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A Simple Method for the Determination of Trace Levels of Alkylphenolic Compounds in Fish Tissue Using Pressurized Fluid Extraction, Solid Phase Cleanup, and High-Performance Liquid Chromatography Fluorescence Detection

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A simple automated extraction method for the determination of alkylphenolic compounds in fish tissue is reported. Pressurized fluid extraction is used to extract ground fish tissue, and the resulting extract is purified on aminopropyl silica (APS) extraction cartridges. With no further sample preparation, nonylphenol (NP) and its ethoxylates, up to nonylphenol pentaethoxylate, are quantitated using normal phase (APS Hypersil) high-performance liquid chromatography with fluorescence detection. The major advantage of this technique is elimination of the conventional gel permeation cleanup step, a lengthy procedure designed to remove fish lipids. Spiked recoveries with lake trout averaged 85% for the six NP and NP ethoxylates that were investigated. Tissue concentrations of NP and NP ethoxylates determined in fish from various locations of the Great Lakes region ranged from 18 to 2075 ng/g, wet weight.

KEYWORDS: Alkylphenol ethoxylates; nonylphenol; non-ionic surfactants; fish

INTRODUCTION

Starting in about 1950, alkylphenol ethoxylates (APEs), a class of nonionic surfactants, have been widely utilized as industrial, agricultural, and household chemicals (*1*, *2*). They are used as detergents, emulsifiers, wetting agents, and dispersing agents in the manufacturing of plastics, textiles, pesticide formulations, and pulp and paper (*3*, *4*). Annual worldwide production is estimated at 500 000 tons, with United States production of 200 000 tons in 1990 (*1*, *5*). About 60% of the total surfactant production enters the aquatic environment (*1*) by way of industrial and municipal wastewater discharges. Sewage treatment plants (STP) biodegrade APEs to more persistent and toxic products, namely, nonylphenol (NP), nonylphenol monoethoxylate (NP1EO), nonylphenol diethoxylate (NP2EO), nonylphenoxyacetic acid (NP1EC), and nonylphenoxyethoxy acetic acid (NP2EC) and octylphenol (OP), octylphenol monoethoxylate (OP1EO), octylphenol diethoxylate (OP2EO), octylphenoxyacetic acid (OP1EC), and octylphenoxyethoxyacetic acid (OP2EC) (*6*).

NP and OP are acutely toxic to fish, invertebrates, and algae in the 17−3000 μg/L range and demonstrate chronic toxicity in some fish and invertebrates at concentrations as low as 3.7−6 μg/L (*7*). Of serious concern is exposure of these weakly estrogenic chemicals to aquatic organisms. With recent reports documenting the estrogenic activity of NP, OP, NP2EO, and NP1EC in fish (*4*, *8*−*10*), alkylphenolic degradation compounds have potentially emerged as a new class of toxic chemicals in the aquatic environment.

Additionally, aquatic organisms bioaccumulate APEs and their metabolites (*11*, *12*); thus, humans have the potential to be exposed via consumption of aquatic fauna. Other sources of human exposure include water supply and sewage sludge used for fertilizers.

To more accurately assess the risk of exposure to wildlife and humans, residues of contaminants in all environmental compartments need to be determined. While the occurrence of APE metabolites in wastewater and sludge from STPs and in freshwater and sediment have been well-studied, such data are lacking for biota. This is due in large part to the fact that over the past 20 years many analytical methods have been developed for determining environmental concentrations of alkylphenolics in wastewater and water but methods for biological samples have not been similarly developed.

Previous methods for analysis of alkylphenolic residues in biota have relied on lengthy extractions or tedious cleanup steps to remove lipids. Lye et al. (*13*) employed steam distillation to extract APEs from fish but were able to only report concentrations of NP, OP, and NP1EO. For mussels, Wahlberg et al. (*14*) used 0.1 N sodium hydroxide and acetonitrile partitioning and

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reported NP, NP1EO, NP2EO, and NP3EO. This procedure may not be suitable for fish tissue containing a higher percentage of fat as compared to mussels.

