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IDENTIFICATION AND QUANTITATION OF NONYLPHENOL ETHOXYLATES AND NONYLPHENOL IN FISH TISSUES OF MICHIGAN, USA

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Timothy L. Keith

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John P Giesy
Major professor

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IDENTIFICATION AND QUANTITATION OF NONYLPHENOL ETHOXYLATES AND NONYLPHENOL IN FISH TISSUES OF MICHIGAN, USA

By

Timothy Lawrence Keith

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ABSTRACT

IDENTIFICATION AND QUANTITATION OF NONYLPHENOL ETHOXYLATES AND NONYLPHENOL IN FISH TISSUES OF MICHIGAN, USA

By

Timothy Lawrence Keith

Persistent metabolites of nonylphenol polyethoxylates (NPE, n=1-18), such as nonylphenol (NP), nonylphenol monoethoxylate (NPE_1) and nonylphenol diethoxylate (NPE_2) are weak estrogenic environmental contaminants and have been implicated in the disruption of endocrine function in wildlife. In order to evaluate bioaccumulation potential and to identify potential risks posed by these chemicals, concentrations of NP and NPE_1, NPE_2, and nonylphenol triethoxylate (NPE_3) were determined in the tissues of fish inhabiting various waters in Michigan, USA. To measure these concentrations, a method was developed to extract samples using exhaustive steam distillation with concurrent liquid extraction. Concentrations of NP among all sites and species ranged from <3.3 to 29.1 ng/g wet wt and varied little statistically among sites. NPE_1 was detectable but at concentrations below the limit of quantitation. NPE_2 and NPE_3 were below their method detection limits of 18.2 and 20.6 ng/g wet wt, respectively.

DEDICATION

I would like to thank Dr. John P. Giesy for his guidance and support. I am grateful for the advice of my committee members, Dr. Robert Huggett and Dr. Susan Masten. I am indebted to Dr. Kurunthachalam Kannan and Dr. Shane Snyder of the Aquatic Toxicology Laboratory, Cheryl Summer from the Michigan Department of Environmental Quality, and my Laboratory Technician Adam Pitt for all their help. Thanks to my colleagues in the Aquatic Toxicology Laboratory and to all my family and friends who have made this possible. Most importantly, I thank my parents, Ray and Joan Keith, for their belief and support throughout the years.

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LIST OF ABBREVIATIONS

4tb = 4-tert-butyl ortho-cresol
ACN = acetonitrile
AP = alkylphenol
APE = alkylphenol ethoxylate
CP = para-cumylphenol
CV = coefficient of variation
DCM = dichloromethane
ECD = electron capture detector
GC = gas chromatograph, gas chromatography
GC/MS = gas chromatograph/mass spectrometer,
gas chromatography/mass spectrometry
GPC = gel permeation chromatography
HP = Hewlett-Packard
HPLC = high pressure liquid chromatograph
high pressure liquid chromatography
IDL = instrumental detection limit
i-C_8 = iso-octane
LC = liquid chromatography
m/z = mass-to-charge ratio
MDL = method detection limit
MeOH = methanol
NP = 4-Nonylphenol
NPE = nonylphenol ethoxylate
NPE_1 = nonylphenol monoethoxylate
NPE_2 = nonylphenol diethoxylate
NPE_3 = nonylphenol triethoxylate
NoP = normal phase
OP = octylphenol
OPE = octylphenol ethoxylates
ppb = part per billion (ng/g, ng/mL)
ppm = part per million (μg/g, μg/mL)
ppt = part per trillion (pg/g, pg/mL)
PE = Perkin Elmer
PDMS = polydimethylsiloxane
RP = reverse phase
SIM = selected ion monitoring
TFAA = trifluoroacetic acid
WWTP = wastewater treatment plant
ww = wet weight

INTRODUCTION

Alkylphenols and alkylphenol ethoxylates (APEs) have numerous applications, including pesticide formulations, petroleum production, cleaning products, pulp and paper manufacturing, and plastics manufacturing (Metcalfe et al., 1996). Approximately 80% of the APEs used are nonylphenol ethoxylates (NPEs), while the remaining 20% are almost entirely octylphenol ethoxylates (OPEs). The hydrophilic moiety is an ethoxylate chain ranging from 1 to 20 ethoxy units while the hydrophobic moiety is a branched alkyl group typically with 8 or 9 carbons. These compounds enter wastewater treatment plants (WWTPs) where they may undergo degradation. The degradation intermediates, nonylphenol and its mono, di and triethoxylates tend to be more persistent, so are often the dominant NPE species in WWTP effluents (Ahel et al., 1993a; McLeese et al., 1981). This degradation resistance is a result of the alkyl branching and the presence of the aromatic ring. They are lipophilic and tend to adsorb to organic surfaces (Metcalfe et al., 1996). Wastewater treatment in the United States generally removes around 95% of all the NPEs entering the plant (Naylor, 1995).

It has been shown that NP is an estrogen mimic and can interfere with the reproduction of fish. NP at low μg/L concentrations can induce the production of vitellogenin in cultured rainbow trout hepatocytes (Jobling and Sumpter, 1993). NP is also capable of inducing cell proliferation in the estrogen-sensitive MCF-7 human breast tumor cells (Soto et al., 1991). The estrogenic potency of NPEs appear to decrease with increasing ethoxylate chain length. However, the water solubility of NPE increases with increasing ethoxylate chain length (Ahel and Giger, 1993b).

The first objective of this study was to develop a reliable, cost-effective, and simple method to sensitively detect and quantify NPE_{1-3} and NP in the tissues of fish using commonly available equipment. The second objective was to measure concentrations of NPE_{1-3} and NP in fish from various rivers of mid-Michigan and the Great Lakes, USA.

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Chapter 1

METHOD DEVELOPMENT

Introduction

When faced with the challenge to develop a reliable, cost-effective and simple method to sensitively detect and quantify nonylphenol (NP), nonylphenol monoethoxylate (NPE_1), nonylphenol diethoxylate (NPE_2), and nonylphenol triethoxylate (NPE_3) in the fish tissues, several obstacles had to be overcome. One was to develop or adapt a pre-existing procedure to prepare the sample for extraction and to extract the analytes from the tissue matrix. The second and most difficult was separation of lipid interferences from the sample extract. The third was to develop a procedure for quantifying the analytes.

Glassware Preparation

All glassware was washed using a quality commercial glassware detergent with deionized water, rinsed three times with high purity acetone, followed by three rinses with high purity hexane and allowed to dry before use.

Test Matrices

Two different matrices facilitated the method development. The sample matrix used for extraction development was homogenated laboratory raised whole body goldfish (*Carassius auratus*). Goldfish were removed from a –20 °C freezer and allowed to thaw. Goldfish were quartered and homogenized using a Sorvall

OmniMixer blender in 400 mL OmniMixer blender cups. Homogenate was stored in glass jars at –20 °C until needed. The homogenate was spiked with external standards of NPE_{1-3} and NP to represent an environmentally exposed sample.

The second matrix used to develop clean up and subsequent quantification was corn oil. Corn oil was used as a surrogate for fish lipids in an attempt to reduce time in sample preparation. To simulate actual samples, a percent lipid of 15% w/w was assumed and the corn oil spiked appropriately. This percentage was chosen since the extracted goldfish homogenate contained 11 to 25% lipid ww.

Soxhlet Extraction

Soxhlet extraction was the first method explored. Ten g of the pre-homogenated tissue sample was placed into an OmniMixer cup with 50 g anhydrous sodium sulfate (Na_2SO_4) and blended for 1 min. The mixture was placed in a –10 °C freezer for 5 min to assist in desiccation. Two more additions of 50 g of sodium sulfate were added to the mixture and homogenized as previously described. The sample was then spiked with 50 μL of a mixture containing 100 μg/mL each of octylphenol (OP) and NP in acetonitrile (ACN) for a total concentration of 500 ng/g. The sample was Soxhlet extracted overnight with 350 mL of dichloromethane (DCM). The extract was then cooled and stored out of light until lipid removal could be performed. The total time for this extraction procedure varied in total from 18-24 hr.

Lipid mass was determined by removing 100 µL of the extract and placing it into a pre-weighed aluminum weigh boat. The sample was then placed into a drying oven at 60 °C until the solvent had evaporated. The sample was weighed again and the percentage lipid calculated. Goldfish tissues ranged from 11 to 25% lipid or 1.1 to 2.5 g per 10 g sample.

Among the advantages of this Soxhlet extraction method were its common availability to analytical laboratories and ease of operation. Samples could remain relatively unattended for the majority of their extraction. Until a lipid clean-up method was developed, this extraction procedure was considered adequate.

Lipid Removal Method 1

Following the Soxhlet extraction, each sample was concentrated using a Labconco TurboVap at 30° C to a final volume of approximately 5 mL. The concentrated sample was transferred with DCM to a 15 mL centrifuge tube and evaporated under nitrogen to a final volume of 6 mL.

Several gel permeation chromatography (GPC) elution profiles were tested. The GPC system consisted of a Rheodyne 7725i injector (Cotati, CA) with a 5 mL sample loop, a quaternary high-pressure liquid chromatography (HPLC) pump (Perkin Elmer, Series 410, Norwalk, CT) and an electronic fraction collector (ISCO Foxy 200, Lincoln, NE). Mass loading, mobile phase flow rates and the number and type of columns were investigated. The largest mass of interferences that could be loaded into the GPC system while maintaining acceptable lipid removal was determined to be 0.5 g. Therefore, three separate injections of 2 mL of the 6 mL extract were introduced through a Rheodyne injection port into an isocratic HPLC system. A Phenomenex Envirosep-ABC 350 mm X 21.20 mm column followed by a Phenomenex Phenogel 5A 300 mm X 21.20 mm column was used to obtain the greatest lipid removal. DCM was used as the mobile phase at a flow rate of 5 mL/min. Ten fractions were collected every 2 min starting at 10 min to determine where the APs would elute. Fractions were evaporated under nitrogen and solvent exchanged into ACN. Extracts were analyzed by reverse phase HPLC using fluorescence detection. Recovery of NP was 71.0% +/- 8%.

During the solvent exchange to ACN, it was observed that residual lipids would precipitate and could be removed by centrifugation. While some lipid did remain in the ACN solution, the addition of 0.5 mL of water resulted in a greater lipid precipitation (removal). This led to an alternative lipid removal procedure summarized in the following section.

Lipid Removal Method 2

Preliminary testing of this method used corn oil as a surrogate for fish lipids in an attempt to reduce time in sample preparation. Experiments were conducted with various mixtures of ACN, ACN/water, methanol (MeOH), and MeOH/water. The following describes some of the more successful trials.

In a 15 mL centrifuge tube 4 mL DCM, 1 mL corn oil and 50 µL of a 100 ppm alkylphenol (AP) mixture in ACN were vortexed for approximately 1 min. Two mL MeOH were added and vortexed for 5 min. The mixture was evaporated under nitrogen to 3 mL and vortexed again. The sample was centrifuged at 3500 RPM for 5 min and the supernatant transferred into another 15 mL centrifuge tube. Two 2 mL of MeOH added to sample, vortexed and centrifuged and supernatant transferred to the same tube. This was repeated. The supernatant was concentrated under nitrogen to 1 mL in ACN and quantitated using the same HPLC method described in Method 1.

This method was then tested with actual fish lipids using Soxhlet extracts from 10 g fish tissue. The extracts were concentrated by rotary and nitrogen evaporation to 5 mL and Lipid Removal Method 2 applied. NP recovery in corn oil was 84.1% +/- 9% and 66.8% +/- 5% in fish homogenate.

Sample Concentration

As all the aforementioned techniques require varying degrees of solvent concentration, different techniques were investigated. For bulk concentration, the Buchi RotoVap and the Labconco RapidVap N_2 Evaporation System were tested and the Organomation 25 position and 100 position N-Evap for fine solvent concentration.

Logistically, the Labconco RapidVap N_2 Evaporation System is superior to the Buchi Rotary Evaporator. The RapidVap is programmable to regulate temperature, vortex speed and evaporation time. The RapidVap sample container is engineered to eliminate the risk of complete solvent evaporation that may cause loss of volatile analytes. In addition, six samples may be simultaneously concentrated with no need for monitoring during operation. Conversely, the Buchi Rotary Evaporator requires monitoring during operation and accommodates only one sample but the concentration occurs much faster. For example, 100 mL of MeOH evaporated to approximately 2 mL at 30 °C requires 2 hr for the RapidVap and only 0.5 hr for the RotoVap.

