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IDENTIFICATION AND QUANTITATION METHOD FOR NONYLPHENOL AND LOWER OLIGOMER NONYLPHENOL ETHOXYLATES IN FISH TISSUES

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Abstract—Substantial research is currently focused on the toxicological effects of alkylphenol ethoxylates (APEs) and alkylphenols (APs) on aquatic animals. Considerable data are available on the concentrations of APEs and APs in river systems in the United States; however, few if any data are available on the tissue concentrations of fish living in these rivers. A reliable method for the analysis of nonylphenol (NP) and lower oligomer nonylphenol ethoxylates ($NPE_{1–3}$) in fish tissues has been developed. Nonylphenol and $NPE_{1–3}$ were extracted from fish tissues using extractive steam distillation. Normal phase high-performance liquid chromatography (HLPC) was used as a cleanup step prior to analysis by gas chromatography with mass selective detection (GC/MSD) using selected ion monitoring. Optimization of this technique resulted in consistent recoveries in excess of 70%, with the exception of NPE_3 (17%). Method detection limits (MDLs) and limits of quantitation using the technique range from 3 to 20 and 5 to 29 ng/g wet weight, respectively. Nonylphenol and NPE_1 were detected in subsamples ($n = 6$) of a single common carp captured in the Las Vegas Bay of Lake Mead (NV, USA) at average concentrations of 184 ± 4 ng/g and 242 ± 9 wet weight, respectively. Nonylphenol $ethoxylates_{2\&3}$ were not detected in the carp collected at Lake Mead.

Keywords—Alkylphenols Method Bioconcentration Fish Tissue

INTRODUCTION

Alkylphenol polyethoxylates (APEs) are nonionic surfactants widely used in various industrial (55% of total demand), institutional (30% of total demand), and household applications (15% of total demand) [1]. In 1998, U.S. usage of APEs was approximately 250 million kg (Chemical Market Reporter, October 18, 1999, p 15). The APEs are primarily used as surfactants, which can function as detergents, wetting agents, dispersants, emulsifiers, solubilizers, and foaming agents. These surfactants are used in many industrial applications, including pulp and paper, textiles, coatings, agricultural pesticides, lubricating oils and fuels, metals, and plastics. Most of the APEs used are of the nonylphenol polyethoxylate (NPE) type that contain a nine-carbon branched isomeric alkyl group [1,2]. The NPEs and the less widely used octylphenol polyethoxylates (OPEs) degrade during wastewater treatment or in the environment through transient intermediates including alkylphenol ethoxycarboxylates (APECs), lower oligomer APEs such as $NPE_{(1–3)}$, and alkylphenols (APs) such as octylphenol (OP) and nonylphenol (NP) (Fig. 1) [2–5]. The type and efficiency of wastewater treatment affects the degree of APE removal during treatment [6].

Several reports have linked APs and certain APEs to adverse effects on aquatic organisms [1,7–9]. Both NP and OP have been shown to be estrogenic in a variety of both in vitro [9–13] and in vivo bioassays [14,15]. Some oligomers of NPE have also been found to be estrogenic in vitro [9,14,16,17] and in vivo [14].

The most comprehensive survey from the United States reported concentrations of NP and NPEs from 30 rivers that are influenced by municipal or industrial wastewater effluents [2]. That study found that 60 to 75% of samples had no detectable levels of NP, NPE_1, or NPE_2 [2]. However, data are scarce on the concentrations of APs and APEs in fish in U.S. waters. Recently, NP and NPE have been reported to occur in the Las Vegas Bay of Lake Mead [18]. Because of this known contamination, it was determined to be a suitable site to capture fish likely to have detectable NP and NPE tissue concentrations.

The objective of this study was to develop a reliable and simple method to sensitively detect and quantify NP and $NPE_{(1–3)}$ in the tissues of fish. Soxhlet extraction followed by gel permeation chromatography was initially investigated; however, all attempts were unsuccessful [19]. Extractive steam distillation has been used previously for the extraction of alkylphenols from sediment, water, and fish tissue [20–22]. Extractive steam distillation minimizes the coextraction of large molecular weight interferences (such as lipids) while efficiently extracting compounds with sufficient volatility [23]. This method also uses far less solvent than would be required for Soxhlet extraction [19]. After extensive trial-and-error-method development [19], an extractive steam distillation coupled with removal of coextractives by preparative normal-phase high-performance liquid chromatography (HPLC) and quantitation using gas chromatography with mass selective detection (GC/MSD) was chosen. This method proved to be reliable and sensitive for the identification and quantitation of NP and $NPE_{(1–3)}$ in fish tissues. Goldfish spiked with various concentrations of the compounds of interest were used for method development. Carp captured from the Las Vegas Bay of Lake Mead were used for method validation.