Gel permeation chromatography (GPC) has been used extensively to separate PCBs and chlorinated pesticides from fish lipids, and this technique has been extended to alkylphenolic compounds (*15*−*17*). Shiraishi et al. (*16*) analyzed for *tert*-pentylphenolic and chlorinated *tert*-pentylphenolic residues in carp from the Detroit River using gas chromatography/mass spectrometry (GC/MS); however, nonyl and octylphenolic compounds were not determined. In analyzing salmon, McLeese et al. (*15*) reported NP levels alone employing a GC method with flame ionization detection. Shang et al. (*17*) could not quantitate any alkylphenolic compounds by liquid chromatography (LC)/MS in fish due to problems of interfering lipid components. This study investigates the suitability of GPC for the analysis of alkylphenolic compounds in fish and reports an automated extraction and rapid solid phase extraction cleanup technique for the analysis of NP and its ethoxylates, up to nonylphenol pentaethoxylate (NP5EO).

EXPERIMENTAL SECTION

Reagents. Standards were purified from commercially available preparations as follows: NP (branched nonyl) was secured from Schenectady International. Distilled NP1EO (95.5% pure) called Surfonic N-10 (7283-78B) and a mixture containing NP1EO to NP4EO, called Surfonic N-10 (7202-96C), were provided by the Huntsman Corporation. These two mixtures required further cleanup as discussed below. NP2EO was supplied by the U. S. Environmental Protection Agency Region V who had it synthesized by Aldrich Chemical Co. POE (4) NP was obtained from Chem Services. This standard was a mixture of ethoxylated NPs containing predominantly NP4EO with lesser quantities of NP5EO and NP3EO. This mixture was the source of NP4EO and NP5EO, which were purified by silica gel chromatography. Safety consideration: all preparations of APEs were conducted in a fume hood employing gloves and safety glasses. Solvents were all pesticide grade (Burdick & Jackson or Fisher, optima grade). Anhydrous sodium sulfate, 10−60 mesh (Fisher), was oven-baked for 4 h at 400 °C.

Purification of Authentic Standards. NP1EO was purified from 300 mg of Surfonic N-10 (7283-78B) by silica gel chromatography. A 21 cm plug of silica gel (60 g, 0.040−0.063 mm) (EM Science) was prepared in ethyl acetate/hexane (20/80) in a flash chromatography glass column (1.9 cm × 46 cm). A 1 cm plug of sea sand (VWR) was placed between the silica gel and a glass wool plug. Two bed volumes of ethyl acetate/hexane (20/80 v/v), the elution solvent, was passed through the column to remove any impurities. Nineteen 12 mL fractions were collected and checked for purity by thin-layer chromatography (TLC) and PMA (phosphomolybdic acid and permanganate) indicator (Aldrich Chemical Co). TLC plates were stained in PMA solution and then charred by a heat gun to reveal spots of the standards and analytes.

High-purity fractions were confirmed by high-performance liquid chromatography (HPLC) fluorescence analysis and then pooled in weighed test tubes, and the solvent was evaporated. NP3EO was similarly purified using 400 mg Surfonic N-10 (7202-96C). NP4EO and NP5EO were purified from 1.5 g POE (4) by flash chromatography using a silica gel (130 g, 0.040−0.063 mm) (EM Science) 3.5 cm × 53 cm glass column. A step gradient elution was performed with ethyl acetate/hexane (50/50 v/v) to ethyl acetate. A second step gradient of ethyl acetate to ethanol eluted NP4EO and NP5EO. Each of the isolated components was confirmed for purity by HPLC fluorescence and GC/MS. Stock standard solutions were prepared using neat laboratory purified standards (NP1EO, NP3EO, NP4EO, and NP5EO), Aldrich purified NP2EO, and commercially available *para*-NP.