Aside from logistics, recoveries of analytes were also investigated. Spike recoveries of NP spiked into 100 mL DCM were approximately 20% greater using the RapidVap than the RotoVap (97.3% to 78.5%). From this information, it was decided to employ the Labconco RapidVap N_2 Evaporation System for bulk solvent concentration.

Spike recoveries comparing the 25 position and the 100 position Organomation N-Evap were nearly identical. This is expected as both these systems utilize the same evaporation technique of gentle streaming nitrogen and a heated water bath. However, beside the apparent advantage of increased sample throughput using the 100 position unit over the 25 position unit, the 100 position unit also allowed for greater control of nitrogen flow. While the 25 position unit uses one nitrogen flow control per sample, the 100 position unit uses one flow control per 10 samples. This ensures that the 10 samples controlled by the same flow control receive the same flow of nitrogen, decreasing sample variability. For this reason, the Organomation 100 position N-Evap was chosen for fine solvent concentration. To test this model's reproducibility, eight spiked samples containing 10 μg of NP in ACN were concentrated from 6mL to 1mL. The average recovery of NP was 95% with a coefficient of variation (CV) less than 10%.

Liquid Chromatography and Detection

Despite its lower resolution and some coelution problems described below, liquid chromatography (LC) is a very versatile analytical technique suitable for compounds with a wide range of polarity, volatility and molecular weights such as the NPEs, as well as the isomers of NP.

As the NPEs and NP have a strong chromophore (phenyl group), one of the most sensitive and selective detection methods is fluorescence. As the NPEs and NP have their excitation and emission spectra maxima at approximately 230 nm and 310 nm respectively, researchers have often used these wavelengths for detection of NPEs and NP.

Another important consideration to the LC method development was the ability to separate NP from OP, another common surfactant found in the environment. If the LC method could not resolve OP from NP, the calculated NP concentration would be suspect as a summed concentration of OP and NP.

HPLC coupled to a programmable fluorescence detector was initially investigated. This HPLC system consisted of a Perkin Elmer (PE) (Norwalk, CT) series 200 autosampler, a PE series 200 binary pump, a Hewlett Packard (HP, Palo Alto, CA) 1046A fluorescence detector, a Degasys (Tokyo, Japan) electronic vacuum degasser, a PE Nelson series 900 interface and PE TurboChrome 4.0 data software package.

Reverse Phase Liquid Chromatography

As all the NPE oligomers have the same hydrophobic moiety, they elute as a single peak using the non-polar solid phase octadecylsilica. As such, this would require an additional analytical procedure to analyze the various oligomers of NPE. Gas chromatography/mass spectrometry (GC/MS) using electron impact ionization was proposed as a possible means to confirm the identification of NP and to identify and quantify the NPEs.

The instrumental profile for the RP-HPLC method that best separated the APs is described below. Elution solvents were reagent water and ACN delivered at a constant flow rate of 1 mL/min. The elution profile was a 20 min gradient (curve = -2) from 50% water/ACN to 2% water/ACN followed by a 10 min isocratic ACN purge. The column was returned to initial conditions by a 10 min isocratic flow of 50% water/ACN. The HPLC injection volume was 10 μL. For all compounds of interest, the fluorescence detector settings were 229 nm excitation and 310 nm emission.

An HP 5890 Series II Plus GC and an HP 5972 electron impact ionization mass spectrometer (MS) were used for further separation and identification. The GC was operated using a 30 m DB-17 column (J&W Scientific, Folsom, CA), starting temperature of 100 ^{O}C for 2 min, ramped to 300 ^{O}C for 10 min at 4 ^{O}C/min. The GC injection volume was 4 μL. To detect the analytes, the MS was operated in selected ion monitoring (SIM) (Table 1).

Table 1. Analyte ions monitored for GC/MS/SIM

Analyte	Ions (m/z) Monitored
NP	107, 135, 149
NPE_1	135, 179, 193
NPE_2	135, 223, 237
NPE_3	135, 267, 281

Preliminary Results

This combined technique of RP-HPLC and GC/MS worked well for standard mixtures of APs. In addition, this profile was sufficient for separation and identification for goldfish homogenate spikes when using large amounts of analytes (approximately 1μg NP/mL extract concentration) (Figure 1). However, this method was insufficient for analytes that were spiked in trace amounts (resulting in an extract concentration of approximately 50 ng NP/mL) (Figure 2).

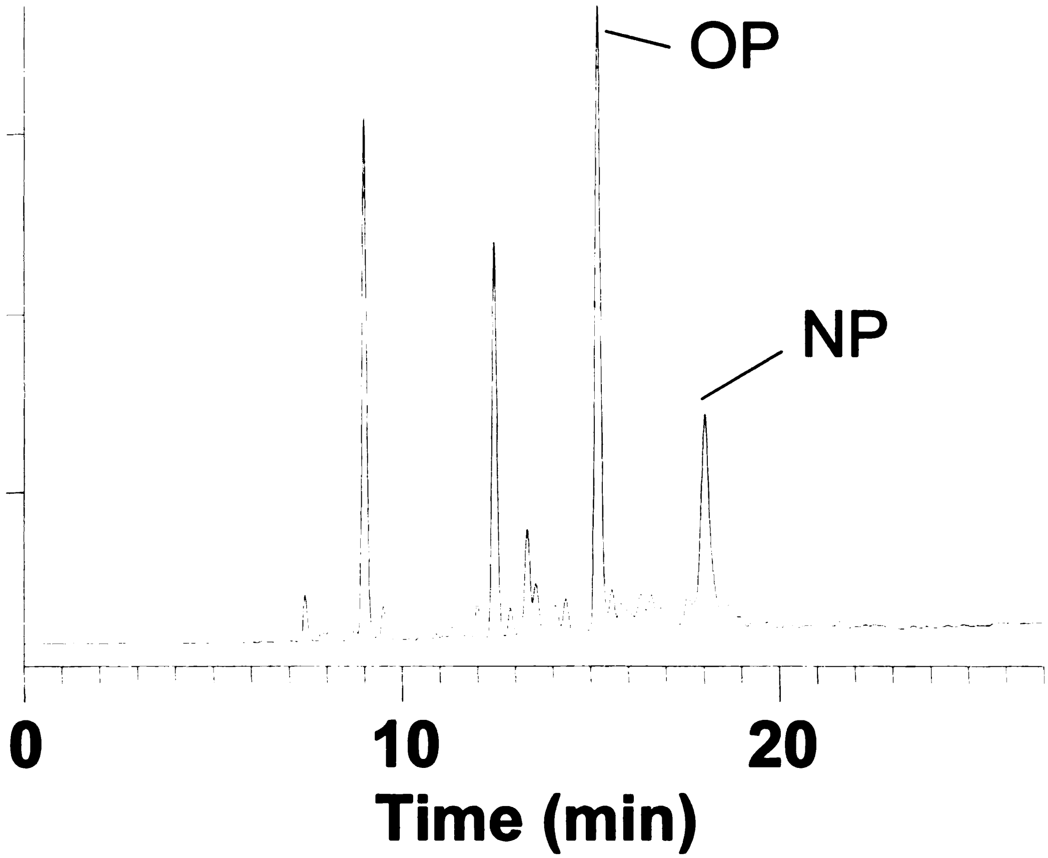


Figure 1. HPLC chromatogram showing goldfish homogenate spike recovery extract using large concentration of analytes (approximately 1μg NP/mL extract concentration).

The remaining interferences, even after lipid removal using the technique described above, caused a high signal-to-noise ratio that prevented any identification of analytes in trace quantities. The problem was compounded by the HPLC operating in reverse-phase, allowing the nonpolar residual lipid to elute over the entire elution.

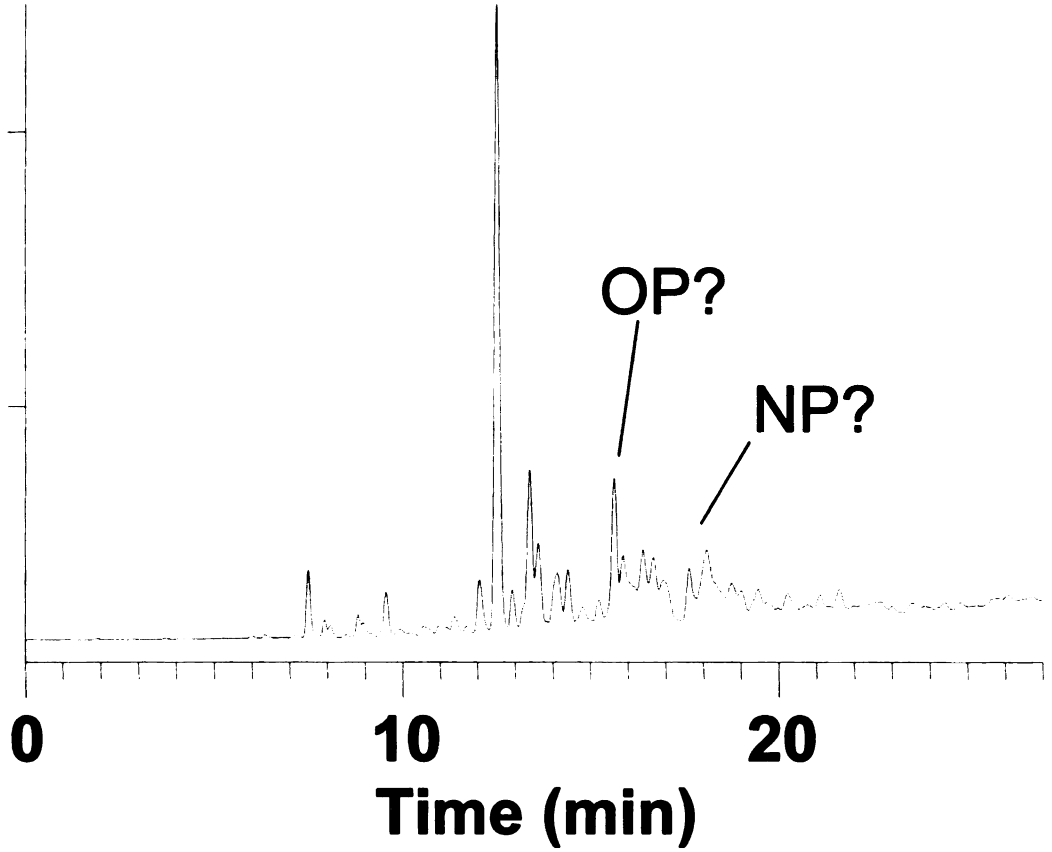


Figure 2. HPLC chromatogram showing goldfish homogenate spike recovery extract using trace concentration of analytes (approximately 50 ng NP/mL extract concentration).

To remedy this, a post-extraction clean-up method needed to be developed. Miniature silica gel columns were constructed using 0.5 g silica gel (100/200 mesh) in a 5¾ inch Pasteur pipette. The column was wetted with 3 mL hexane and the extract loaded. The extract was first eluted with 3 mL DCM, then 3 mL MeOH. This second fraction was again analyzed by GC/MS. Concentration of lipids remained too high and quantitation by GC/MS was determined unsuitable. An additional approach was required.

Steam Distillation with Concurrent Liquid-Liquid Extraction

Steam distillation separates chemicals based on vapor pressure differences over water (Veith and Kiwus, 1977). Veith and Kiwus developed a modified steam distillation apparatus that allows steam distillation with concurrent solvent extraction. This allows an exhaustive and continuous extraction of the matrix of interest. The flask containing the sample is heated to produce a vigorous boil. Steam distillate passes through an inner tube and condenses on the walls of the cooling jacket. Condensate flows down the condenser and through a layer of non-polar solvent of lower density than water. The water passes through this solvent layer and is recycled through an overflow tube back into the boiling flask and the cycle repeats. Once the desired amount of time has passed, the solvent can be removed through a side arm on the base of the condenser.

This technique allows direct analysis of most extracts without additional concentration and clean up while using very little solvent. Although this method has utility for water, sediment, and sludge samples, few researchers have investigated this method for biological matrices. One group reported successful attempts using this technique (Ahel et al., 1993).

