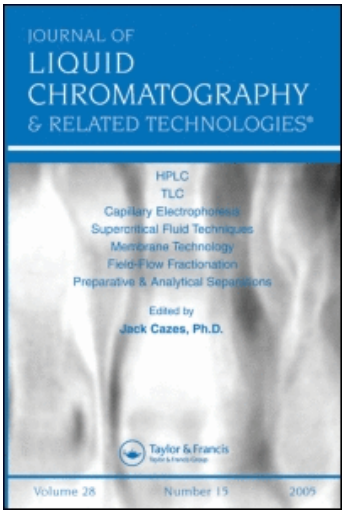

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Renata Gadzała-Kopciuch[a]; Anna Filipiak[a]; Beata Berecka[b]; Piotr Gomułka[c]; Bogusław Buszewski[a]
[a] Department of Environmental Chemistry and Bioanalytics Faculty of Chemistry, Nicolaus Copernicus University, Toruń, Poland [b] Department of Chemistry, Faculty of Environmental Management and Agriculture, University of Warmia and Mazury, Olsztyn, Poland [c] Department of Ichthiology, Faculty of Environmental Sciences & Fisheries, University of Warmia and Mazury, Warmia and Mazury, Olsztyn, Poland

To cite this Article Gadzała-Kopciuch, Renata , Filipiak, Anna , Berecka, Beata , Gomułka, Piotr and Buszewski, Bogusław(2009) 'Selection of Extraction Method for the Estimation of the Bioaccumulation Factor of 4-N-Nonylphenol and 4-Tert-Octylphenol in an Aquatic System', Journal of Liquid Chromatography & Related Technologies, 32: 7, 971 — 983

To link to this Article: DOI: 10.1080/10826070902790777

URL: http://dx.doi.org/10.1080/10826070902790777

Journal of Liquid Chromatography & Related Technologies®, 32: 971–983, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070902790777

Selection of Extraction Method for the Estimation of the Bioaccumulation Factor of 4-N-Nonylphenol and 4-Tert-Octylphenol in an Aquatic System

Renata Gadzała-Kopciuch,[1] Anna Filipiak,[1] Beata Berecka,[2] Piotr Gomułka,[3] and Bogusław Buszewski[1]

[1]Department of Environmental Chemistry and Bioanalytics Faculty of Chemistry, Nicolaus Copernicus University, Toruń, Poland
[2]Department of Chemistry, Faculty of Environmental Management and Agriculture, University of Warmia and Mazury, Olsztyn, Poland
[3]Department of Ichthiology, Faculty of Environmental Sciences & Fisheries, University of Warmia and Mazury, Warmia and Mazury, Olsztyn, Poland

Abstract: A few extraction methods for the reverse-phase high performance liquid chromatography (RP-HPLC) determination of alkylphenolic compounds in fish tissues were examined. The best results of the isolation of 4-nonylphenol (4-*n*-NP) and 4-tert-octylphenol (4-*t*-OP) from Rainbow trout were obtained using soxhlet extraction; the recoveries reached $64.64 \pm 7.26\%$ and $65.26 \pm 5.29\%$, respectively. The subsequent solid phase extraction (SPE) cleanup process of fish extract was optimized using four different sorbents and the highest recoveries of the analytes were obtained for aminopropyl silica cartridges. Their biological concentration factors (BCF) expressing the ratio between the concentration in Rainbow trout and in water were determined for two temperatures, 15 and 20°C.

Keywords: 4-*n*-Nonylphenol, 4-tert-Octylphenol, Endocrine disruptor, Liquid chromatography, Sample preparation

Correspondence: Renata Gadzała-Kopciuch, Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, 7 Gagarin St, 87-100 Toruń, Poland. E-mail: rgadz@chem.uni.torun.pl