GPC. Two GPC column setups were employed. Column 1 was a glass column (70.0 cm × 2.5 cm) packed with approximately 60 g of Bio-Beads SX-3 (Bio-Rad). Sample was introduced with a Rheodyne four-way rotary valve injector equipped with a female luer adaptor and a 5 mL sample loop. The mobile phase of cyclohexane/methylene chloride (50/50 v/v) was pumped at 5 mL/min. Column 2 was a low-pressure Teflon column of 2 cm × 32 cm dimensions (OI Analytical) prepared with 24 g SX-3. This column was operated on an automated GPC cleanup system, ABC Autoevap AS 2000 with a 2.5 mL sample loop. Cyclohexane/methylene chloride (50/50 v/v) at 4.5 mL/min served as the mobile phase. UV detection at 230 nm was achieved with a Waters 490 Multiwavelength detector. Components tested were NP, *tert*-OP, Surfonic N-10 (7283-78), POE (4) NP, POE (3) *tert*-OP (Chem Service), and POE (5) *tert*-OP (Chem Service). Methylene chloride extracts of lake trout were rotoevaporated to approximately 1 mL and then diluted in the mobile phase of the GPC column. Recovery experiments with the automated GPC cleanup method were determined in 2.5 g lake trout extracts spiked with 0.95 μg/g NP. Fractions containing NP were pooled and rotoevaporated to 5 mL before quantitation by HPLC.

Sample Preparation and Automated Extraction. Frozen fish was thawed for 12 h and homogenized in a Hobart VCM 40 Vertical Chopper following the method of Hesselberg (*18*). All equipment rinses were done without detergent to avoid sample contamination. Fish homogenate was placed in 125 mL I-Chem glass jars and stored frozen. Thawed homogenized fish tissue was mixed with Na_2SO_4 (1/4 w/w) in an industrial chopper (Robot Coupe, Scientific Industrial Division). A 35 g sample of this ground mixture was packed into a stainless steel cell for accelerated solvent extraction (Dionex) using the following conditions: static 10 min, pressure 6.9 MPa, cycles 3, flush volume 90%, purge 200 s, methylene chloride extraction. Methylene chloride extracts were rotoevaported to approximately 1 mL. Concentrated fish extracts were first diluted to 7.0 mL in hexane. A 0.5 mL aliquot was separated for percent lipid determination. Hatchery-reared lake trout from Jordan River, MI served as "clean" fish for spike and recovery studies.

Solid Phase Extraction. Two cleanup cartridge systems were tested; (i) diol (Varian), 500 mg, 3 mL, and (ii) aminopropyl silica (APS) (Supelco) also 500 mg, 3 mL. Cartridges were cleaned and conditioned with 3 mL each of acetone, methylene chloride, and hexane. The 6.5 mL fish extract was divided into three equal portions (∼2.2 mL) and loaded onto three conditioned cartridges. This step was identical for both cleanup cartridge systems. For diol cleanup, cartridges were conditioned with 3 mL of methylene chloride/hexane (9/1 v/v). After the fish extract was passed through the cartridge, a 1 mL rinse of hexane was added. The cartridge was eluted with 6 mL hexane/2-propanol (90:10 v/v). Eluants from the three cartridges were pooled and evaporated under a stream of nitrogen to a final volume of 5 mL. For the APS cleanup, a 1 mL hexane rinse was also added to the cartridge, and an additional 3 mL of hexane was used to wash the cartridge. Analytes of interest were eluted with 7 mL (90/10 v/v) hexane/2-propanol. Eluants from the three cartridges were pooled and nitrogen-evaporated to a 4 mL final volume.

HPLC. Initial HPLC analyses were performed by a Hewlett-Packard HPLC system consisting of a 1050 series pump, 1050 autosampler, 1046A fluorescence detector, and LC Chemstation. A 25 μL sample was chromatographed isocratically on a Hypersil APS (Hewlett-Packard) normal phase column, 4.6 mm × 100 mm i.d., 5 μm particle size with hexane:ethanol (98/2 v/v) pumped at a flow rate of 1.1 mL/min. Fluorescence detection was achieved by 230 nm excitation wavelength and 300 nm emission. Subsequent HPLC analyses were performed on the same column but using a Waters HPLC system consisting of a 616 pump, 600S Controller, 717 plus Autosampler, 474 scanning fluorescence detector, and Millennium 32 workstation. A 25 μL sample was chromatographed at 1.0 mL/min under gradient conditions: initial conditions, hexane/2-propanol (98/2 v/v) for 0−10 min; hexane/ 2-propanol (90:10 v/v) from 10 to 25 min; and return to initial conditions for 10 min. Detector settings were unchanged. HPLC does not separate the individual isomers of the nonyl group of NP and NP ethoxylates as does GC.