Aside from the benefit of no projected extract clean-up, this method offers several other advantages. Homogenization efficiency is greatly increased since this method uses water as the primary solvent. As the matrix is suspended in water causing the tissue to remain in continuous contact with the blender blades, dry homogenization requires frequent pauses to remove the sample from the container walls and back into the blender blades. In addition, there is no need for sample drying. This method is also environmentally friendly since it utilizes only tens of milliliters of non-aqueous solvent.

Experiments as to the suitability of normal phase liquid chromatography (NoP-LC) were conducted since the extract would be solvated in a non-polar solvent and with the complications noted earlier using RPLC.

Normal Phase Liquid Chromatography

Since NPE oligomers differ from each other by the length of their polyethoxylate chain, they are best separated by NoP-LC. For NoP-LC, the order of elution is a function of number of ethoxylate groups: the greater the number of ethoxylates the longer the residence on the LC column.

Two columns were investigated: a Phenomenex Phenosphere 5µ CN 80A (250 mm x 4.6 mm) and a Phenomenex Luna 5µ silica (250 mm x 4.6 mm). Mixtures containing APs and APEs were prepared. These mixtures contained OP, NP and NPE_1 at varying concentrations.

Cyano Column

Initially, a generic NoP-HPLC method using a cyano column was employed on spiked fish extracts. The elution profile was a 30 min linear curve from hexane to MeOH at 1 mL/min. This method proved to be hindered by matrix interferences and was unable to resolve OP from NP and NPE_1. The complete resolution of OP was considered an important requirement. Along with NP, OP is another common environmental contaminant. If OP was not resolved from NP, any calculated concentration of NP had the possibility to be the summed concentration of NP and OP. A less aggressive solvent elution was necessary to allow the lipids to elute before the APs and APEs. Elution solvents considered were hexane combined with various percentages of 30% 2-proponal/MeOH, 20% DCM/MeOH, and 20% MeOH/DCM using curves ranging from –2 to 2 and flow rates between 0.5 to 1.5mL/min. These methods met with limited success: APs and APEs could be separated from the lipid interferences but couldn't be resolved from each other, or APs could be somewhat resolved from APEs but were hindered by matrix interferences. As the method became less aggressive in order to provide adequate separation, the NP peak began to split into two peaks and eluted over a long period of time (approximately 3-4 min).

OP could not be completely resolved from NP. Standards of OP and NP were injected independently and when chromatograms of the two were superimposed, the column showed complete resolution. Injection of mixtures using the same method resulted in incomplete resolution. It was necessary to resolve OP from NP; however, complete resolution was never achieved using this column deeming it unsuitable for this study.

Silica Gel Column

Knowing silica gel has a much higher binding ability than cyano, it was hoped that silica gel could provide sharp, resolved peaks of all compounds and lipid interferences. The approach to using the silica column was the same as the cyano: slow flow rates and non-aggressive solvents to allow the APs and APEs adequate time to separate from the lipids. In early methods, the first 3 min were pure hexane to bleed the lipids off the column, then ramped up at various flow rates and solvent mixtures to elute the APs and APEs. This approach provided much sharper peaks with a higher detector response than did the cyano, but could not resolve OP from NP from NPE_1. Again, when OP and NP were injected independently, superimposed chromatograms showed complete resolution. Mixtures of OP and NP at varying concentrations were injected and resulted in a single peak, roughly the average of the two individual retention times and sum of the individual areas.

It was discovered that when the silica column is initially filled with hexane, APs could not be resolved from the APEs. When the column is initially filled with a mixture of hexane and 20% MeOH/DCM and eluted isocratically, APs could be sufficiently resolved from the APEs. Still, OP could not be resolved from NP.

It was decided that resolution of OP from NP was not necessary for this study. Efforts were redirected to optimize a method using silica gel to resolve NP and NPE_1 from fish lipid.

The elution profile that was found best to resolve NP from NPE_1 with no interference from the fish matrix was an isocratic elution of 12% 1:4 MeOH/DCM and 88% hexane at a flow rate of 0.65 mL/min with fluorescence detection at 229 nm excitation and 310 nm emission.

Extraction

Experiments were initially designed to reproduce the method from Huntsman Corp. (method #ST-38.34-94, Austin, TX) which reported 100% recoveries of NP from spiked water with relative standard deviation of 2.5% (n=4). The method states that it is applicable to waste water and river water as well as "solid matrices such as sediments, sludge and biological tissues". When applying this technique to fish, it was believed that the extracts would not require additional clean up as the higher molecular weight lipids will not distill, therefore not contaminating the extraction solvent.

Experimental parameters for this method called for 20 g sodium chloride (NaCl), 1 L water, boiling chips, 2 mL hexane and cooling jacket temperature at 5 °C. Ten g of pre-homogenated sample, NP and NPE standard mixtures in hexane were spiked directly into the 1 L of water. The temperature-controlling rheostat was set to 100% heating.

This method produced severe foaming which caused excess material to foam over and accumulate in the organic solvent rendering it unusable for analysis. Careful temperature regulation could prevent the sample from foaming over but would be impractical for a high-throughput method. Spike recoveries of NP and NPE_1 were 65.1% and 60.8%, respectively, for those extractions that did not foam significantly.

To address the problem of sample foam-over, varying amounts of polydimethylsiloxane (PDMS), an anti-foaming agent, were added at the beginning of extraction. While PDMS did suppress sample foaming, recoveries of NP fell to 53.3%, a decrease of 11.8%.

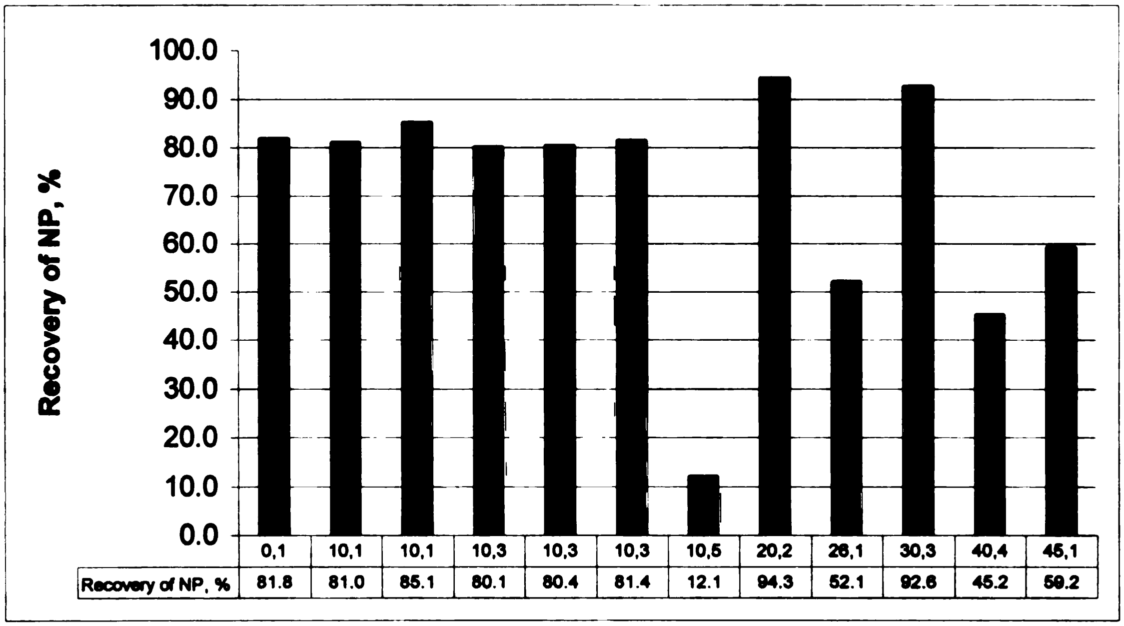
Attempts to contact Dr. Ahel regarding the 100% recoveries of NP from fish tissues he reported in 1993 were unsuccessful (Ahel et al., 1993). Dr. Veith was contacted in mid-October 1998 and recommended the addition of small amounts (1 – 5 mL) of concentrated sulfuric acid to suppress the sample foaming. Initial trials using sulfuric acid indicated that foaming was eliminated and recoveries improved to 68.0%.

The addition of acid caused severe bumping despite the addition of various boiling chips, including Quartz, glass beads, crushed glass, and graphite chips. The bumping problem was solved by continuous mixing using a magnetic stir bar in the boiling flask by a stir plate placed beneath the heating mantle. The stir bar suppressed the bumping well enough as to remove the need for boiling chips of any kind.

With the method developed where recoveries of NP were consistent at 65-70%, method improvement began by conducting a sensitivity analysis on the reagents and parameters. These included:

- condenser temperature
- heating temperature
- extraction time
- number of extractions
- solvent type
- solvent volume
- sulfuric acid volume
- sample mass
- sodium chloride mass

From these sensitivity analyses, different heating temperature showed no significant differences (provided the water came to a boil). Twenty g of salt recovered the greatest amount of analyte. Recoveries differed with varying fish tissue mass to amount of acid ratio (Figure 3). The trend in Figure 3 shows the greatest recoveries are achieved with 1-1.5 mL acid per 10 g fish homogenate. It could also be concluded that iso-octane (i-C_8) exhibited greater analyte recovery than hexane or cyclohexane.



	0,1	10,1	10,1	10,3	10,3	10,3	10,5	20,2	26,1	30,3	40,4	45,1
Recovery of NP, %	81.8	81.0	85.1	80.1	80.4	81.4	12.1	94.3	52.1	92.6	45.2	59.2

Figure 3. Fish weight to acid volume comparison. For the first row of numbers, the first value represents the mass of fish homogenate weight in g and the second value represents the volume of concentrated sulfuric acid in mL.

Of particular concern was the cooling water temperature, as the laboratory ambient temperature of this water was 13-15 °C. It was unknown if this temperature was too great to sufficiently condense the steam before loss occurred through the apparatus top. A recirculating-chiller using an ethylene glycol/water mixture producing cooling water between -5 and 15 °C was tested to see the effect on spike recoveries. Recoveries did not increase with cooler temperatures and in the extreme case actually decrease with cooling temperatures at –5 °C. This is believed to occur by condensing the steam so quickly as to not pass over the inner tube and the analytes never have the opportunity to flow through the organic solvent layer.

In addition, two 1½ hr extractions showed greater recoveries than one 3 hr extraction. The sample that was extracted for 3 hr recovered 56.2% of the spiked NP, while the sample that was extracted twice for 1½ hr recovered 49.6% first extraction and another 19.8% the second extraction.

Glassware Cleaning

Cleaning procedures were studied since the steam distillation glassware was in frequent use. The columns are rinsed with acetone and hexane after each extraction. With the problems of sample foaming described earlier, the inner tube become soiled. Upon arrival of two new steam distillation units, an experiment was designed to compare the recoveries of NP between a new unit and a heavily soiled unit (Table 2).

Table 2. Comparison in NP recovery between soiled and new extractors.

Column Condition	Percent Recovery of NP	Percent Coefficient of Variation
Soiled	38.6	1.7
Soiled	39.7	
Soiled	39.8	
New	59.0	0.55
New	58.9	
New	58.4	

Please note the samples were only extracted once since the trend could readily be seen. This resulted in lower recoveries when compared to samples extracted twice. Soaking the columns for one hr in 17% nitric acid returned the column to near original condition. Extractions using the same parameters shown in Table 2 were run in triplicate after the columns were soaked in acid. The extractions returned a mean recovery of 84.8% for NP with a CV of 5.8%. For information on steam distillation apparatus cleaning, please refer to Appendix A.

Final Extraction Method

From the sensitivity analyses, an optimal extraction and quantitation method was believed to be found. Twenty g of fish homogenate is homogenated in 600 mL deionized water and transferred to a 2 L boiling flask. Four hundred additional mL of deionized water is used to quantitatively transfer the remaining fish homogenate from the blender cup to the boiling flask. Then, 20 g sodium chloride, 3 mL concentrated sulfuric acid, a small amount of Quartz boiling chips and a Teflon coated magnetic stir bar are added to the flask. Two to three mL of deionized water are added through the top of the steam distillation apparatus followed by 10 mL high-purity i-C_8 and the column capped with aluminum foil. The 2 L boiling flask containing the sample homogenate and reagents are attached to the apparatus and placed in the heating mantle. Using the stir plate, a gentle vortex (approximately 50% maximum stir) is created, the heating mantle powered to HIGH and the cooling water flow is set to maximum flow. The sample is extracted twice at 1½ hr each extraction. The extract is then concentrated, fractionated by NoP-HPLC and quantitated by GC/MS. For further information, please refer to Appendix B.

