NP was detected by both methods, but could be quantified by LC–MS–MS only because of the low levels present (13–18 ng/g). Sample S4 is a good example of the limitation of LC–Fl versus LC–MS–MS at low analyte levels in complex matrices. NP was detected by LC–Fl at a relatively high concentration but this was a false positive value. Fluorescence identification, based on retention time matched to known standards is not specific enough for samples containing impurities, even with the selective extraction method and extensive cleanup applied. Thus, for the analysis of compounds present in complex matrices and concentrations near the method detection limits, it is better to rely on a more selective identification tool such as MS–MS. Compared to historic and contemporary concentrations of bioaccumulative organochlorine pesticides and polychlorinated biphenyls (PCBs) in bird eggs, levels of NP, OP and their ethoxylates in these osprey eggs are quite low. These findings suggest that there is no transfer of these compounds from the osprey to eggs, but a more complete study, including the analysis of fish and the osprey itself, would be necessary to evaluate this fully. There are no other data available for field-collected eggs to compare with ours. Guenther et al. [23] reported a concentration of 1.5 ng/g for NP in chicken eggs. Levels in chicken egg analyzed during our method development were around 7 ng/g, similar to levels found in procedural blanks (6–8 ng/g).

3.2.2. Application to fish samples from the Great Lakes area

Six predatory fish (walleye), collected in 1998 from the Mississipi River, near St. Paul, MN, USA, were extracted using the DCM ASE method, and then analyzed by LC–Fl and LC–MS–MS. The average lipid content was 8.2±3.4% (SD). These samples (results in Fig. 4) were highly contaminated with an average of total APEs of 4.9 μg/g, with NP2EO being the most abundant at 2.4 μg/g. The sampling site was located in the outfall channel of the St. Paul Metropolitan wastewater treatment plant, which would explain the high levels of APEs. No OP was measured above the MQL (20 ng/g), and total OPs represented 3% of the total NPs. This percentage is lower than that expected from the use trend of OPs versus NPs (15–20%) [25,39]. Average MS values for the six samples were slightly lower (73 to 90%) than the average fluorescence values for the NPs, suggesting a good correlation between the two methods. No false positives were observed by LC–Fl for these fish at these high concentrations. A

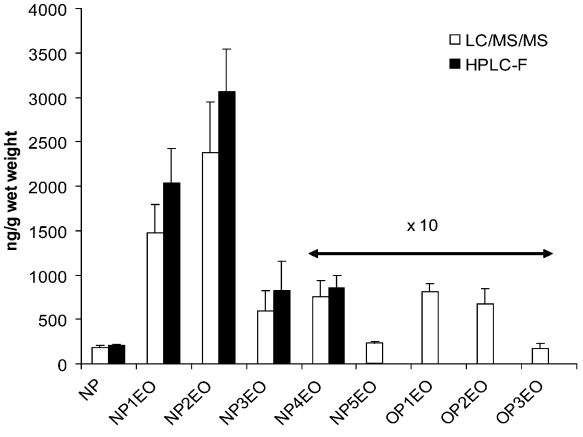

Fig. 4. Comparison of APE concentrations in six walleye fish from Mississipi River, MN, obtained by LC–MS–MS and LC–Fl (concentrations were multiplied by 10 from NP4EO to OP3EO; the standard error of the mean is indicated as error bars).