Standards were prepared in clean fish matrix extracts that came from the procedural blank extracts. This was done to compensate for possible enhacements or suppressions to response that may be caused by the

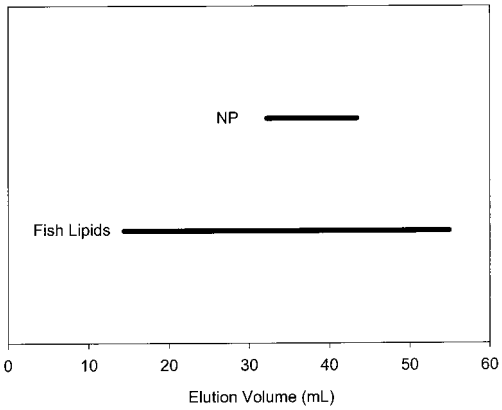

Figure 1. GPC elution profile of NP and fish lipids on a 24 g SX3-Bio-Beads, 2 cm × 32 cm column, cyclohexane:methylene chloride, flow 4.5 mL/min.

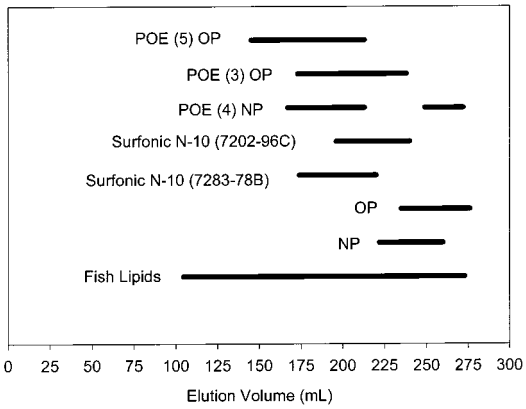

Figure 2. GPC elution profiles of selected alkylphenol surfactants and fish lipids on 60 g SX-3 Bio-Beads, 55 cm × 2.5 cm column, cyclohexane: methylene chloride, flow 5.0 mL/min.

coextracted matrix materials. Quantitation was achieved using an external standard method.

GC/MS. GC/MS was used for confirmation of the LC analytes. Purified standards and sample extracts were derivatized with pentafluorobenzoyl chloride according to a procedure modified from Wahlberg et al (*14*). The major modification involved replacing 0.1 N sodium hydroxide base with a buffered 0.1 M sodium borate. This reduced emulsion formation with the residual fish lipids in the extracts. Pentafluorobenzoyl derivatives of NP and the ethoxylates were confirmed by NCI/GC/MS using a Hewlett-Packard 5890A gas chromatograph and Hewlett-Packard 5989A mass spectrometer. A J&W Scientific DB-17MS column (30 m × 0.25 mm i.d., film thickness of 0.25 μm) was employed. Methane was the ionizing gas at 1.8 Torr and source-operated at 250 °C. The other heated zones were set as follows: injector at 250 °C and transfer line at 280 °C. The GC oven temperature program is as follows: 130 °C for 4 min programmed to 170 °C at 20 °C/min and then 7 °C/min to 250 °C followed by a 10 °C/min to 300 °C with 20 min hold, for a total run time of 42.43 min. The analytes were determined by selective ion monitoring methods, including the verification (14−35%) of the significant $n + 1$ *m/z* for each of the analytes, for example, 414/415 *m/z* for NP, 458/459 *m/z* for NP1EO, 502/503 *m/z* for NP2EO, 546/547 *m/z* for NP3EO, 590/591 *m/z* for NP4EO, and 634/635 *m/z* for NP5EO.

RESULTS AND DISCUSSION

GPC. Automated GPC to remove lipids from the extracts was initially investigated. Chromatograms of the NP standard and the lipid extract in **Figure 1** show substantial overlap of NP and the fish lipid bands indicating the inadequacy of this method for cleanup. To determine NP recovery, spiked (0.95 μg/g) lipid extract from 2.5 g of lake trout tissue was chromatographed by GPC followed by HPLC. Percent recovery was high, 114 ± 8 ($n = 3$); however, the NP fraction was visibly yellow due to the presence of fish lipids.