To achieve greater sample throughput, six steam distillation extractors were operated simultaneously. Cooling water temperature was measured to lessen approximately 1 °C after flowing through one extractor operating at maximum temperature. To reduce the variability between different extractors, two separate cooling water feeds supplied three extractors each. Six replicate spike recovery experiments indicated that sample variability was not significantly greater for six extractors operating simultaneously than one extractor operated alone six times.

Attempted Derivatization

Derivatization of the analytes was investigated for additional quantitation. To test the suitability of derivatized samples detected by an electron capture detector (ECD), NP was acylated by trifluoroacetic acid (TFAA) converting the active hydrogen into a fluorinated ester. Standards of derivatized NP showed increased detectability using ECD than GC/MS. Experiments were then conducted to derivatize environmental extracts. One approach was to attempt derivatization during extraction by adding TFAA into the organic solvent layer before extraction. Another approach was to derivatize the sample after extraction. The TFAA derivatized lipid interferences in both experiments and resulted in an extract unsuitable for ECD analysis.

Internal Standard

Internal standards were employed to correct for extraction variability and improve data quality. Upon suggested by Carter Naylor of Huntsman, Corp., *p*-cumylphenol (CP) was investigated as a surrogate standard for NP. Recoveries of *p*-cumylphenol to measure extraction recovery were 89.0% mean recovery with an 11.3% CV as measured by NoP-HPLC with fluorescence detection. This allowed for correction of NP concentrations due to loss by extraction, concentration or injection into the HPLC.

Method Validation

As a test for the methods ability to analyze environmental samples, common carp (*Cyprinus carpio*) from Lake Mead, Nevada were obtained and processed. While using a matrix of goldfish homogenate in laboratory spikes proved the method reliable in separating the compounds of interest from the matrix interferences, an analysis of these carp samples proved to the contrary. That is, the type and/or amount of interferences from one species of fish are not necessarily the same interferences from another. Thus, further steps had to be taken to assure that the analytes could reliably be separated and quantitated.

An alternative method of quantitation was implemented to resolve this problem. Using the original method, quantitation was achieved using NoP-HPLC with fluorescence detection using an extract that underwent no clean-up. With the improved method, the NoP-HPLC is now used as a fractionating step removing the bulk of the interferences from the compounds of interest while GC/MS separates and quantitates the analytes.

While this added step required approximately two additional hours laboratory processing per sample, the identification of analytes is now accomplished by not only retention time matching but also mass spectral identification. This means of identification is also beneficial since the presence of any polyaromatic hydrocarbons, which have the possibility to interfere with analysis using only fluorescence detection, will not complicate data interpretation. For further information, please see Appendix B.

With the method now employing NoP-HPLC for fractionation and GC/MS for identification and quantitation, further method development was required. With the previous method, NoP-HPLC quantitated NP and CP and GC/MS quantitated the NPEs. The new method required the GC/MS to separate and quantify CP as well. A fraction from the HPLC collected between 7-16 min would not collect the bulk lipids while collecting NP, NPEs and CP (Figure 4).

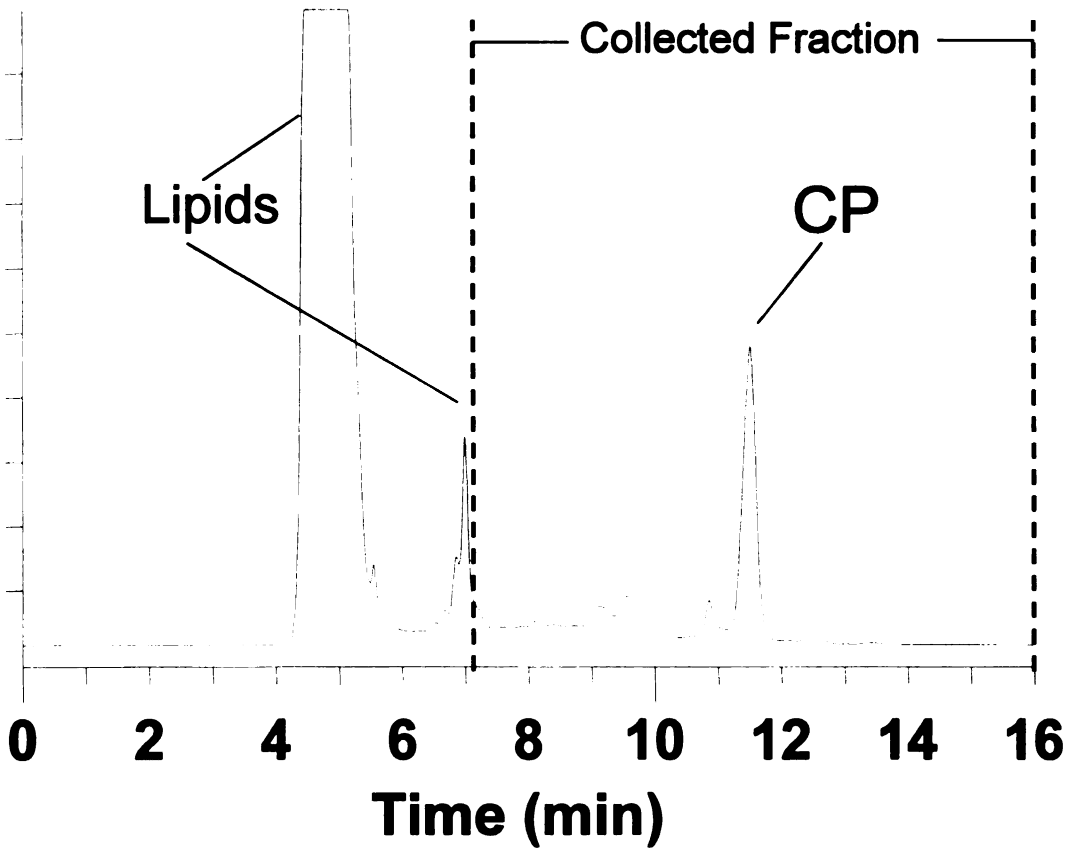


Figure 4. Normal phase HPLC chromatogram showing collected fraction.

As this work progressed, however, it became apparent that CP could not be adequately separated from NP by GC/MS. The GC/MS method worked well for NPE and NP quantitation. The inability to separate CP from NP was overcome using an additional internal standard and not SIM the CP. This internal standard would measure the loss of analyte due to solvent concentration and subsequent injection into the GC. For this internal standard, 4-tert-butyl ortho-cresol (4tb) was investigated. Spike recoveries of 4tb using the same volume of solvent that would elute for a fractionation to simulate an actual sample were conducted. Recoveries of the internal standard 4tb are a 92.2% mean recovery with a 10.9% CV. See Figure 5 for GC/MS chromatogram showing 4tb, NP, NPE_1 and NPE_2.

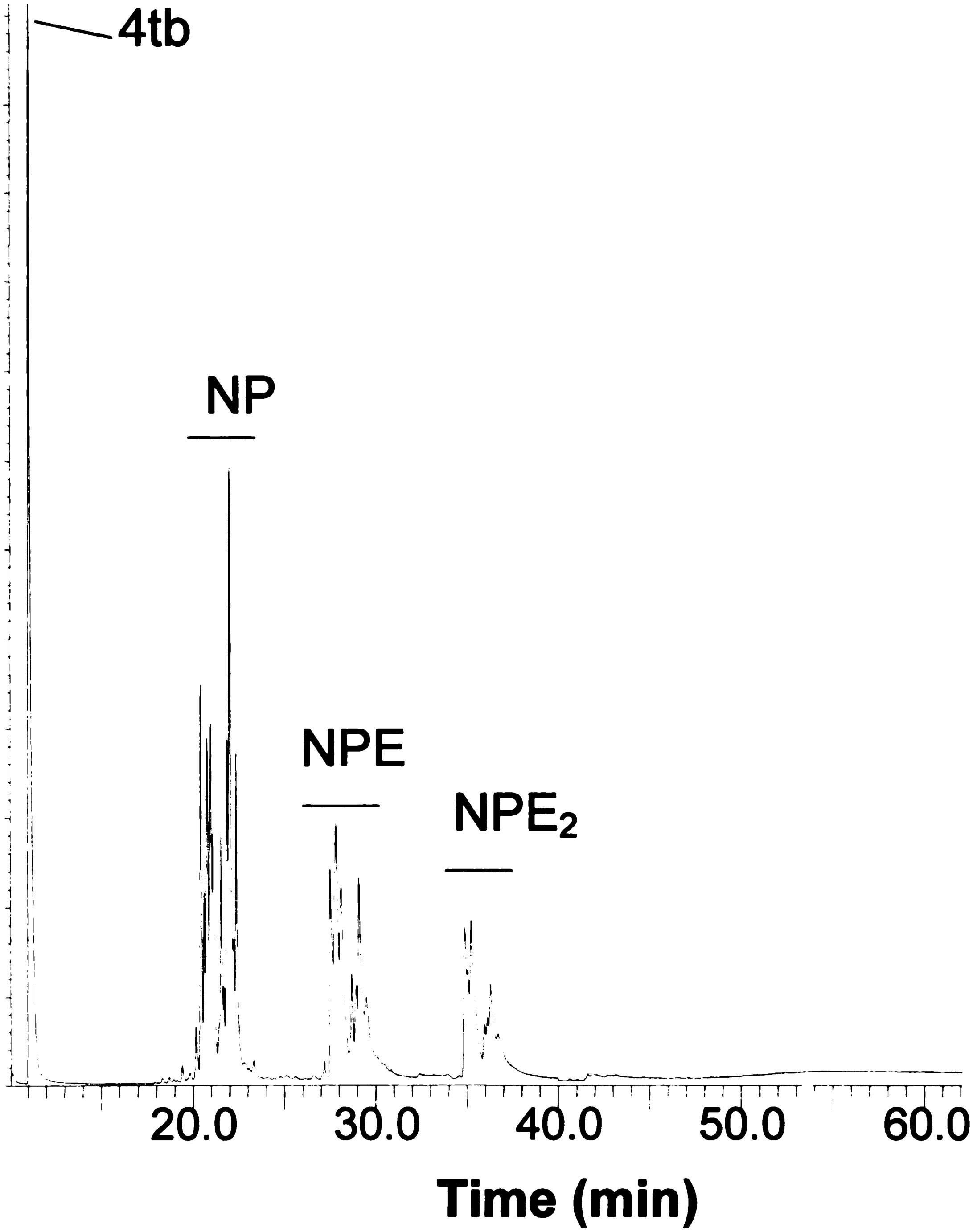


Figure 5. GC/MS chromatogram showing standard solution of 4tb, NP, NPE_1 and NPE_2.

With the improved separation and detection method, NP and NPE_1 were detected in the Lake Mead carp at average concentrations of 184 +/- 4 ng/g and 242 +/- 9 ng/g, wet weight, respectively. NPE_{2-3} were not detected in any carp collected at Lake Mead.

Recovery and Precision

Recoveries of NP and NPE_{1-2} were greater than 70% with CVs less than 20%. NPE_3 was found not to have sufficient volatility to have adequate recovery (Table 3).

Table 3. Recoveries of spiked analytes.

Analyte	Percent Recovery	Percent Coefficient of Variation
NP	78.1	9.2
NPE_1	76.1	9.8
NPE_2	69.4	13.0
NPE_3	17.0	20.1

Instrumental detection limits (IDLs) were determined by analyzing dilute standards (near the estimated IDL) and calculating the signal-to-noise for each standard concentration. Linear regression of the signal to noise ratios against concentrations was then used to determine the IDL (signal to noise ratio = 3). Seven replicates of homogenized goldfish tissues were spiked with NP and NPE_{1-3} at the estimated method detection limits (MDLs) to determine recovery and precision (Table 4). MDLs were calculated by multiplying the standard deviation of the recovered concentrations by a *t*-value of 3.1427 (for n=7 replicates).

Table 4. Instrumental and Method Detection Limits.

Analyte	IDL (ng)	MDL (μg/kg)
NP	5.1	3.3
NPE_1	15.5	16.8
NPE_2	17.3	18.2
NPE_3	112	20.6

Conclusion

This method was shown reliable to identify and quantify NP and $NPE_{1\text{-}2}$ in trace amounts and NPE_3 if present in sufficient quantity in fish tissues. With a reliable method now developed, the next facet of the study was to apply this method to determine concentrations of NP and $NPE_{1\text{-}3}$ in fish tissues in Michigan rivers and lakes.