INTRODUCTION

Over the past years, the release of various organic chemicals into the environment has attracted the great attention of scientists due to their widespread use and toxicity.[1] Alkylphenols ethoxylates (APEs), nonionic surfactants, constitute one of the compound classes of environmental concern. They have a wide rage of applications and are commonly used in various industries including textiles, tanneries, plastics, pulp, and paper production. APEs are added to metal working and metal finishing fluids, as well as to pesticide formulations, personal care, and cleaning products, paints, and lacquers. The most common alkylphenol ethoxylates are the tri- and tetra-ethoxylates of p-octylphenol and p-nonylphenol. Their biodegradation, similarly as of other APEs, leads to the formation of alkylphenols (AP), which are even more toxic than the parent compounds. Due to their lipophilic character, both nonylphenols (NP) and octylphenols (OP) produced during biodegradation can bioaccumulate in aquatic organisms when the compounds enter the aquatic system.[2] Moreover, NP and OP have an ability to mimic natural hormones (estrogens) and induce estrogenic effects in aquatic organisms, and as such they are listed as endocrine disrupting compounds (EDCs).[3] They bind to the hormone receptor (17-β-estrogen) inducing synthesis of vitellogenin (Vtg) and zona radiata protein (Zrp). Endocrine disrupting chemicals cause modifications of sexual development and reproductive functions in wildlife.[4–7] It is also believed that EDCs induce many effects in the human body, for example, thyroid dysfunction, lower sperm counts, undescended testicles, and early puberty.[8]

Nonylphenols have the ability to bioaccumulate in aquatic organisms, especially in fish. These contaminants and their derivatives can occur in various organs. Depending on the type of exposure, different organs are most liable to be affected. In the case of the contact of an organism body with water, nonylphenols and their metabolites were detected in skin and surficial epithelium of the oral cavity and pharynx (gill arches). When the exposure was oral (water and food), the xenobiotics were detected in a stomach, intestine, and gallbladder. The occurrence of these compounds was also confirmed in liver, kidney, brain, muscle, blood, and fat tissues. The major rout of their excretion is with bile and urine. However, the mechanism of metabolism is not well recognized and described. It is believed that the main metabolites are glucuronidated and/or hydroxylated derivatives.[9,10]

Due to the high toxicity of NP and OP, it is advisable to control their levels in the environment. To ensure sensitive and quantitative determination of 4-nonylphenol (4-NP) and 4-tert-octylphenol (4-*t*-OP) in both water and aquatic organisms, such as fish, the reliable and accurate

method of sample preparation should be developed. The aims presented in these studies involved i) the selection of the most appropriate technique for isolation of 4-NP and 4-*t*-OP from water and fish muscles, ii) optimization of cleanup procedure for fish extracts and iii) determination of their bioaccumulation factors (BFC) in an aquatic system using rainbow trout as a model aquatic organism.

EXPERIMENTAL

Reagents

Solutions of 4-*n*-nonylphenol (Lancaster, Morecambe, England) and 4-*tert*-octylphenol (Sigma Aldrich Chemie, Steinheim, Germany) in ethanol were prepared and used as standards. For SPE and chromatographic analyses, organic solvents of HPLC grade purchased from Labscan (Ireland) and deionised water (Mili-Q, Milipore, El Passo, TX, USA) were employed. Anhydrous sodium sulphate (POCh, Gliwice, Poland) was used as a drying agent.

Equipment

HPLC analyses were carried out using an 1100 Agilent Technologies chromatograph equipped with a diode array detector and an autosampler. Separations were achieved on Supelcosil LC-C18-DB column (250×4.6 mm ID, 5 μm particle size). For SPE isolations, polymeric SDB1, aluminum oxide, octadecyl C_{18} cartridges purchased from J.T. Baker, as well as graphitized carbon black tubes from Carbograph (Alltech) and copolymer poly(divinylbenzene-co-N-vinylpyrrolidone) (Waters) were tested. Tubes were processed using a vacuum manifold (J.T. Baker, Groß-Gerau, Germany). Soxhlet and ultrasonic extractions were carried out with a soxhlet extractor manufactured by Agat (Szczecin, Poland) and in an ultrasonic bath providing about 50 W power (Model UM-2, Unitra-Unima, Olsztyn, Poland), respectively.