MATERIALS AND METHODS

Standards and reagents

All standards and reagents used were of the highest purity commercially available. High-purity standards (~96% or

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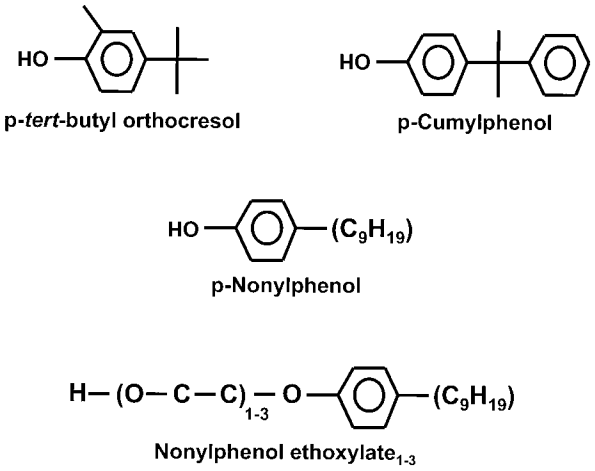

Fig. 1. Structures of compounds of interest.

greater purity) of *p*-nonylphenol (NP), *p*-cumylphenol, and 4-*tert*-butyl orthocresol (Fig. 1) were obtained from Schenectady International (Schenectady, NY, USA). Standards of $NPE_{(1–3)}$ were obtained from Huntsman Corporation (Austin, TX, USA). Pesticide residue-grade hexane, dichloromethane, methanol, and isooctane were obtained from Burdick and Jackson (Muskegon, MI, USA). Reagent water was first purified by reverse osmosis followed by Nanopure™ (Barnstead, Dubuque, IA, USA) treatment.

Sample collection and preservation

Laboratory-raised goldfish (*Carassius auratus*) were chemically euthanized using tricaine methane sulphonate (Finquel™, Argent Chemical Laboratories, Redmond, WA, USA), then frozen until analysis. Several frozen goldfish (5–20 g each) were thawed prior to homogenization (Blender 700, Waring, New Hartford, CT, USA) to form a pool of tissues from which subsamples were removed for extraction. Common carp (*Cyprinus carpio*) from Las Vegas Bay of Lake Mead were captured in June of 1999 via gill nets, euthanized with tricaine methane sulphonate, then frozen until analysis. An approximately 150-g centered cross-section from a large carp (~1 kg) captured from the Las Vegas Bay of Lake Mead was homogenized.

Extraction

Twenty grams of the fish homogenate was blended with 350 ml of laboratory water for 2 min. The resulting mixture was added to a 2-L boiling flask with 20 g sodium chloride (ACS reagent grade, J.T. Baker, Phillipsburg, NJ, USA), 3 ml concentrated sulfuric acid (GR grade, EM Science, Gibbstown, NJ, USA), several glass boiling chips (3 mm diameter, Pyrex, Big Flats, NY, USA), and a Teflon™-coated stir bar. An additional 650 ml of laboratory water was added to the homogenizer in small portions to rinse and transfer any remaining homogenate into the 2-L boiling flask. Five hundred nanograms of *p*-cumylphenol was added to the boiling flask mixture as a surrogate standard.

The mixture described above was placed onto a heating mantle with a magnetic stir plate below the mantle. A Nielsen–Kryger improved version steam-distillation column (Ace Glass, Vineland, NJ, USA) was attached to the round-bottom flask containing the sample. Three milliliters of laboratory water and 10 ml of isooctane were added to the steam-distillation column. Tap water was circulated through the condenser at a fast flow rate. The heating mantle was operated on the maximum setting and the stir plate speed was set to approximately 50%. The mixture was boiled for 1.5 h and the water layer in the distillation column discarded. The isooctane layer was collected in a 15-ml centrifuge tube. An additional 3 ml of laboratory water and 10 ml of isooctane were added to the distillation column. The mixture was then boiled again for 1.5 h and the isooctane layer combined with the initial extract. The combined extract was concentrated to 1 ml at 30°C under a gentle stream of purified nitrogen and stored at −20°C until analysis.