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Chapter 2

FIELD APPLICATION

Introduction

The most comprehensive survey of nonylphenol from the United States reported water and sediment concentrations of NP and NPEs from 30 rivers that are influenced by municipal or industrial wastewater effluents (Naylor et al., 1992). That study found that 60-75% of water samples had no detectable levels of NP, NPE_1, or NPE_2 while 30% of sediments were non-detect. However, reports on the concentrations of APs and APEs in fish in the U.S. waters are scarce. Recently, NP and NPE have been detected in water of the Las Vegas Bay of Lake Mead, Nevada (Snyder et al., 2000a).

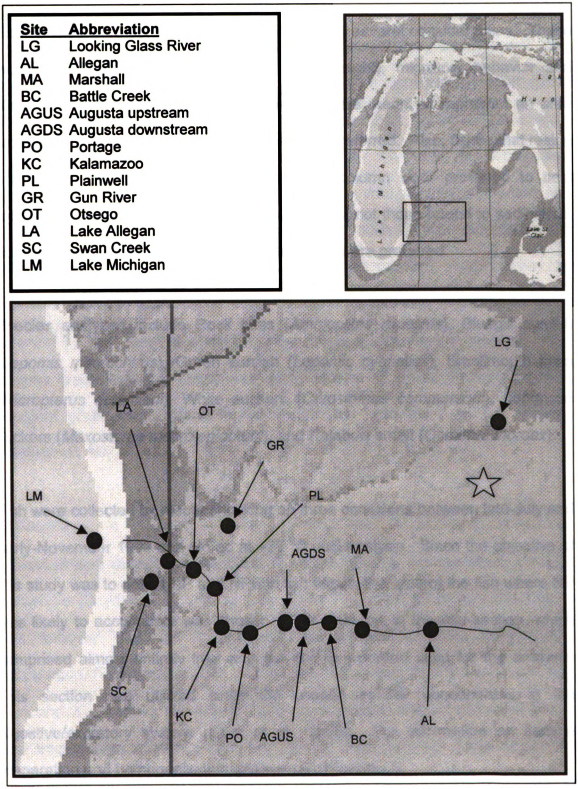
While information on the occurrence of NP in water and sediments is available, due to the lack of suitable analytical techniques, very few studies have examined the occurrence of nonylphenol in fish. Monitoring of NP in fish is important to assess the potential for dietary exposure of humans and wildlife. A method was developed for the analysis of NP and its ethoxylates in fish (Snyder et al., 2000b). In this study, the method was applied to determine concentrations of NP and $NPE_{1\text{-}3}$ in fish from Michigan waters.

The objective of this study was to measure concentrations of NP and $NPE_{1\text{-}3}$ in fish from various rivers in Mid-Michigan and the Great Lakes, USA. Since these fish were not caged, samples from a particular "site" actually represent a segment of water that may range approximately 3 km up or downstream from the sampling location. Thus, analyte concentrations should be associated with a river segment, rather than a specific point. This information will prove valuable to guide further studies.

Overview of Study Area

Sampling sites were chosen to represent the ambient environmental concentrations of the compounds of interest (COI) in fish. Fish were collected with assistance from the Michigan Department of Environmental Quality from two major regions: the Kalamazoo River Basin, MI and Lake Michigan near the mouth of the Kalamazoo River (Figure 6). The Kalamazoo River flows through both urban areas and rural areas and receives secondary and tertiary WWTP effluent and industrial discharges including those of paper manufacturing facilities. For information on sample transfer from the Michigan Department of Environmental Quality, please see Appendix C.

Sampling along the Kalamazoo River was conducted up and downstream of WWTPs whenever possible. Fish were captured within approximately 40 m of the WWTP effluents. The Kalamazoo WWTP and the Battle Creek WWTP have tertiary treatment. The Portage, Allegan, Marshall, Gun Lake, Augusta, Albion and Otsego WWTPs employ secondary treatment.



Site	Abbreviation
LG	Looking Glass River
AL	Allegan
MA	Marshall
BC	Battle Creek
AGUS	Augusta upstream
AGDS	Augusta downstream
PO	Portage
KC	Kalamazoo
PL	Plainwell
GR	Gun River
OT	Otsego
LA	Lake Allegan
SC	Swan Creek
LM	Lake Michigan

Figure 6. Location of sampling sites.

Fish and Sampling

Fish species were selected for analysis based on several considerations. These include availability at sampling sites, size (weight), migratory behavior and placement in the food chain. Since the fish were meant to represent an area within a river, only less migrating species were preferred. Also, fishes that reside primarily in the middle depths of the water column were preferred to best represent the exposure to dissolved analytes and not those bound to sediments. Further, species that are classified as game fish were preferred.

Species analyzed include Rock bass (*Ambloplites rupestris*), Bluegill sunfish (*Lepomis macrochirus*), Green sunfish (*Lepomis cyanellus*), Smallmouth bass (*Micropterus dolomieui*), White suckers (*Catostomus commersoni*), Longnose suckers (*Maxostoma macrolepidotum*), and Rainbow smelt (*Osmerus mordax*).

Fish were collected by electroshocking on three occasions between late-July and early-November 1999 and stored at –20 ^{0}C until analysis. Since the objective of this study was to detect NP and NPE in fish tissue, the area of the fish where NP was likely to accumulate was chosen. Fish were cut at the mid-section, which comprised almost entirely liver and gut and this portion used for the analysis. This section was utilized since the chosen as NP concentrates in the digestive/excretory system (Liber et al., 1999). For information on sample preparation and homogenization, please see Appendix D.

Standards and Reagents

High purity standards ($\geq$96% purity) of *p*-nonylphenol (NP), *p*-cumylphenol (CP), and 4-*tert*-butyl orthocresol (4tb) were obtained from Schenectady International (Freeport, TX). Standards of NPE_{1-3} were obtained from Huntsman Corporation (Austin, TX). High purity pesticide residue grade *n*-hexane, dichloromethane (DCM), and iso-octane were obtained from Burdick and Jackson (Muskegon, MI). Organic-free water was obtained by purification of reverse osmosis treated water followed by Nanopure^{TM} (Barnstead, Dubuque, IA) treatment. All glassware and stainless steel homogenization equipment was rinsed with organic-free water followed by high purity pesticide residue grade acetone and *n*-hexane. ACS reagent grade sodium chloride was obtained from JT Baker (Phillipsburg, NJ). Reagent grade concentrated sulfuric acid was obtained from EM Science (Gibbstown, NJ).

Extraction

Extraction and quantitation methods of nonylphenolics are described in detail elsewhere (Snyder et al., 2000b). Briefly, a 20 g representative cross-section was removed from the sample and homogenized (Blender 700, Waring Corporation, New Hartford, CN). This homogenate was transferred to a boiling flask and 20 g sodium chloride and 3 mL concentrated sulfuric acid were added. This homogenate was then extracted using a Nielsen-Kryger improved version steam-distillation column (Ace Glass, Vineland, NJ) for 3 hrs. The resulting extract was concentrated to 1 mL in iso-octane using a Nitrogen Evaporator

(Organomation Associates, Inc., Berlin, MA). To further remove lipids from the sample extract, a Perkin-Elmer (Norwalk, CT) series 200 autosampler and binary pump and a Hewlett Packard (HP) (Palo Alto, CA) 1046A fluorescence detector was employed to separate lipids from the compounds of interest. Eight hundred □L of the iso-octane extract was separated using a Phenomenex Luna 5 μm silica column (250 mm x 4.6 mm, Torrance, CA) by a 0.65 mL/min isocratic elution using 12% 1:4 MeOH:DCM and 88% hexane. Fluorescence detection was used to determine surrogate recovery during this fractionation. A fraction of HPLC effluent was collected between 7 and 16 min, 3.0 μg 4-*tert*-butyl orthocresol added as an internal standard and concentrated under nitrogen to 100 μL iso-octane. 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 ID, 0.15 μm film, J&W Scientific, Folsom, CA). The GC was held at 100 °C for 2 min and ramped to 300 °C for 10 min at 4 °C/min. The MSD was operated in selected ion monitoring (SIM) mode with 3 ions monitored for each compound of interest. Internal standard recoveries were greater than 80 %. Matrix spike recoveries were greater than 70 % for NP and NPE_{1-2}, 17 % for NPE_3. MDLs for NP, NPE_1, NPE_2, and NPE_3 were 3.3, 16.8, 18.2 and 20.6 ng/g, ww, respectively.

Results and Discussion

Concentrations of NP greater than the MDL were found in 75 of 197 (38%) samples across all sites and species with a mean concentration, excluding non-detects, of 12.0 ng NP/g, wet weight (ww) with a range of 3.3 ng NP/g, ww to 29.1 ng NP/g, ww (Figure 7). If non-detects are included, the mean NP concentration across all sites and species is 4.0 ng NP/g, ww. NPE_1 was found in 21 samples but all at concentrations below the calculated MDL (16.8 ng NPE_1/g, ww). NPE_2 and NPE_3 were not detected in any of the samples.

Five of the seven species contained detectable concentrations of NP. Rock bass (*Ambloplites rupestris*) contained the greatest average detectable NP concentration, 8.1 ng NP/g, ww, while the rainbow smelt (*Osmerus mordax*) exhibited the second greatest detectable tissue concentration at 7.7 ng NP/g, ww (Table 5). Longnose sucker (*Maxostoma macrolepidotum*) and green sunfish (*Lepomis cyanellus*) contained no detectable concentrations of NP.

There were no significant differences in concentrations of NP among species so the samples were pooled for comparison among sites (ANOVA with a Type I error of 0.1 followed by a Tukey's Studentized Range Test). However, there were significant differences in concentrations of NP in fish among sampling sites (Figure 7).

The greatest concentrations were found in fish from the section of the river near Kalamazoo and Battle Creek (Table 6). While these cities employ advanced wastewater treatment technologies, industrial discharges, either through the municipal WWTP or direct effluent discharge may have contributed to greater concentrations of nonylphenolic compounds in these cities.

Table 5. Detectable Concentrations of NP in Tissue Across Species.

Species	Mean tissue concentration (ng/g, ww)	Tissue concentration range (ng/g, ww)	Number of Samples <MDL
Longnose Sucker (*Maxostoma macrolepidotum*)	<3.3	ND	2/2 (100%)
Green Sunfish (*Lepomis cyanellus*)	<3.3	ND	4/4 (100%)
Bluegill Sunfish (*Lepomis macrochirus*)	5.7	<3.3 – 16.6	12/36 (33%)
Smallmouth Bass (*Micropterus dolomieui*)	5.8	<3.3 – 16.2	11/29 (38%)
White Sucker (*Catostomus commersoni*)	7.2	<3.3 – 20.3	23/60 (38%)
Rainbow Smelt (*Osmerus mordax*)	7.7	<3.3 – 10.1	2/5 (40%)
Rock Bass (*Ambloplites rupestris*)	8.1	<3.3 – 22.5	31/48 (65%)

ND = non-detectable

Table 6. Range of NP tissue concentrations by site and specie.

Location	Specie	Number of Samples Analyzed	NP Tissue Concentration Range (ng/g, ww)
LG	RB	8	ND
	WS	11	ND
OT	RB	9	<3.3 – 6.1
	GS	4	ND
LA	BG	8	<3.3 – 6.4
AGDS	SM	9	<3.3 – 9.5
LM	RS	5	<3.3 – 10.1
GR	RB	7	<3.3 – 11.2
PO	WS	20	<3.3 – 11.9
AL	RB	6	<3.3 – 13.1
	LNS	2	ND
	BG	2	<3.3 – 7.1
MA	SM	8	<3.3 – 16.2
PL	WS	18	<3.3 – 20.3
	RB	2	ND
SC	SM	5	<3.3 – 16.4
	WS	11	<3.3 – 21.4
KC	RB	10	<3.3 – 22.5
BC	BG	26	<3.3 – 26.6
	SM	2	<3.3 – 14.0
AGUS	SM	3	<3.3 – 15.8
	RB	7	<3.3 – 29.1

Species: WS = White sucker, RB = Rock bass, GS = green sunfish, LNS = Longnose sucker, BG= Bluegill sunfish, SM = Smallmouth bass. See Figure 6 for site abbreviations. ND = non-detactable.