Experimental Setup

The Local Ethics Committee of the University of Technology and Agriculture in Bydgoszcz, Poland has approved the study of contaminants in fish tissue. To estimate the bioaccumulation factor (BCF) of 4-*n*-NP and 4-*t*-OP in an aquatic system, the farming of rainbow trout was established

in the Department of Ichtiology (UWM Olsztyn). The fish was placed in 100 L aquariums, which were mechanically aerated and thermostated at 15 or 20°C, as required. After the adaptation period, the water in the aquariums were spiked with the mixture of 4-*n*-NP and 4-*t*-OP in ethanol to achieve the concentrations of analytes ca 0.05 ppm; no pollutants were added to the control aquariums to determine their background levels in water and fish (blank samples). After seven days, the water was sampled for analysis and the fish was subjected to processing preceding analysis of its tissue for 4-*n*-NP and 4-*t*-OP contamination.

Treatment of Water Samples

Isolations of 4-*n*-nonylophenol and 4-*tert*-octylphenol from water samples were carried out by solid phase extraction using two sorbents, graphitized carbon black (Carbograph) and polymeric SDB1. The optimized extraction procedure is shown in Figure 1.

Treatment of Biological Samples

In order to select an appropriate extraction method for the isolation of 4-*n*-NP and 4-*t*-OP from the biological samples, rainbow trout without innards and head was bought at the local food market and stripped of its skin. Then the fish tissues were homogenized and stored deep frozen until analysis time. Before analysis the fish tissues were thawed, spiked with the standards, 4-*n*-nonylophenol and 4-*tert*-octylphenol, and placed for 24 h in a refrigerator.

Three methods of 4-*n*-NP and 4-*t*-OP isolation from fish tissues were examined: matrix solid-phase dispersion (MSPD),[11] soxhlet extraction,[12] and ultrasonic extraction.[13]

MSPD Procedure

Homogenized fish tissues, 0.5 g, was added to 2 g of prewashed with methanol C_{18} powder. The mixture was ground with a coated pestle and placed on 3 g of aluminium oxide, which was washed with 10 mL of methanol and then deactivated with demineralized water (5% w/w) before introducing to a column. The analytes were eluted with 10 mL then 8 mL and 5 mL of methanol. The eluates were combined together and the volume of extract was reduced to 1 mL by solvent evaporation. The scheme of a MSPD extraction column is presented in literature.[11]