Table 1. Ions monitored and recovery data ($n = 7$)[a]

Compound	Ions (m/z)	Spike (ng/g)	Recovery % CV
NP	107, 135, 149	15.4	78.1 ± 9.21
NPE_1	135, 179, 193	74.8	76.1 ± 9.84
NPE_2	135, 223, 237	67.2	69.4 ± 13.0
NPE_3	135, 267, 281	40.0	17.0 ± 20.1
TBC	149, 164, 121	NA	NA

[a] NP = nonylphenol; NPE_1 = nonylphenol monoethoxylate; NPE_2 = nonylphenol diethoxylate; NPE_3 = nonylphenol triethoxylate; TBC = 4-*tert*-butyl orthocresol; CV = coefficient of variation; NA = not applicable.

Normal-phase HPLC fractionation

The normal-phase HPLC fractionation system consisted of a Perkin-Elmer (Norwalk, CT, USA) series 200 autosampler, a 200 binary pump (Perkin-Elmer), a Hewlett-Packard ([HP] Avondale, PA, USA) 1046A fluorescence detector, and TurboChrome 4.0 data software package (Perkin-Elmer). An 800-μl injection volume of the isooctane extract was separated using a Phenomenex Luna 5-μm silica column (250 mm × 4.6 mm, Torrance, CA, USA) by isocratic elution with a binary mixture of 88% hexane and 12% 1:4 methanol:dichloromethane at a flow rate of 0.65 ml/min. Fluorescence detection was used to determine surrogate recovery during this fractionation at an excitation wavelength of 229 nm and an emission wavelength of 310 nm. The HPLC effluent containing the analytes of interest was collected during the time period of 7 to 16 min. To this fraction, 3.0 μg of 4-*tert*-butyl orthocresol was added as an internal standard. This fraction was then concentrated using a gentle stream of purified nitrogen to 100 μl isooctane.

Identification and quantitation

Compounds of interest were identified and quantified using a HP 5890 series II plus GC and a HP 5972 MSD. Separation was accomplished using a 30-m DB-17MS capillary column (0.25-mm i.d., 0.15-μm film, J&W Scientific, Folsom, CA, USA) and a 4-μl injection volume. The GC was programmed with a starting temperature of 100°C for 2 min followed by a 4°C/min temperature ramp to a final temperature of 300°C that was held for 10 min. The MSD was operated in selected ion monitoring mode with three ions monitored for each compound of interest (Table 1). Identification was made by proper retention time and ion ratios (Fig. 2). Peak areas were determined by electronic integration, and concentrations were determined using external calibration and Microsoft Excel spreadsheets (Version 7; Microsoft, Redmond, WA, USA). Quantitation for NP and NPE was based on the sum of the peak areas for all isomers for each compound (Fig. 2). Five point calibration

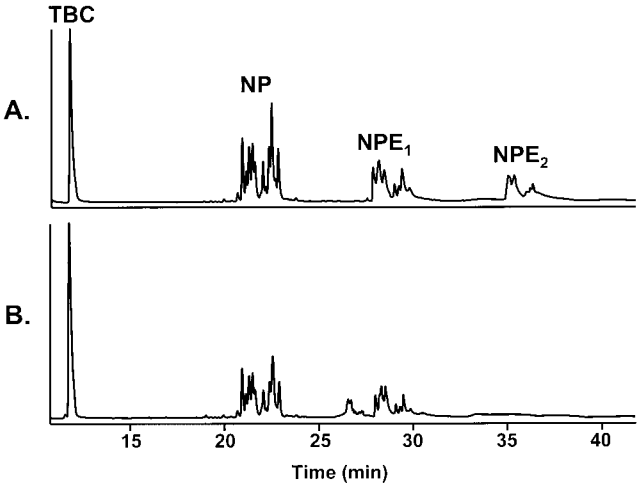

Fig. 2. Chromatograms of nonylphenol (NP), nonylphenol mono- and diethoxylate ($NPE_{(1–2)}$), and 4-*tert*-butyl orthocresol (TBC). (**A**) Standards. (**B**) Las Vegas Bay carp tissue extract.

curves were linear ($r^2 > 0.98$) across the range of concentrations used to bracket the sample concentrations. Where necessary, NP and NPE results were adjusted for recovery of the internal standard.

Recovery and detection limits

Instrument detection limits were defined as the minimum mass of analyte required to yield a signal to noise ratio of three. Seven replicates of homogenized goldfish tissues were spiked with NP and $NPE_{(1–3)}$ (dissolved in 50 μl of methanol) at the estimated method detection limits (MDLs) to determine recovery and precision (Table 2). The MDLs were calculated by multiplying the standard deviation of the recovered concentrations by a *t* value of 3.1427 (for $n = 7$ replicates) [24]. The limits of quantitation were calculated by adding five standard deviations to the MDL.