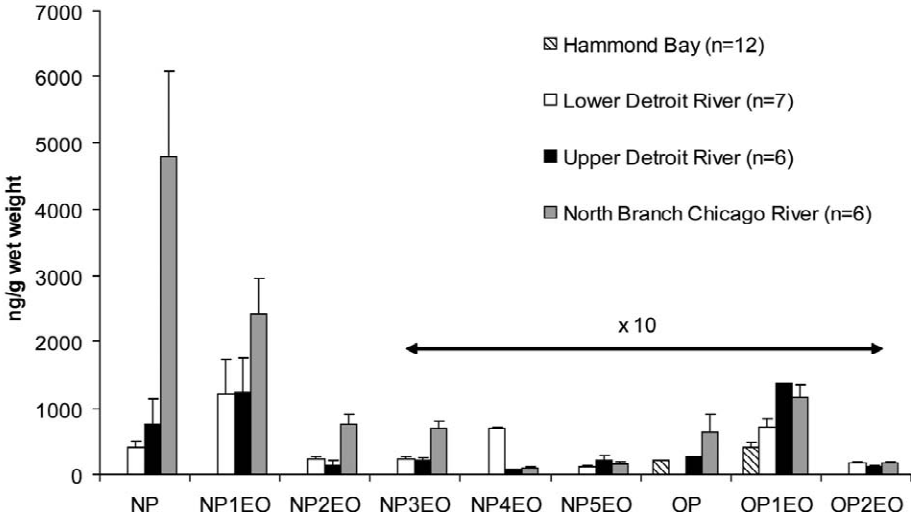

Fig. 5. APE concentrations in carp from the Great Lakes area (*n* is the number of samples; concentrations were multiplied by 10 from NP3EO to OP2EO; the standard error of the mean is indicated as error bars).

possible explanation for the higher LC–Fl values could be the coelution of OPs with the corresponding NPs. We observed that adding OPs to NP standards in concentrations representing 5 and 10% of the NPs resulted in 10 and 25% overestimation of the NPs concentration.

Carp samples from various effluent dominated waterways were extracted using acetonitrile ASE and analyzed by LC–MS–MS only (results in Fig. 5). Carp lipid concentrations were around 20% in North Branch Chicago River, IL, USA, and around 14% in upper Detroit River, MI, USA. The most contaminated site was North Branch Chicago River, located near the North Side sewage treatment plant for the district of Greater Chicago (capacity around 1300 million liters per day). Total APEs were 8.2 μg/g, among the highest concentrations found in the USA. Samples from Detroit River presented lower APEs concentrations of most compounds. In contrast to walleye, the most abundant homologues in carp were NP (Chicago River) or NP1EO (Detroit River). Total OPs in carp occurred also in lower than expected amounts (2 to 8% of the total NPs). Carp from the control site (Hammond Bay, MI, USA) showed residual levels of OP and OP1EO, which was unexpected and somewhat surprising because of the absence of NPs and requires additional confirmation.

4. Conclusions

New methods have been developed and validated for quantitation of individual alkylphenols and alkylphenolethoxylates in egg and fish matrices. These methods could be extended to other biological matrices using ASE followed by an adapted solid-phase cleanup. LC–MS–MS brings the high sensitivity and specificity required to determine these analytes in complex biological matrices and an accurate quantitation with the use of the appropriate ^{13}C-internal standards. The methods have been routinely applied in the laboratory to conduct studies on bioaccumulation of APEs in biota. Additional data are provided for osprey egg, carp and walleye contamination in the USA, especially regarding the higher ethoxylates and the OPs. These data have to be correlated to biological markers, such as vitellogenin levels, to evaluate the influence of APEs in fish. As for the osprey, a more complete study would have to be pursued to evaluate whether food chain buildup exists or not for APEs in osprey species.

Acknowledgements

This work was supported by the US Environmen-

tal Protection Agency, Region V, Great Lakes National Program Office; the USGS BEST (Biomonitoring of Environmental Status and Trends) Program); and J.E.L.-R. was supported by a Conacyt-Fulbright/Garcia-Robles scholarship provided by the Mexican and American governments. Most experimental work was performed at the Environmental Quality Laboratory, ARS, USDA. We would like to thank Steve Smith, Linda Begnoche, Dora Passino-Reader, Elizabeth Rice and Seema Datta for collecting the fish samples and Pamela C. Toschik, Jonathen B. Cohen and Robert Barber-Delach for collecting the osprey egg samples. We also thank P.L. Ferguson for kindly providing us with the internal standards.