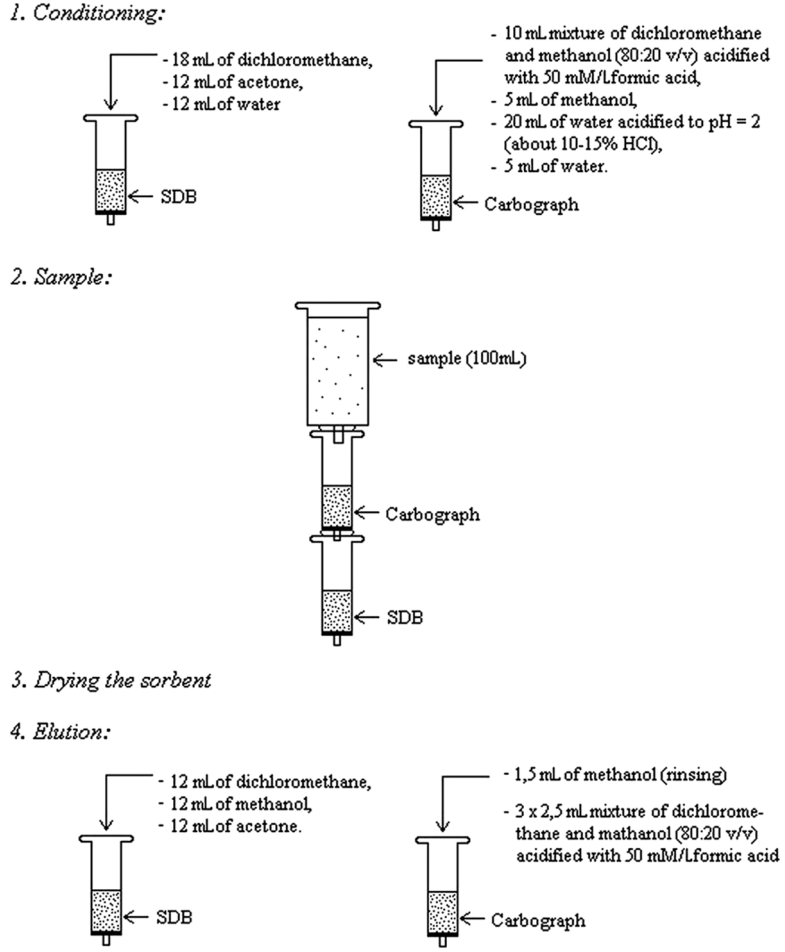

Figure 1. The stages of analytical proceeding for aqueous samples.

Soxhlet Extraction

Of homogenized fish tissue, 5 g was mixed with anhydrous sodium sulphate and extracted with 150 mL of dichoromethane for 4 h in a soxhlet extractor. The volume of extract was reduced to 0.5 mL and then the extract was cleaned up by solid phase extraction, using aluminum oxide (Al_2O_3) cartridges, according to the procedure described by Blackburn et al.[12] The analytes were eluted with two aliquots of 5 mL 1:1 hexane/acetone. The solvent was evaporated to dryness and the sample was dissolved with 1 mL of methanol.

Ultrasonic Extraction

Fish tissues of 5 g was macerated in 50 mL of methanol, and 100 mL liquid extract was centrifuged at 3000 rpm for 10 min. The extract supernatant was cleaned up using poly(divinylbenzene-co-N-Vinylpyrrolidine) cartridges conditioned with 1 mL of methanol and equilibrated with 1 mL of ultra pure water, acidified to pH 3 with acetic acid (0.1% v/v). Prior to introducing to a SPE cartridge the fish extract was diluted with acidified acetic acid ultra pure water (pH 3) so that the final concentration of methanol did not exceed 10% v/v. The analytes were eluted from the SPE cartridges with 2 mL of methanol. The volume of cleaned up fish extract was reduced to 1 mL by solvent evaporation.

Optimization of Cleanup Procedure for Fish Extract

In order to select the appropriate cleanup process for fish extracts, the recoveries of 4-*n*-NP and 4-*t*-OP from four SPE cartridges were tested: aminopropyl silica (APS), diol, aluminum oxide, and poly(divinylbenzene-co-N-vinylpyrrolidone).

For aluminium oxide cartridges, the procedure was as follows: mixture of standards (4-*n*-NP and 4-*t*-OP) was added to 0.5 mL of dichloromethane and introduced onto the sorbent. Column washing, conditioning, and elution were carried out as described in the section for Soxhlet extraction.

For poly(divinylbenzene-co-N-vinylpyrrolidone) cartridges, the mixture of standards (4-*n*-NP and 4-*t*-OP) was spiked with methanol and water, acidified to pH 3 with acetic acid (0.1% v/v). Column washing, conditioning, and elution were carried out as described in the section for ultrasonic extraction.

The mixture of standards (4-*n*-NP and 4-*t*-OP) was added to 1 mL of dichloromethane and 6 mL of hexane before the introduction to aminopropyl silica (APS) and diol cartridges, respectively. SPE columns washing, conditioning, and elution were carried out according to the procedure described by Patta et al.[14]

HPLC Analysis

Chromatographic separations were carried out on Supelcosil LC-C18-DB column (250 × 4.6 mm ID, 5 μm particle size) using the mixture of acetonitrile, water, and methanol (43/14/43% v/v) as a mobile phase. The flow rate was 1 mL/min. The injection volumes were 20 and 100 μL for the extracts from water and fish tissues, respectively. The identification of

4-*n*-NP and 4-*t*-OP was performed at 226 and 277 nm. For quantification the chromatograms registered at 277 nm were used.

RESULTS AND DISCUSION

In the applied reverse-phase mode of liquid chromatography, the less hydrophobic 4-tert-octylphenol eluted before 4-nonylphenol (retention time 6.12 and 11.09, respectively). The analysis of series of 4-*n*-NP and 4-*t*-OP standard solutions of increasing concentrations in the range of 0.1 to 25 ppm permitted plotting their calibration curves, the parameters of which are presented in Table 1. High values of regression coeficients confirmed the linear correlation between HPLC peak areas and compound concentrations in the examined concentration range. Limits of detection (LOD) and quantifications (LOQ) were determined and are also shown in Table 1. The results indicated that the developed HPLC method with UV detection at 277 nm may be applied for the determination of the analytes at low concentrations.