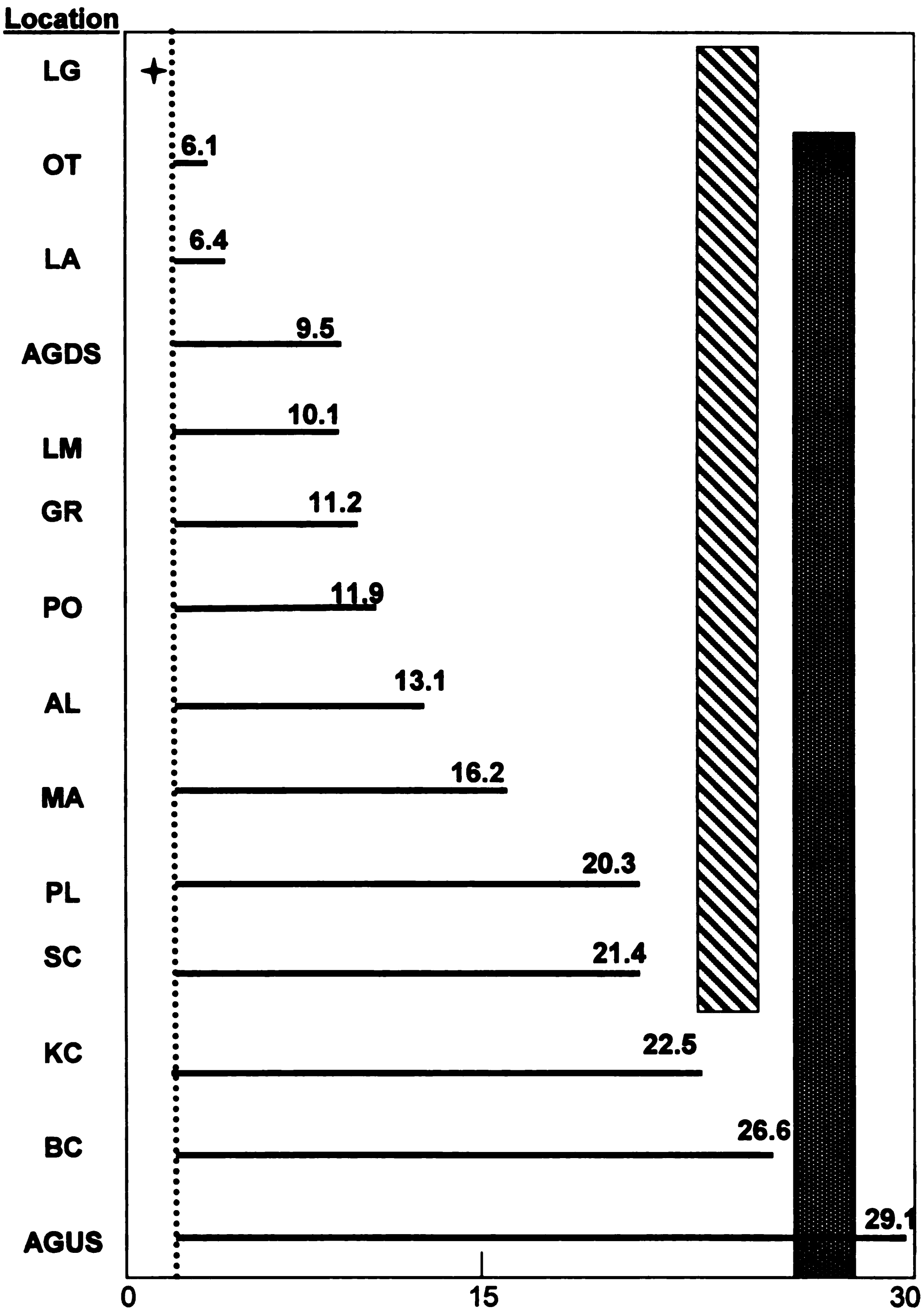


Figure 7. Range of NP Tissue Concentration, ng/g tissue, wet weight. ♦ = All samples below MDL. Vertical bars represent sites that are not significantly different. See Figure 6 for site abbreviations.

Estimated Water Concentrations and Bioconcentration Factors

Based on the concentrations of NP measured in fish and an assumed BCF, concentrations of biologically available NP were estimated. BCFs for NP have been reported earlier (Staples et al., 1998; Liber et al., 1999; Snyder et al., 2000c; Brooke, 1993; Ward and Boeri, 1991). The BCF value of 300 was used as an approximate estimate for various species. The BCF for bluegill sunfish is 220 (Brooke, 1993) and that for the fathead minnow is 271 (Ward and Boeri, 1991). To validate this BCF value, fathead minnows (*Pimephales promelas*) were exposed to 10 ng NP/mL for 28-30 d. These fish were prepared and extracted as described above. The resulting cross-section tissue NP concentration was 2.93 μg/g, ww (n=4), which corresponds to a BCF of 293.

By applying a BCF of 300 and the least and greatest observed tissue concentrations of 3.3 and 29.1 ng/g, ww, respectively, the estimated water concentrations ranged from 0.011 to 0.097 ng NP/mL, or 11 to 97 ng NP/L, which is comparable to the values reported by others (6, 14).

Ranges of Possible Concentrations

Since concentrations of NP in some fish were less than the MDL, there were uncertainties associated with presenting actual concentration ranges. Assigning a value of zero for those samples containing concentrations less than the MDL would underestimate overall mean values, while assigning MDL values for the non-detectable estimates would possibly overestimate the actual concentrations.

Thus, a range between maximum and minimum possible values was estimated. Proxy values were substituted for samples for which the actual concentrations were less than the MDL. These values are chosen such that the greatest possible ranges of concentration were calculated. Therefore, samples for which concentrations were less than the MDL were first assigned the least possible concentration of zero ng/g, then the greatest possible non-detectable concentration, which is the MDL for that analyte. Samples that had detectable concentrations were not altered. A non-parametric Wilcoxins Rank Test was performed on each site and species to determine a range of possible concentrations (Figure 8). When using this proxy value substitution, tissue concentrations among all species and sites ranged from 4.4 – 6.4 ng NP/g ww. Concentrations of NPE_{1-3} were all less than their respective MDLs. The estimated ranges were 0.0 - 16.8, 0.0 - 18.2, and 0.0 - 20.6 ng/g, ww, respectively for the 3 NPEs.

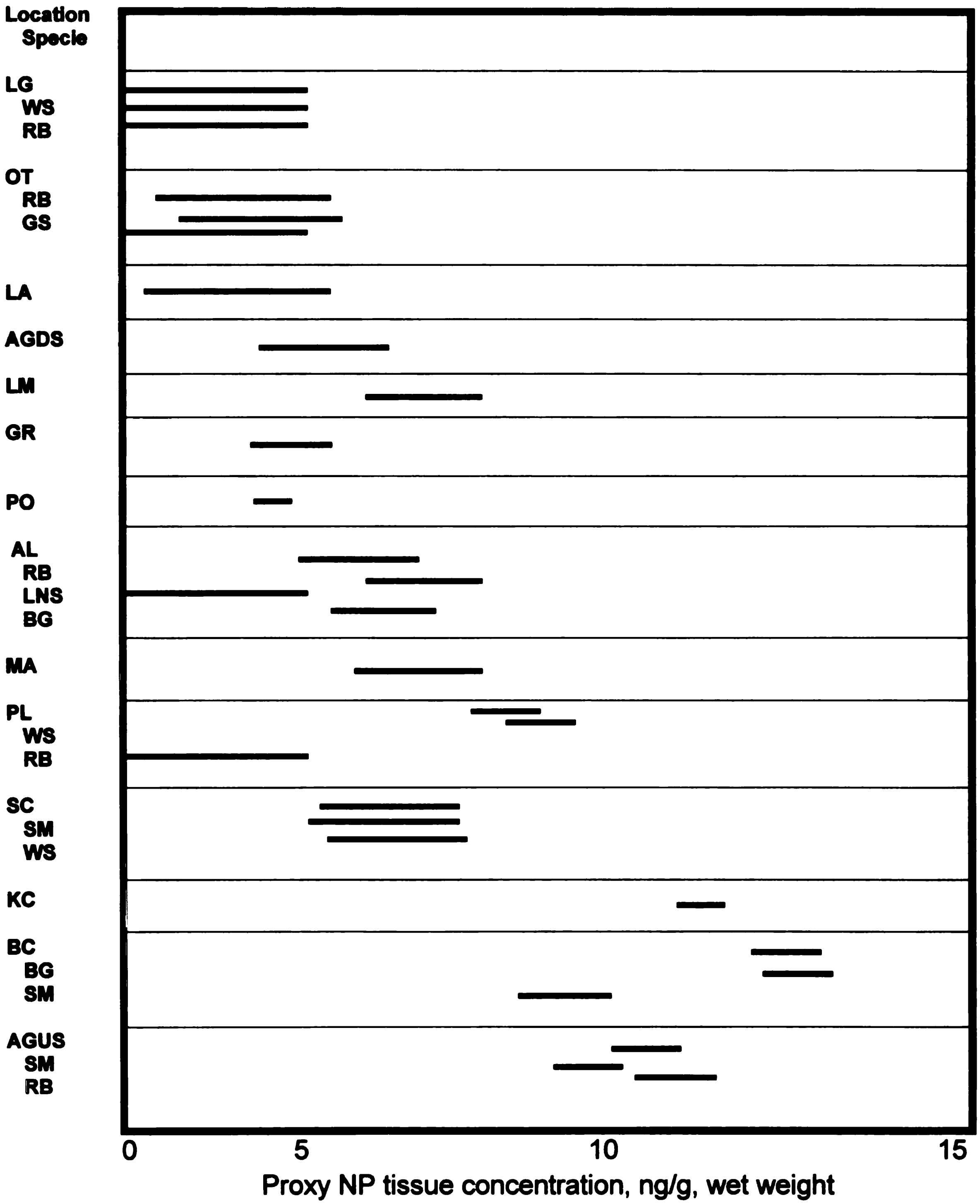


Figure 8. NP tissue concentration using proxy values for non-detects. Species: WS = White sucker, RB = Rock bass, GS = Green sunfish, LNS = Longnose sucker, BG= Bluegill sunfish, SMB = Smallmouth bass. See Figure 6 for site abbreviations.

Conclusion

The results of this study suggest the presence of nonylphenolics in fish, but at relatively low concentrations. NP is the predominant compound, with concentrations of NPEs less than those of NP. Fishes collected near WWTP effluent discharge sites contain relatively greater concentrations than those collected from more remote areas. Future research is needed at those areas where fish tissue NP concentration is great to determine biological implications and routes of exposure.

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APPENDICES

APPENDIX A

GLASSWARE CLEANING: STREAM DISTILLATION APPARATUS

Michigan State University
National Food Safety and Toxicology Center
Aquatic Toxicology Laboratory

Standard Operating Procedure

Glassware Cleaning:
Stream Distillation Apparatus

Version 2 June 19, 1999

Timothy L. Keith, Shane A. Snyder and John P. Giesy

Correspondence to:
218C National Food Safety and Toxicology Center
Michigan State University
East Lansing, MI 48824-1222
USA
T: 517.353.1957
F: 517.432.2310

DEFINITIONS AND ACRONYMS

APERC	Alkylphenol Ethoxylates Research Council
ATL	Aquatic Toxicology Laboratory
MSU	Michigan State University
QA	Quality Assurance
SOP	Standard Operating Procedure

1.0 PURPOSE

This standard operating procedure (SOP) specifies the procedure for cleaning the Nielsen-Kryger improved version steam-distillation apparatus.

2.0 SCOPE AND APPLICATION

Extraction of organic contaminants at trace levels requires extremely clean handling of glassware. Due to the intricate nature of the steam-distillation apparatus and the difficulty of cleaning by traditional means, this SOP will be used to clean the steam-distillation apparatus while SOP 212 will be used for all other glassware.

3.0 SAFETY CONSIDERATIONS

The solvents, acids, and bases used in this glassware cleaning procedure are hazardous. Care should be taken to minimize exposure according to institutional guidelines (refer to the Safety Manual for the Aquatic Toxicology Laboratory at Michigan State University), medium should be collected in a liquid trap for disposal as hazardous waste.