References

[1] C.G. Naylor, J.P. Mieure, W.J. Adams, J.A. Weeks, F.J. Castaldi, L. Ogle, R.R. Romano, J. Am. Oil Chem. Soc. 69 (1992) 695.
[2] M. Ahel, J. McEvoy, W. Giger, Environ. Pollut. 79 (1993) 243.
[3] M.R. Servos, Water Qual. Res. J. Can. 34 (1999) 123.
[4] R. White, S. Jobling, S.A. Hoare, J.P. Sumpter, M.G. Parker, Endocrinology 135 (1994) 175.
[5] S. Gimeno, A. Gerritsen, T. Bowner, Nature 384 (1996) 221.
[6] K.M. Nichols, E.M. Snyder, S.A. Snyder, S.L. Pierens, S.R. Miles-Richardson, J.P. Giesy, Environ. Toxicol. Chem. 20 (2001) 510.
[7] J. Schwaiger, O.H. Spieser, C. Bauer, H. Ferling, U. Mallow, W. Kalbfus, R.D. Negele, Aquat. Toxicol. 51 (2000) 69.
[8] C.A. Staples, J. Weeks, J.F. Hall, C.G. Naylor, Environ. Toxicol. Chem. 17 (1998) 2470.
[9] E.R. Bennett, C.D. Metcalfe, Environ. Toxicol. Chem. 17 (1998) 1230.
[10] D.T. Bennie, Water Qual. Res. J. Can. 34 (1999) 79.
[11] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, Environ. Sci. Technol. 36 (2002) 1202.
[12] S.A. Snyder, T.L. Keith, C.G. Naylor, C.A. Staples, J.P. Giesy, Environ. Toxicol. Chem. 20 (2001) 1870.
[13] S. Datta, J.E. Loyo-Rosales, C.P. Rice, J. Agric. Food Chem. 50 (2002) 1350.
[14] M.A. Blackburn, S.J. Kirby, M.J. Waldock, Mar. Pollut. Bull. 38 (1999) 109.
[15] C.M. Lye, C.L.J. Frid, M.E. Gill, D.W. Cooper, D.M. Jones, Environ. Sci. Technol. 33 (1999) 1009.
[16] S. Tavazzi, E. Benfenati, D. Barceló, Chromatographia 56 (2002) 463.
[17] T. Tsuda, A. Takino, M. Kojima, H. Harada, K. Muraki, J. Chromatogr. B 723 (1999) 273.
[18] F. Ferrara, F. Fabietti, M. Delise, A.P. Bocca, E. Funari, Environ. Sci. Technol. 35 (2001) 3109.
[19] E.R. Bennett, C.D. Metcalfe, Environ. Toxicol. Chem. 19 (2000) 784.
[20] T. Tsuda, K. Suga, E. Kaneda, M. Ohsuga, J. Chromatogr. B 746 (2000) 305.
[21] S.A. Snyder, T.L. Keith, S.L. Pierens, E.M. Snyder, J.P. Giesy, Chemosphere 44 (2001) 1697.
[22] S.N. Pedersen, C. Lindholst, J. Chromatogr. A 864 (1999) 17.
[23] K. Guenther, V. Heinke, B. Thiele, E. Kleist, H. Prast, T. Raecker, Environ. Sci. Technol. 36 (2002) 1676.
[24] J.E. Loyo-Rosales, I. Schmitz-Afonso, C.P. Rice, A. Torrents, Anal. Chem., in press.
[25] P.L. Ferguson, C.R. Iden, B.J. Brownawell, Anal. Chem. 72 (2000) 4322.
[26] P.L. Ferguson, C.R. Iden, B.J. Brownawell, J. Chromatogr. A 938 (2001) 79.
[27] D.Y. Shang, M.G. Ikonomou, R.W. Macdonald, J. Chromatogr. A 849 (1999) 467.
[28] N. Furusawa, K. Okazaki, S. Iriguchi, H. Yamaguchi, M. Saitoh, J. AOAC Int. 81 (1998) 1033.
[29] G. Zweig (Ed.), Analytical Methods for Pesticides and Plant Growth Regulators, Vol. .XI, Academic Press, New York, 1980, p. 408.
[30] S.J. Lehotay, A.R. Lightfield, J.A. Harman-Fetcho, D.J. Donoghue, J. Agric. Food Chem. 49 (2001) 4589.
[31] S.A. Volz, J.J. Johnston, J. Sep. Sci. 25 (2002) 119.
[32] Solid Waste Test Methods, SW-846 with update III, CD-ROM 8000B-41 Revision 2, US EPA, 1996.
[33] C. Wahlberg, L. Renberg, U. Wideqvist, Chemosphere 20 (1990) 179.
[34] M. Petrovic, D. Barceló, J. Mass Spectrom. 36 (2001) 1173.
[35] M. Petrovic, A. Diaz, F. Ventura, D. Barceló, Anal. Chem. 73 (2001) 5886.
[36] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[37] F. Houde, C. Deblois, D. Berryman, J. Chromatogr. A 961 (2002) 245.
[38] E.W. Cromartie, W.L. Reichel, L.N. Locke, A.A. Belisle, T.E. Kaiser, T.G. Lamont, B.M. Mulhern, R.M. Prouty, D.M. Swineford, Pestic. Monit. J. 9 (1975) 11.
[39] R.T. Pedersen, E.M. Hill, Environ. Sci. Technol. 36 (2002) 3275.