Selection of Extraction Method for Biological Sample

To select the extraction method for isolation of 4-*n*-NP and 4-*t*-OP from fish tissues, three sample preparation methods matrix-solid phase desorption (MSPD), soxhlet extraction, and ultrasonic extraction (UE) were examined and the recoveries of the compounds were determined and compared. The results are presented in Figure 2. The very low (and null) recoveries obtained using ultrasonic extraction indicated that the method is inappropriate for the isolation of 4-*t*-OP and 4-*n*-NP

Table 1. Parameters of calibration of the 4-*n*-NP and 4-*t*-OP determined by HPLC

	Value	
Parameters	4-*n*-NP	4-*t*-OP
Concentration range (ng/μL)	0.125–24.9	0.129–25.75
Equation of regression line (for peak area)	$y = 48.452 \times -7.5715$	$y = 46.276 \times -3.5525$
Concentration levels (n)	5	5
Correlation coefficient	0.9984	0.9989
Limit of detection (ng/μL)	0.06	0.04
Limit of determination (ng/μL)	0.18	0.16

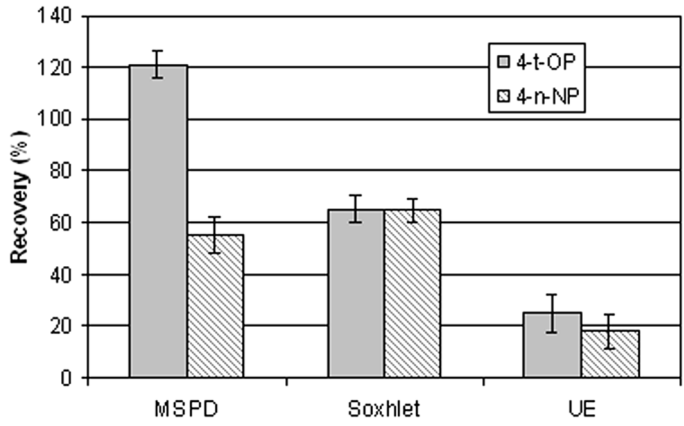

Figure 2. Comparison of recovery for 4-*n*-NP and 4-*t*-OP received using different methods: matrix solid phase dispersion (MSPD), soxhlet extraction and ultrasonic extraction for preparation the fish sample.

from fish tissues. Considerably high values of recovery for both analytes were obtained when soxhlet extraction was employed (65.26 ± 5.29 and $64.64 \pm 4.26\%$ for 4-*t*-OP and 4-*n*-NP, respectively). Matrix-solid phase dispersion (MSPD) appeared to deliver the results of questionable quality, the determined recovery of 4-*t*-OP reached $121.37 \pm 5.04\%$. It seams that the MSPD cleanup process was not sufficient and the extract contained the interfering compounds that coeluted with the analyte. On the base of collected data it can be concluded that the best method for isolation of 4-*n*-NP and 4-*t*-OP from fish tissues is soxhlet extraction.

The examples of chromatograms obtained after injections of a standard solution of 4-*n*-NP and 4-*t*-OP and of the extracts from fish tissues contaminated with the analytes are presented.

The examples of chromatograms of tissue prepared by different methods are placed in Figures 3 and 4.

Optimization of Cleanup Process

To examine the applicability of SPE for isolation of 4-*n*-NP and 4-*t*-OP in the cleanup process of fish extract, the recoveries of the analytes were studied using aminopropyl silica (APS), diol, aluminum oxide, and poly (divinylbenzene-co-N-vinylpyrrolidone). It was found that the highest recoveries of 4-*n*-NP and 4-*t*-OP were obtained with aminopropyl silica sorbent $99.54 \pm 4.24\%$ and $104.83 \pm 8.22\%$, respectively.