4.0 EQUIPMENT, MATERIALS, AND REAGENTS

- Alconox or Liquinox – a quality commercial glassware detergent.
- High purity acetone and hexane.
- Clean drying rack or cart.
- Barnstead or equivalent water purification system.
- Nitric acid.
- Sodium hydroxide tablets.

5.0 METHOD, PROCEDURES, AND REQUIREMENTS

- Fill a sink with hot water and add enough detergent to form a good bath and soak the glassware. Brush clean. ***(Be careful of the apparatus's drain spout; it is easily broken while being handled).***
- Rinse the glassware clean of soap detergent with tap water. Then, rinse 3 times with deionized water and drain.
- Soak the apparatus in a 17% nitric acid (HNO_3) bath for 1 hour.
- Carefully drain the apparatus and rinse with deionized water.
- Soak the apparatus in a 1 M sodium hydroxide (NaOH) bath for 1 hour.
- Again, carefully drain the apparatus and rinse with deionized water 3 times.
- Set aside in the clean glassware cart and allow to dry.
- Rinse the apparatus 3 times with high purity acetone, followed by 3 times with high purity hexane and allow to dry before reuse.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

6.1 Records and Documentation

The primary analyst shall document any anomalies and/or deviations from the specific method in a bound, serially numbered, laboratory notebook with tear-out carbon copies.

The technical reviewer will record any problems noted during the technical review. The technical reviewer will return the items to the analyst for corrections prior to inclusion in the data package. The technical reviewer will sign and date all forms as the reviewer.

7.0 RESONSIBILITES

The primary analyst will complete the glassware cleaning as specified in the SOP and provide documentation of any anomalies.

8.0 REFERENCES

8.1 MSU-ATL SOP 212

APPENDIX B

EXTRACTION AND QUANTITATION OF ALKYLPHENOL AND ALKYLPHENOL ETHOXYLATES IN TISSUE SAMPLES

Michigan State University
National Food Safety and Toxicology Center
Aquatic Toxicology Laboratory

Standard Operating Procedure

Extraction and Quantitation of Alkylphenol and Alkylphenol Ethoxylates in Tissue Samples

Version 2 February 25, 2000

Timothy L. Keith, Shane A. Snyder, K. Kannan and John P. Giesy

Correspondence to:
218C National Food Safety and Toxicology Center
Michigan State University
East Lansing, MI 48824-1222
USA
T: 517.353.1957
F: 517.432.2310

DEFINITIONS AND ACRONYMS

APERC	Alkylphenol Ethoxylates Research Council
ATL	Aquatic Toxicology Laboratory
COC	Chain of Custody
MSU	Michigan State University
ORCBS	Office of Radiation, Chemical, and Biological Safety (MSU)
PMAPERC	Program Manager Alkylphenol Ethoxylates Research Council
PPE	Personal Protective Equipment
SOP	Standard Operating Procedure
STS	Sample Tracking Sheet

1.0 PURPOSE

This standard operating procedure (SOP) specifies the procedure for extraction and quantitation of alkylphenol and alkylphenol ethoxylates in samples collected by the Michigan Department of Environmental Quality (MDEQ) and the Aquatic Toxicology Laboratory (ATL) for the Alkylphenol Ethoxylates Research Council (APERC) Project.

2.0 SCOPE AND APPLICATION

This SOP applies to samples to be analyzed for the APERC. Samples will be sent under chain of custody (COC) from the MDEQ to MSU for homogenization and subsequent analysis. This SOP will describe extraction and quantitation procedures only. See MSU-ATL SOP 223 for homogenization procedures.

3.0 SAFETY CONSIDERATIONS

All safety considerations will be in accordance with MSU-ORCBS procedures.

3.1 Personal Protective Equipment

Personal protective equipment (PPE) consisting of lab coats, safety glasses, and latex gloves will be worn at all times when handling samples.

3.2 Waste Management

All waste will be managed and disposed in accordance with MSU-ORCBS regulations. Waste management practices will include the control of all standards and solutions. This means that expired or used standards and associated solvents will be disposed of in labeled waste containers and ORCBS will be notified for waste pick up.

3.3 Sample Decontamination

If a spill occurs in the laboratory, ORCBS will be notified immediately. This area where the spill occurred will be evacuated and marked.

4.0 EQUIPMENT, MATERIALS, AND REAGENTS

Equipment for extraction

- 2 L round bottom boiling flask (Ace Glass, Vineland, NJ)

- Nielsen-Kryger improved version steam-distillation apparatus (Ace Glass, Vineland, NJ)
- Heating mantle capable of accommodating 2 L round bottoms (Glass-Col, Terre Haute, IN)
- Magnetic stir plate (VWR Scientific, So. Plainfield, NJ)
- Rheostat temperature controller (Glass-Col, Terre Haute, IN)
- Cooling water system
- Nitrogen evaporator for sample concentration (Organomation Associates, Inc., Berlin, MA)
- 15 mL centrifuge tubes (Ace Glass, Vineland, NJ)
- Amber autosampler vials and Teflon lined caps

Equipment for quantitation
- High pressure liquid chromatograph binary pump with autosampler (Perkin Elmer series 200, Norwalk, CT)
- Programmable fluorescence detector (Hewlett Packard 1046A fluorescence detector, Palo Alto, CA)
- Phenomenex Luna 5μ silica (250 mm x 4.6, Torrance, CA) analytical chromatography column or equivalent
- Gas chromatograph (5890 Series II Plus, Hewlett Packard, Palo Alto, CA)
- Mass spectrometer (5972 MSD, Hewlett Packard, Palo Alto, CA)

Materials for extraction
- High purity iso-octane
- Reverse osmosis laboratory grade water
- Concentrated sulfuric acid

Materials for quantitation
- High purity methanol
- High purity dichloromethane
- High purity hexane
- High purity standards of nonylphenol (NP), nonylphenol monoethoxylate (NPE_1), nonylphenol diethoxylate (NPE_2), nonylphenol triethoxylate (NPE_3), para-cumylphenol (CP), and 4-tert-butyl-*o*-cresol (4tb).

5.0 METHODS, PROCEDURES, AND REQUIREMENTS

APs and APEs can be identified and quantified by high-pressure liquid chromatography with fluorescence detection (HPLC) and gas chromatography/mass spectrometry (GC/MS). Each technique has advantages and disadvantages. Therefore, it is suggested that if HPLC-fluorescence is used to quantification, GC/MS be used to verify the identity and purity of the analytes.

5.1 Standard Curve Generation for HPLC-fluorescence

5.1.1 Prepare standards of NP, NPE_1, NPE_2, NPE_3, CP, and 4tb in iso-octane at concentrations varying from 0.100 ng/mL to 100 µg/mL.

5.1.2 Prepare a 1 L mixture of 20% methanol/dichloromethane by adding 800 mL of dichloromethane to 200 mL methanol (hereafter referred to as **Solvent A**; hexane is referred to as **Solvent B**).

5.1.3 Set the fluorescence detector to 229 nm excitation, 310 nm emission, PMT gain of 12, lamp time of -1, response time of 2 sec, stop time at 30 min, gate and delay at zero.

5.1.4 Equilibrate the chromatography column by purging 12% Solvent A and 88% Solvent B with a flow rate of 2.0 mL/min for 30 minutes.

5.1.5 After solvent purge is complete, program the binary pump to elute at 12% Solvent A/88% Solvent B at 0.65 mL/min for 30 minutes upon sample injection.

5.1.6 Inject each standard in triplicate and average the peak area at each concentration.

5.1.7 For each compound, perform a linear regression to develop the Standard Curve. If the coefficient of determination, R^2, is 0.90 or less, new standards must be prepared and reanalyzed.

5.2 Standard Curve Generation for GC/MS

5.2.1 Inject 4µL of each standard prepared above in triplicate at each concentration using a DB-17 column, starting temperature of 100°C for 2 min, ramp to 300°C for 10 min at 4° C/min. To detect the analytes, operate in SIM mode monitoring the following m/z values:

Analyte	m/z value
NP	107, 135, 149
NPE_1	135, 179, 193
NPE_2	135, 223, 237
NPE_3	135, 267, 281
4tb	121, 149, 164

5.2.2 Average the peak areas at each concentration and for each compound perform a linear regression to develop the Standard Curve. If the coefficient of determination, R^2, is 0.90 or less, new standards must be prepared and reanalyzed

5.3 Sample Extraction

5.3.1 Homogenize sample as described in MSU-ATL SOP 223 and add 3 mL concentrated sulfuric acid to the homogenate.

NOTE: *Addition of sulfuric acid to water too rapidly may cause bumping and pose a safety risk. Acid should be added* ***slowly****.*

5.3.2 Place round bottom flask on heating mantle and secure.
5.3.3 Place magnetic stir plate below heating mantle and adjust to a rapid stir.
5.3.4 Add 50μL of 10 ppm CP as an internal standard.
5.3.5 Attach the steam-distillation apparatus to the round bottom.
5.3.6 Add 3 mL reverse osmosis water through the top of the steam-distillation apparatus, then 10 mL high purity iso-octane, then cap the column with aluminum foil.
5.3.7 Turn cooling water on to full flow.
5.3.8 Adjust the Rheostat temperature control to HIGH.
5.3.9 At first sign of boiling, time the extraction for 1 ½ hr.
5.3.10 After the 1½ hr has expired, turn the Rheostat to OFF and wait for boiling to cease.
5.3.11 Decant the lower water layer in the steam-distillation apparatus into waste.
5.3.12 Decant the iso-octane layer into a 15 mL centrifuge tube and record volume.
5.3.13 To the apparatus, add an additional 3 mL reverse osmosis water, then 10 mL iso-octane and cap with aluminum foil.
5.3.14 Turn the Rheostat temperature control to HIGH.
5.3.15 At first sign of boiling, time the extraction for 1 ½ hr.
5.3.16 While second extraction is running, place the 15 mL centrifuge tube containing the first 10 mL of iso-octane in a nitrogen evaporator set to a temperature of 30 °C and concentrate under a gentle stream of nitrogen to 3 mL.
5.3.17 Cap the centrifuge tube and wait for extraction to complete.
5.3.18 After the 1 ½ hr has expired, turn the Rheostat to OFF and wait for boiling to cease.
5.3.19 Decant the lower water layer in the steam-distillation apparatus into waste.
5.3.20 Decant the iso-octane layer into the same 15 mL centrifuge tube as the first extraction and record volume.
5.3.21 Concentrate under a gentle stream of nitrogen to 1 mL.
5.3.22 Transfer to an amber autosampler vial and cap with Teflon sealed autosampler vial cap.
5.3.23 Store in a –20 °C freezer until quantitation can proceed.

5.4 Sample Chromatography for High Pressure Liquid Chromatography

5.4.1 Prepare a 1 L mixture of 20% methanol/dichloromethane by adding 800 mL of dichloromethane to 200 mL methanol (hereafter referred to as **Solvent A**; hexane is referred to as **Solvent B**).

5.4.2 Set the fluorescence detector to 229 nm excitation, 310 nm emission, PMT gain of 12, lamp time of -1, response time of 2 sec, stop time at 30 min, gate and delay at zero.

5.4.3 Equilibrate the chromatography column by purging 12% Solvent A and 88% Solvent B with a flow rate of 2.0 mL/min for 30 min.

5.4.4 After solvent purge is complete, program the binary pump to elute at 12% Solvent A/88% Solvent B at 0.65 mL/min for 30 min upon sample injection.

5.4.5 Inject 800 μL of the extract and collect the eluent between the times of 7 min to 16 min.

5.4.6 The HPLC chromatogram will be used to calculate the recovery of the spiked CP, indicative of the recovery efficiency of the extraction.

5.4.7 Concentrate eluent under nitrogen to 1mL, add 30 μL of 100 ppm 4tb as an internal standard to the eluent and concentrate to 100 μL. Transfer to an autosampler vial and proceed with GC/MS quantitation.

5.5 Sample Chromatography for Gas Chromatography/Mass Spectrometry

5.5.1 Using the same GC/MS method described above, inject 4 μL of the sample extract and record data. The GC/MS chromatograms will be used to calculate the concentrations of NP and NPE_{1-3}, confirm analyte identity as well as the recovery of the spiked 4tb to determine the loss, if any, of analytes during concentration or sample injection.

5.6 Calculations

5.6.1 Using the Standard Curve for each analyte generated above, calculate the concentration of each target analyte present in the extract by solving for *x* using the relationship:

$$y = mx + b$$

where y is the integrated peak area, m is the calculated slope, x is the concentration of the target analyte, and b is the calculated intercept.