RESULTS AND DISCUSSION

It was determined that two successive distillations with fresh isooctane of 1.5 h each were more efficient than one 3-h distillation [19]. Recoveries of the compounds of interest using two distillations were consistently greater than 70%, with the exception of NPE_3 (Table 1). The coefficient of variation in recoveries was less than 20% (Table 1). The lesser recovery of NPE_3 is a function of its lesser volatility and lipophilicity. Initial method development with steam distillation resulted in extreme foaming that caused contamination of the isooctane extract. While silicon-based antifoaming agents successfully reduced foaming, recoveries were reduced and inconsistent. The addition of concentrated sulfuric acid reduced foaming and did not negatively affect recovery or precision. Although cooling the distillation condensers resulted in slightly greater recovery, the improvement was small.

Table 2. Detection limits for compounds of interest ($n = 7$)[a]

Compound	IDL (ng)	MDL (ng/g)	LOQ (ng/g)
NP	5.12	3.30	4.82
NPE_1	15.5	16.8	18.5
NPE_2	17.3	18.2	20.6
NPE_3	112	20.6	28.9

[a] NP = nonylphenol; NPE_1 = nonylphenol monoethoxylate; NPE_2 = nonylphenol diethoxylate; NPE_3 = nonylphenol triethoxylate; IDL = instrument detection limit; MDL = method detection limit; LOQ = limit of quantitation.

Table 3. Average concentrations of NP and NPEs in Lake Mead (NV, USA) carp tissues ($n = 6$)[a]

Compound	ng/g wet wt	% CV
NP	184	4.4
NPE_1	242	9.3
NPE_2	ND	NA
NPE_3	ND	NA

[a] NP = nonylphenol; NPE_1 = nonylphenol monoethoxylate; NPE_2 = nonylphenol diethoxylate; NPE_3 = nonylphenol triethoxylate; CV = coefficient of variation; MDL = method detection limit; ND = not detectable at MDL; NA = not applicable.

Nonylphenol and NPE_1 were detected in the tissue from a carp captured in Las Vegas Bay (Table 3). The variability in concentrations of NP and NPE_1 among the subsamples from a single fish was small (Table 3). These concentrations observed are reasonable, based on reported water concentrations ranging from 160 to 750 ng/L for NP and 3,180 to 4,850 ng/L for NPE in Las Vegas Bay [18] and published bioconcentration factors of 100 to 300 for NP [8,25,26]. Although concentrations of NP and NPE in sediments of Las Vegas Bay are unknown, it is possible that sediment-bound NP and NPE affected the carp tissue concentrations since carp are bottom feeders and these compounds are known to accumulate in sediments [5,20,27–29].

The method presented here was successful for sensitive and reproducible measurements of concentrations of NP and $NPE_{(1,2)}$ in fish tissues. Because NP and NPE are structurally similar to lipids, conventional methods that would coextract lipids require extensive clean-up to be amenable to GC/MSD analysis. The method presented here minimizes coextraction of lipids while providing detection limits sufficient to determine toxicologically relevant concentrations. This analytical method is amenable for use in most environmental laboratories with access to HPLC and GC/MSD instrumentation.

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REFERENCES

1. Talmage SS. 1994. *Environmental and Human Safety of Major Surfactants: Alcohol Ethoxylates and Alkylphenol Ethoxylates.* Lewis, Boca Raton, FL, USA.
2. Naylor CG, Mieure JP, Adams WJ, Weeks JA, Castaldi FJ, Ogle LD, Romano RR. 1992. Alkylphenol ethoxylates in the environment. *JAOCS* 69:695–703.
3. Field JA, Reed RL. 1996. Nonylphenol polyethoxy carboxylate metabolites of nonionic surfactants in U.S. paper mill effluents, municipal sewage treatment plant effluents, and river waters. *Environ Sci Technol* 30:3544–3550.
4. Rudel RA, Melly SJ, Geno PW, Sun G, Brody JG. 1998. Identification of alkylphenols and other estrogenic phenolic compounds in wastewater, septage, and groundwater on Cape Cod, Massachusetts. *Environ Sci Technol* 32:861–869.
5. Bennie DT, Sullivan CA, Lee HB, Peart TE, Maguire RJ. 1997. Occurrence of alkylphenols and alkylphenol mono- and diethoxylates in natural waters of the Laurentian Great Lakes basin and the upper St. Lawrence River. *Sci Total Environ* 193:263–275.
6. Naylor CG. 1995. Environmental fate and safety of nonylphenol ethoxylates. *Textile Chem Colorists* 27:29–33.