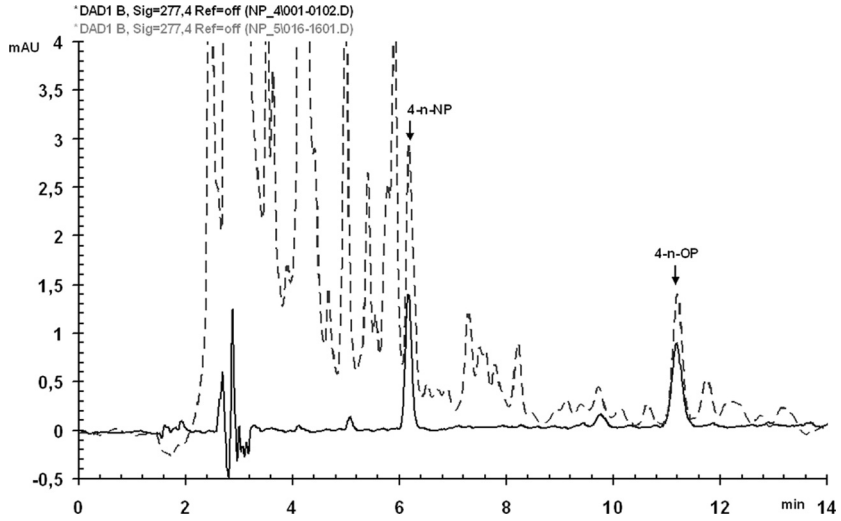

Figure 3. Chromatogram of standard mixture 4-*n*-NP and 4-*t*-OP (a) (1 ng/μL) and fish tissue (spiked with mixture of standards: 4-*n*-NP and 4-*t*-OP) after matrix solid phase dispersion (b).

Both analytes were also isolated well when diol and aluminium oxide cartridges were employed; the recoveries surpassed 60%. Poly(divinylbenzene-co-N-vinylpyrrolidine) sorbent appeared to be inappropriate for

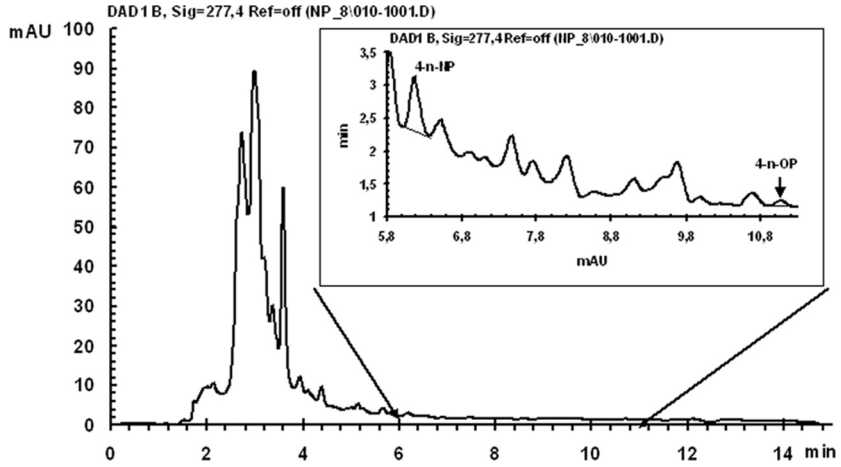

Figure 4. Chromatogram of fish tissue (spiked with mixture of standards: 4-*n*-NP and 4-*t*-OP) after an extraction in soxhlet apparatus and cleanup process by means of aminopropyl silica.

SPE extraction of the analytes of interest for the cleanup of fish extract, since their recoveries were too low, in the case of 4-*t*-OP it reached only $43.63 \pm 1.42\%$ and 4-*n*-NP – $59.35 \pm 4.43\%$.

Bioaccumulation of 4-*n*-NP and 4-*t*-OP in Aquatic System

In order to examine bioaccumulation of 4-*n*-NP and 4-*t*-OP in fish tissues, the isolations of 4-*n*-NP and 4-*t*-OP from aqueous samples collected from aquariums were carried out by SPE, using polymeric SDB1 and graphitised carbon black cartridges (Carbograph) according to procedure described in Figure 1, as well as from the tissues of the fish kept in aquariums, for which soxhlet extraction followed by the cleanup procedure using aminopropyl silica were employed.