In an effort to improve efficiency of separation, a larger Bio-Beads column operated on a nonautomated setup was employed. Chromatographic separation of a number of alkylphenol polyethoxylates and a lake trout lipid extract representing 7 g of fish are shown in **Figure 2**. Although there is improved separation of the NP and the fish extract band, the two components still overlap. Other alkylphenolic mixtures were characterized including ethoxylates of NP (Surfonic N-10 (7283-78B) and Surfonic N-10 (7202-96C)), POE (4) NP, POE (3) *tert*-OP, POE (5) *tert*-OP, and *tert*-OP. **Figure 2** illustrates coelution of all of these compounds with fish lipid.

Results of both the automated and the nonautomated GPC for cleanup of fish lipids for the analysis of NP and other alkylphenol polyethoxylates indicate that this method is inadequate for removing interfering lipids from the analytes of interest. Moreover, GPC even with automation is an expensive and time-consuming step that requires high consumption of solvents and, therefore, cannot be justified as a technique for routine analysis of alkylphenolics in fish tissue. Another limitation is the amount of sample that can be analyzed. The high-efficiency columns available for the automated GPC system have restricted loading of lipid material. The sample extract should contain <0.5 g of lipid. This means that a sample of only up to 5 g of fish tissue containing 10% fat could be cleaned up by this method. For residue analysis, this could be particularly limiting.

Table 1. Spike, Recovery, LOD,[a] MDLs,[b] Relative Standard Deviations, and Linearity of Detector Response[c] of Nonylphenolic Compounds in Spiked Matrix[d]

component	spike (ng/g)	recovery (%)	LOD (ng/mL)	MDL (ng/g)	RSD (%)	corr coeff
NP	360	103	4	5	3	0.999
NP1EO	270	94	3	8	7	0.991
NP2EO	360	125	3	20	10	0.999
NP3EO	900	89	15	21	6	0.986
NP4EO	1350	100	15	32	6	0.999
NP5EO	1350	74	7	44	10	0.955

[a] LOD values correspond to an instrument signal/noise ratio of three. [b] MDLs calculated based on $\alpha = 0.99$. Calibration curves made with spiked matrix containing the following concentrations: 80, 96, 120, and 140 ng/mL for NP and NP2EO; 60, 72, 90, and 120 ng/mL for NP1EO; 200, 240, 300, and 400 ng/mL for NP3EO; and 300, 360, 450, and 600 ng/mL for NP4EO and NP5EO. [c] Linearity of detector response to concentration of calibration standards expressed as correlation coefficient (r^2) for the least squares equation. [d] All analytical results with the exception of the correlation coefficient are based on eight replicates.

Detection Limits, Precision, and Linearity. The diol cartridges were found to be not as effective in removing lipid as the APS cartridges as demonstrated by the presence of interfering lipid components in the HPLC chromatograms. APS cartridges were subsequently employed for all recovery and quantitation work. **Table 1** summarizes the recoveries of nonylphenolic compounds from spiked fish matrix. Hatchery-reared lake trout served as a clean blank for recovery studies. Spiked and unspiked hatchery lake trout HPLC chromatograms are shown in **Figure 3**. Recoveries for eight replicates were 74−125%. High precision was achieved using an external matrix calibration procedure as indicated by the low relative standard deviations, ≤10%, for all analytes tested. On a wet tissue basis, the method detection limits (MDLs; $n = 8$) for NP and the NP

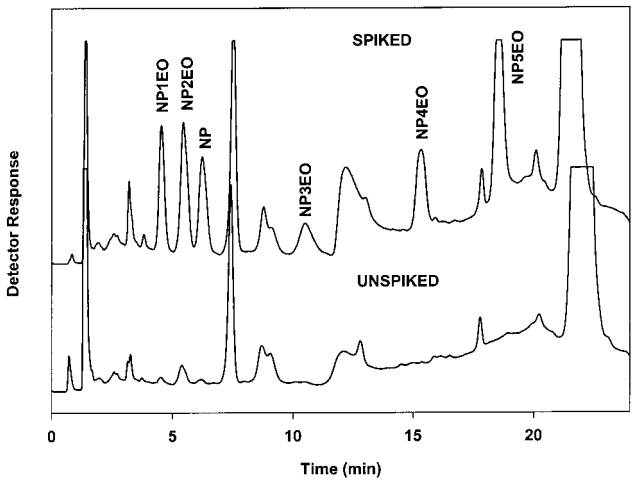

Figure 3. HPLC chromatograms of spiked and unspiked hatchery lake trout.