7. Weeks JA, Adams WJ, Guiney PD, Hall JF, Naylor CG. 1998. Risk assessment of nonylphenol and its ethoxylates in U.S. river water and sediment. *Alkylphenols Alkylphenol Ethoxylates Rev* 1:64–74.
8. Staples CA, Weeks J, Hall J, Naylor C. 1998. Evaluation of aquatic toxicity and bioaccumulation of C_8- and C_9-alkylphenol ethoxylates. *Environ Toxicol Chem* 17:2470–2480.
9. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. 1994. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrine* 135:175–182.
10. Mueller GC, Kim UH. 1978. Displacement of estradiol from estrogen receptors by simple alkylphenols. *Endocrine* 102:1429–1435.
11. Soto AM, Lin TM, Justicia H, Silvia RM, Sonnenschein C. 1992. An ''in culture'' bioassay to assess the estrogenicity of xenobiotics (e-screen). In Colburn T, Clement C, eds, *Chemically Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection—Advances in Modern Environmental Toxicology*, Vol 21. Princeton Scientific, Princeton, NJ, USA, pp 295–310.
12. Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ Sci Technol* 32:1549–1558.
13. Jobling S, Noylan M, Tyler CR, Brighty G, Sumpter JP. 1998. Widespread sexual disruption in wild fish. *Environ Sci Technol* 32:2498–2506.
14. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 15:194–202.
15. Nimrod AC, Benson WH. 1996. Environmental estrogenic effects of alkylphenol ethoxylates. *Crit Rev Toxicol* 26:335–364.
16. Jobling S, Sumpter JP. 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat Toxicol* 27:361–372.
17. Routledge EJ, Sumpter JP. 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. *J Biol Chem* 272:3280–3288.
18. Snyder SA, Keith TL, Verbrugge DA, Snyder EM, Gross TS, Kannan K, Giesy JP. 1999. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environ Sci Technol* 33:2814–2820.
19. Keith TL. 2000. Identification and quantitation of nonylphenol ethoxylates and nonylphenol in fish tissues of Michigan, USA. MSc thesis. Michigan State University, East Lansing, MI, USA.
20. Lye CM, Frid CLJ, Gill ME, Cooper DW, Jones DM. 1999. Estrogenic alklphenols in fish tissues, sediments, and waters from the U.K. Tyne and Tees estuaries. *Environ Sci Technol* 33:1009–1014.
21. Kubeck E, Naylor CG. 1990. Trace analysis of alkylphenol ethoxylates. *JAOCS* 67:400–405.
22. Ahel M, McEvoy J, Giger W. 1993. Bioaccumulation of the lipophilic metabolites of nonionic surfactants in freshwater organisms. *Environ Pollut* 79:243–248.
23. Veith GD, Kiwus LM. 1977. An exhaustive steam-distillation and solvent-extraction unit for pesticides and industrial chemicals. *Bull Environ Contam Toxicol* 17:631–636.
24. Glaser JA, Foerst DL, McKee GD, Quave SA, Budde WL. 1981. Trace analyses for wastewaters. *Environ Sci Technol* 15:1426–1435.
25. Liber K, Gangl JA, Corry TD, Heinis LJ, Stay FS. 1999. Lethality and bioaccumulation of 4-nonylphenol in bluegill sunfish in littoral enclosures. *Environ Toxicol Chem* 18:394–400.
26. Snyder SA, Keith TL, Pierens SL, Snyder EM, Giesy JP. 2001. Bioaccumulation of nonylphenol in fathead minnows (*Pimephales promelas*). *Chemosphere* (in press).
27. Long JLA, House WA, Parker A, Rae JE. 1998. Micro-organic compounds associated with sediments in the Humber rivers. *Sci Total Environ* 210/211:229–253.
28. Khim JS, Villeneuve DL, Kannan K, Giesy JP. 1999. Characterization and distribution of trace organic contaminants in sediment from Masan Bay, Korea. 2. In vitro gene expression assays. *Environ Sci Technol* 33:4206–4211.
29. Khim JS, Kannan K, Villeneuve DL, Koh CH, Giesy JP. 1999. Characterization and distribution of trace organic contaminants in sediment from Masan Bay, Korea. 1. Instrumental analyses. *Environ Sci Technol* 33:4199–4205.