The following concentrations of 4-*n*-NP and 4-*t*-OP were recorded in water samples:

Temperature 15°C:
4-*t*-OP: $0.01\ \text{ppm} \pm 1\%$
4-*n*-NP: $0.04\ \text{ppm} \pm 1.2\%$

Temperature 20°C:
4-*t*-OP: $0.03\ \text{ppm} \pm 1\%$
4-*n*-NP: $0.04\ \text{ppm} \pm 1\%$

The results indicated that biodegradation of 4-*t*-OP is higher in lower temperature (15°C). In the case of 4-*n*-NP, no significant changes in its concentration were observed in neither of the two temperatures examined. The large measurement error for the concentration determination of 4-*n*-NP in 15°C may be attributed to the presence of interfering substances coeluting with the analyte during the chromatographic analysis.

In research related to bioaccumulation, a biological concentration factor (BCF)[15] is of a great importance and significance. For the aquatic system, the BCF, which is a concentration ratio between a toxin accumulated in a living organism and present in the environment, can be expressed as:

$$BCF = \frac{concentration\ in\ organism\ in\ whole\ fish}{concentration\ in\ water}$$

The measured concentrations of 4-*n*-NP and 4-*t*-OP in fish tissues and relevant BCFs calculated were as follows:

Temperature 15°C:
4-*t*-OP: $3.07 \pm 0.80\ \text{mg/kg} \Rightarrow \text{BCF} = 307$
4-*n*-NP: $20.75 \pm 4.26\ \text{mg/kg} \Rightarrow \text{BCF} = 518$

Temperature 20°C:
4-*t*-OP: 22.44 ± 6.71 mg/kg $\Rightarrow$ BCF = 748
4-*n*-NP: 25.43 ± 1.03 mg/kg $\Rightarrow$ BCF = 636

It is apparent that the higher amounts of 4-*t*-OP, as well as 4-*n*-NP, were bioaccumulated in fish in higher temperature (20°C). The concentration of 4-*t*-OP in the fish kept in 15°C was significantly lower (3.07 mg/kg) than that in fish kept in 20°C. The similar and high values of BCF for 4-*n*-NP in both temperatures suggest that this xenobiotic penetrated easily into the bodies of animals living in aqueous ecosystem and bioaccumulated in them. The previous research demonstrated that 4-*n*-NP can penetrate into organisms, which are in contact with contaminated water or sludge by a dermal and trophic way.[16]

CONCLUSIONS

Sample pretreatment is a very important step in the analysis of APs in water and especially in biological tissues. A technique of choice should be time efficient and effective. In this paper, three different techniques for isolation of of 4-nonylphenol and 4-tert-octylphenol from fish tissues were described along with the SPE procedure for the cleanup of fish extracts. The biological concentration factors (BCF) were determined for these compounds in the aquatic ecosystem using rainbow trout as a model animal. The comparison of extraction recoveries showed the soxhlet extraction as the most appropriate method for the pretreatment of biological samples. The best recoveries of the analytes from SPE cartridges were obtained using aminopropyl silica sorbent, which were $104.83 \pm 8.22\%$ and $99.59 \pm 4.24\%$ for 4-*t*-OP and 4-*n*-NP, respectively. During bioaccumulation studies the higher biodegradation of 4-*t*-OP in lower (15°C) than in higher (20°C) temperature was observed. The biodegradation of 4-*n*-NP was slow. The larger amounts of both 4-*n*-NP and 4-*t*-OP bioaccumulated in rainbow trout tissues in the higher temperature (20°C). In both examined temperatures 4-*n*-NP was accumulated in an aquatic organism to a higher extent than 4-*t*-OP.

ACKNOWLEDGMENT

This research was financially supported by a grant (project No. R05 037 03) from the Ministry of Science and Higher Education (Warsaw, Poland).

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Received July 18, 2008
Accepted December 1, 2008
Manuscript 6400