5.6.2 Divide the extract concentration by the sample weight to determine the concentration of target analytes in the whole fish.

5.7 Glassware Cleaning

5.7.1 After extraction has completed, **thoroughly** rinse the inside of the steam-distillation apparatus, first with high purity acetone and then high purity hexane.

5.7.2 This means of cleaning can only be done a maximum of three times for one given batch or until the batch has been completed, whichever comes first, before MSU-ATL SOP 222 (Apparatus Cleaning) takes priority.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

6.1 Records and Documentation

Documentation will be consistent with procedures outlined in MSU-ATL SOPs 803 and 402. All samples will be labeled at all times and will be accompanied by COC, STS, and an extraction worksheet.

6.2 QC Requirements

With every batch of samples (samples belonging to a collection site) a method blank will be performed. This blank will be performed by following the extraction and quantitation procedure, MSU-ATL SOP 803, with every component except a homogenized sample.

7.0 RESPONSIBILITIES

The primary analyst will complete the analysis as specified in this SOP and provide documentation of raw data, any anomalies, and data to the data analyst who will perform data calculations in accordance with MSU-ATL SOP 224.

The technical reviewer will determine if data quality objectives were met and notify the analyst if any problems were found.

8.0 REFERENCES

8.1 MSU-ATL SOP 222

8.2 MSU-ATL SOP 223

8.3 MSU-ATL SOP 803

8.4 MSU-ATL SOP 402

APPENDIX C

REQUIREMENTS FOR COLLECTION AND TRANSFER OF SAMPLES OBTAINED BY THE MDEQ

Michigan State University
National Food Safety and Toxicology Center
Aquatic Toxicology Laboratory

Standard Operating Procedure

Requirements for Collection and Transfer of Samples Obtained by the MDEQ

Version 2 June 19, 1999

Timothy L. Keith, Shane A. Snyder and John P. Giesy

Correspondence to:
218C National Food Safety and Toxicology Center
Michigan State University
East Lansing, MI 48824-1222
USA
T: 517.353.1957
F: 517.432.2310

DEFINITIONS AND ACRONYMS

APERC	Alkylphenol Ethoxylates Research Council
ATL	Aquatic Toxicology Laboratory
COC	Chain of Custody
MDEQ	Michigan Department of Environmental Quality
MSU	Michigan State University
ORCBS	Office of Radiation, Chemical, and Biological Safety (MSU)
PMAPERC	Program Manager Alkylphenol Ethoxylates Research Council
PPE	Personal Protective Equipment
SOP	Standard Operating Procedure
STS	Sample Tracking Sheet

1. PURPOSE

This standard operating procedure (SOP) specifies the procedure for the collection and transfer of samples collected by the Michigan Department of Environmental Quality (MDEQ) and the Aquatic Toxicology Laboratory (ATL) for the Alkylphenol Ethoxylates Research Council (APERC) Project.

2. SCOPE AND APPLICATION

This SOP applies to the MDEQ and ATL for samples to be analyzed for the APERC. Samples will be sent under chain of custody (COC) to Michigan State University (MSU) for homogenization and subsequent analysis. This SOP will describe the collection and transfer procedures only.

3. SAFETY CONSIDERATIONS

All safety considerations will be in accordance with MSU-ORCBS procedures. Personnel at the MDEQ will follow all prudent safety procedures they deem necessary. The following guidelines apply to personnel at the MSU-ATL.

3.1 Personal Protective Equipment

Personal protective equipment (PPE) consisting of lab coats, safety glasses, and latex gloves will be worn at all times when handling samples.

3.2 Waste Management

All waste will be managed and disposed in accordance with MSU-ORCBS regulations. Waste management practices will include the control of all standards and solutions. This means that expired or used standards and associated solvents will be disposed of in labeled waste containers and ORCBS will be notified for waste pick up.

3.3 Sample Decontamination

If a spill occurs in the laboratory, ORCBS will be notified immediately. This area where the spill occurred will be evacuated and marked.

4.0 EQUIPMENT, MATERIALS, AND REAGENTS

Equipment for collection procedure include:
- All equipment the MDEQ requires in their SOP for fish collection.

Materials for collection procedure include:
- Freezer bags with secure seal, small size
- Freezer bags with secure seal, large size
- Small strips of paper suitable for label making
- Pencil
- Permanent marker
- Watch or clock for timekeeping
- Multiple large coolers or similar containers filled with ice

Reagents for collection procedure include:
- No reagents are required for sample collection

5.0 METHODS, PROCEDURES, AND REQUIREMENTS

5.1 Sample Collection

5.1.1 Fish are electroshocked using the SOP developed at the MDEQ and performed by MDEQ personnel.
5.1.2 Upon retrieval of stunned fish, a visual determination of approximate fish weight will be done by MDEQ personnel.
5.1.3 If fish is approximated to be greater than 400 g or less than 20 g, the fish will not be collected for subsequent analysis and released.
5.1.4 If fish is approximated to weigh between 20 g and 400 g, the fish is placed in a small freezer bag. A strip of paper detailing the location of site and time of collection written in pencil is also placed in the freezer bag.
5.1.5 The freezer bag is sealed and the location of site and time of collection written in permanent marker on the outside of the freezer bag.
5.1.6 The sample is placed in a large freezer bag with samples collected only at the same site and placed in a large cooler or similar container filled with ice.
5.1.7 At the end of the sampling trip, personnel at the ATL will be notified for sample pick-up or delivery.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

6.1 Records and Documentation

Documentation will be consistent with procedures outlined in MSU SOP 402 (Sample Management) and MDEQ SOP 48. All samples will be labeled at all times and will be accompanied by a COC form.

6.2 QC Requirements

There are no QC requirements for sample collection.

7.0 RESPONSIBILITIES

Personnel for the MDEQ will follow the MDEQ SOP 48 and MSU SOP 221 (this one) during fish collection and will properly complete the COC forms and document any anomalies during collection.

The MSU-ATL will review and complete COC forms as detailed in MSU SOP 221.

8.0 REFERENCES

8.1 MSU-ATL SOP 402
8.2 MDEQ SOP 48

APPENDIX D

STANDARD OPERATING PROCEDURE FOR HOMOGENIZATION OF TISSUE SAMPLES FOR MEASUREMENT OF ALKYLPHENOLS AND ALKYLPHENOL ETHOXYLATES

Michigan State University
National Food Safety and Toxicology Center
Aquatic Toxicology Laboratory

Standard Operating Procedure

Homogenization of Tissue Samples for Measurement of Alkylphenols and Alkylphenol Ethoxylates

Version 2 February 25, 2000

Timothy L. Keith, Shane A. Snyder, K. Kannan and John P. Giesy

Correspondence to:
218C National Food Safety and Toxicology Center
Michigan State University
East Lansing, MI 48824-1222
USA
T: 517.353.1957
F: 517.432.2310

DEFINITIONS AND ACRONYMS

APERC	Alkylphenol Ethoxylates Research Council
ATL	Aquatic Toxicology Laboratory
COC	Chain of Custody
MDEQ	Michigan Department of Environmental Quality
MSU	Michigan State University
ORCBS	Office of Radiation, Chemical, and Biological Safety (MSU)
PMAPERC	Program Manager Alkylphenol Ethoxylates Research Council
PPE	Personal Protective Equipment
SOP	Standard Operating Procedure
STS	Sample Tracking Sheet

1.0 PURPOSE

This standard operating procedure (SOP) specifies the procedure for homogenization of samples collected by the Michigan Department of Environmental Quality (MDEQ) and the Aquatic Toxicology Laboratory (ATL) for the Alkylphenol Ethoxylates Research Council (APERC) Project.

2.0 SCOPE AND APPLICATION

This SOP applies to the ATL for samples to be analyzed for the APERC. Samples will be sent under chain of custody (COC) from the MDEQ to MSU for homogenization and subsequent analysis. This SOP will describe homogenization procedures only. See MSU-ATL SOP 224 for the methods for extraction and quantitation.

3.0 SAFETY CONSIDERATIONS

All safety considerations will be in accordance with MSU-ORCBS procedures.

3.1 Personal Protective Equipment

Personal protective equipment (PPE) consisting of lab coats, safety glasses, and latex gloves will be worn at all times when handling samples.

3.2 Waste Management

All waste will be managed and disposed in accordance with MSU-ORCBS regulations. Waste management practices will include the control of all standards and solutions. This means that expired or used standards and associated solvents will be disposed of in labeled waste containers and ORCBS will be notified for waste pick up.

3.3 Sample Decontamination

If a spill occurs in the laboratory, ORCBS will be notified immediately. This area where the spill occurred will be evacuated and marked.

4.0 EQUIPMENT, MATERIALS, AND REAGENTS

Equipment for homogenization procedures include:

- Analytical balance to determine sample weight (Mettler, Type BB2400, Hightstown, NJ)
- Stainless steel 1 Liter Omni-Mixer cup and base for homogenization (Blender 700, Waring Corporation, New Hartford, CN or equivalent).
- Freezer at –20°C (for sample storage)
- Stainless steel serrated knife or saw
- 2 L round bottom boiling flask

Reagents for homogenization procedures include:

- Reverse-osmosis laboratory grade water

Materials for cleaning and storing of equipment used in the homogenization procedure include:

- Alconox soap (or equivalent)
- Tap and deionized water
- Teflon brushes
- Acetone, laboratory grade
- Hexane, laboratory grade
- Solvent waste jars
- Drying rack

5. METHODS, PROCEDURES, AND REQUIREMENTS

5.1. Sample Homogenization

5.1.1. Inspect sample for integrity and note any degradation or loss of sample container integrity.

5.1.2. All samples to be analyzed for the APERC are initially weighed before homogenization and the weight recorded. If sample mass is less than 20 g, the project manager will be notified.

5.1.3. Weigh and pour into a 2 L round bottom boiling flask 20 g ACS certified laboratory grade sodium chloride (NaCl).

5.1.4. Place one 2 cm Teflon coated magnetic stir bar and five to ten quartz boiling chips into the 2 L boiling flask.

5.1.5. If sample is greater than 20 g, a representative cross section weighing 20 g is cut from the sample and weight recorded.

5.1.6. Sample and 350 mL reverse-osmosis (RO) laboratory grade water are added to the Omni-Mixer cup and blended for 2 minutes.

NOTE: *Blender cup lid must be securely placed on the blender cup so that homogenate does not leak during blending.*

5.1.7. Sample homogenate is poured into the 2 L round bottom boiling flask.
5.1.8. Another 650 mL of RO laboratory grade water is added to the blender cup in small portions to rinse and transfer any remaining homogenate into the 2 L boiling flask.
5.1.9. Homogenate is immediately extracted by the methods given in MSU-ATL SOP 224.

5.2. Cleaning Procedure for Homogenization

5.2.1. Dismantle the Omni-Mixer between samples.
5.2.2. Scrub components with a Teflon brush in a hot water solution of Alconox soap (or equivalent) to remove solid particles or other residues.
5.2.3. Rinse 3X with hot tap water to remove soap residues.
5.2.4. Rinse 3X with deionized or RO water.
5.2.5. Rinse 3X with laboratory grade acetone.
5.2.6. Rinse 3X with laboratory grade hexane.
5.2.7. Allow components to air dry.
5.2.8. At the end of the day all components of the Omni-Mixer will be cleaned and placed on tin foil away from all sources of contamination.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

6.1 Records and Documentation

6.1.1 Documentation will be consistent with procedures outlined in MSU-ATL SOPs 803 (Data Analysis) and 402 (Sample Management). All samples will be labeled at all times and will be accompanied by COC, STS, and an extraction worksheet.

6.2 QC Requirements

6.2.1 With every batch of samples (samples belonging to a collection site) a method blank will be performed. This blank will be performed by following the extraction procedure, MSU-ATL SOP 224, with every component except a homogenized sample.

7.0 RESPONSIBILITIES

The primary analyst will complete the analysis as specified in this SOP and provide documentation of raw data, any anomalies, and data to the data analyst who will perform data calculations in accordance with MSU-ATL SOP 224.

The technical reviewer will determine if data quality objectives were met and notify the analyst if any problems were found.

8.0 REFERENCES

8.1 MSU-ATL SOP 224
8.2 MSU-ATL SOP 803
8.3 MSU-ATL SOP 402