Table 2. Concentrations of Nonylphenolic Compounds in Fresh Water Fish (ng/g wet wt)[a]

sample	location	% lipid	NP	NP1EO	NP2EO	NP3EO
carp 41	Detroit River, MI	17.9	1842	2075	567	402
carp 79	Grand Calumet, IL	2.6	223	700	295	nd
carp 91	Des Plaines River, IL	7.2	248	248	473	nd
yellow perch	Milwaukee Harbor, WI	na	119	87	18	nd

[a] nd = not detected and na = not available.

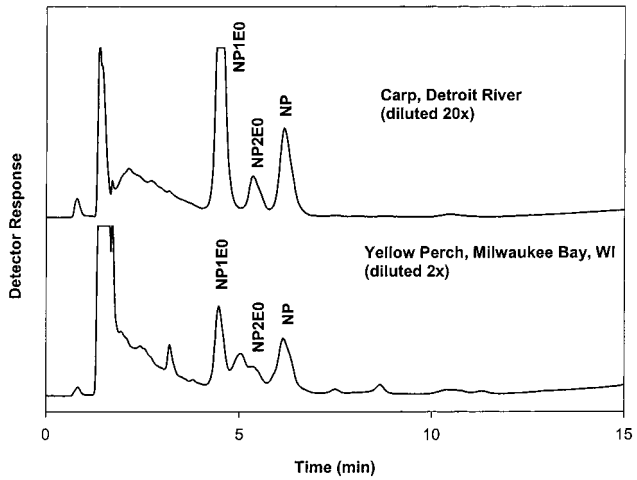

Figure 4. HPLC chromatograms of carp (diluted 20×) from the Detroit River and yellow perch (diluted 2×) from Milwaukee Bay, WI.

ethoxylates ranged from 5 to 44 ng/g fish. The limits of detection (LOD) (*19*) corresponded to the instrument signal-to-noise ratio of three. Estimated LODs of 4−15ng/mL (or 34−171 ng/g) were achieved for the nonylphenolic compounds. All calibration curves were prepared in fish matrix. Linearity of the calibration curve was investigated over the range of 60−140 ng/mL for NP, NP1EO, and NP2EO; 200−400 ng/mL for NP3EO; and 300−600 ng/mL for NP4EO and NP5EO. With the exception of NP5EO, the correlation coefficients were all better than 0.986.

Environmental Samples. Residues of NP and its ethoxylates, up to NP5EO, were determined in lake trout, common carp, and yellow perch from various locations in the Great Lakes region. Concentrations of NP, NP1EO, NP2EO, and NP3EO in four fish samples ranged from 18 to 2075 ng/g wet weight (**Table 2**). HPLC chromatograms of typical common carp and yellow perch are found in **Figure 4**. The total concentration of NP compounds measured in freshwater fish from the more polluted sites was summed to be as high as approximately 5 μg/g wet weight. Results of elevated levels of nonylphenolic compounds in fish suggest that these compounds are persistent, hydrophobic, and bioaccumulating in aquatic organisms. Having simple and rapid analytical methods for measuring environmental contaminants becomes important in monitoring studies to determine their geographic distribution, sources, trends, environmental fate, and biological significance.

CONCLUSIONS

A more rapid and simple method for NP and NP ethoxylates analysis using solid phase extraction instead of GPC residue cleanup is described. NP binds to APS, allowing facile cleanup of fish lipids. Elution with a polar solvent mixture elutes the analytes. This procedure can be used for routine analysis and monitoring of NP and its ethoxylates in fish tissue. Although not all lipid material is removed with the cartridge system, selectivity of the fluorescence detection overcomes interference problems associated with the matrix. Furthermore, this HPLC technique avoids the use of more costly MS detection and the more involved steps necessary for GC/MS analysis such as derivatization steps.

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