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Short communication

Determination of 4-nonylphenol, nonylphenol monoethoxylate, nonylphenol diethoxylate and other alkylphenols in fish and shellfish by high-performance liquid chromatography with fluorescence detection

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Abstract

A simple and highly sensitive method is described for the HPLC determination of 4-nonylphenol (NP), 4-nonylphenol mono- (NP1EO) and diethoxylates (NP2EO) in fish and shellfish together with bisphenol A (BPA), 4-*tert*.-butylphenol (BP) and 4-*tert*.-octylphenol (OP). The NP, NP1EO, NP2EO and other alkylphenols in the samples are extracted with acetonitrile and the lipid in the sample extract is eliminated by partitioning between hexane and acetonitrile. After Florisil PR clean-up the sample extract is analyzed by HPLC with a fluorescence detection. Recoveries in Japanese smelt, carp and corbicura are 81.8–84.3% for NP, 83.5–84.3% for NP1EO, 90.5–96.2% for NP2EO, 70.7–72.9% for BPA, 71.0–73.4% for BP and 77.1–83.2% for OP spiked at 0.5 μg each chemical per 5 g of the fish and shellfish samples. The detection limits are 2 ng/g for NP, NP1EO and NP2EO, and 1ng/g for BPA, BP and OP.

Keywords: 4-Nonylphenol; Nonylphenol ethoxylate; Alkylphenols

1. Introduction

Alkylphenol polyethoxylates (APEOs) have been widely used in the last 50 years for a variety of industrial, household and commercial applications. It is well known that the hydrophilic polyethoxylate chains of the APEOs are shortened during biodegradation and that 4-alkylphenol di- (AP2EO), monoethoxylates (AP1EO) and 4-alkylphenol are formed as persistent metabolites during activated sludge treatment and discharged into the environment. Field investigations on rivers [1–4] have revealed that 4-nonylphenol di- (NP2EO), monoethoxylates (NP1EO) and 4-nonylphenol (NP) are major constituents in the river water. Because of their estrogenic activities to fish [5,6], their contamination in fish or shellfish is much concerned. However, field investigations on the aquatic organisms [7,8] are very few. In the survey by Ahel et al. [7], tissue samples were extracted, employing concurrent steam-distillation and solvent extraction in a specially designed apparatus and the extracts were analyzed by normal-phase HPLC. On the other hand, in the survey by Wahlberg et al. [8], NP, NP1EO, NP2EO and NP3EO in mussels were analyzed as their pentafluorobenzoates by GC–MS or ECD-GC

In the above methods [7,8], analyses of the chemicals in the biological samples are possible but

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special apparatus is necessary [7] or the operation of derivatization is tedious and time-consuming [8].

Recently, several studies [9,10] have been reported for liquid chromatographic–mass spectrometric (LC–MS) determination of these chemicals in river water. However, application to biological samples is under way and there is still a large demand for an improved HPLC method.

Therefore, we have developed a simple and highly sensitive HPLC method for the determination of NP, NP1EO and NP2EO in the biological samples such as fish and shellfish together with bisphenol A (BPA), 4-*tert*.-butylphenol (BP) and 4-*tert*.-octylphenol (OP). In the proposed method, an altered sample preparation technique is adopted and the chemicals in the sample solution are well separated and high-sensitively detected using HPLC column and operating conditions with reference to the studies by Kojima et al. [3] and Ahel et al. [7].

2. Experimental

2.1. Apparatus

The HPLC systems LC-10ADGH-I (Shimadzu, Japan) were equipped with a fluorescence detector RF-550A (Shimadzu, Japan). As HPLC column, Inertsil PH, 150×4.6 mm I.D. (GL Sciences, Japan) was used.

2.2. Operating conditions

Fluorescence detector, Ex 275 nm, Em 300 nm; column temperature, 40°C; flow-rate, 1.0 ml/min; mobile phase, A=water, B=methanol. Time program: linear gradient (0 min A–40%, B–60%, 25 min A–20%, B–80%, 30 min A–40%, B–60%, 40 min stop).

HPLC column and operating conditions were fixed with reference to the studies by Kojima et al. [3] and Ahel et al. [7]. That is, NP, NP1EO, NP2EO, BPA, BP and OP were well separated and high-sensitively detected using the same HPLC column (Inertsil PH) as in Kojima et al. [3] and almost the same spectrofluorimetric detection at 275/300 nm as in Ahel et al. [7].

2.3. Reagents

4-Nonylphenol (NP), a mixture of compounds with branched side chains, 4-*tert*.-butylphenol (BP), purity more than 98%, 4-*tert*.-octylphenol (OP), purity more than 93%, and bisphenol A (BPA), purity more than 99%, were purchased from Tokyo Chemical Industry (Tokyo, Japan). Nonylphenol monoethoxylate (NP1EO), purity 99.9%, and nonylphenol diethoxylate (NP2EO), purity 99.5%, were purchased from Hayashi Pure Chemical (Osaka, Japan). Florisil PR from Wako Pure after activation at 130°C for 16 h were used for column clean-up. HPLC-grade methanol and nanopure-grade water were used for HPLC mobile phase. Pesticide-grade solvents and chemicals were used for sample preparations.

2.4. Standard solution

Standard stock solutions (1000 μg/ml) were prepared by dissolving 100 mg of each NP, NP1EO, NP2EO, BPA, BP and OP in 100 ml of methanol. Working standards of mixtures of the chemicals (0.1–1.0 μg/ml) were prepared by diluting aliquots of the stock solutions in methanol.

2.5. Determination of NP, NP1EO, NP2EO, BPA, BP and OP in fish and shellfish

About 5 g of the homogenized tissue sample (water content 76–85%) were suspended twice with 30 ml of acetonitrile by high-speed homogenizer after addition of 5 g of anhydrous sodium sulfate, and the organic layer was filtered through anhydrous sodium sulfate. The combined filtrate was rotary-vacuum evaporated just to dryness at 40°C. The residue was dissolved in 10 ml of hexane and shaken 5 min twice with 30 ml of acetonitrile saturated with hexane. The combined acetonitrile layer was rotary-vacuum evaporated just to dryness at 40°C. The residue was dissolved in 5 ml of hexane and passed through a 30×1.0 cm I.D. glass clean-up column containing 5 g of hexane-rinsed Florisil PR and 1 g of anhydrous sodium sulfate. The column was washed with 50 ml of hexane, eluted with 80 ml of ethyl ether and hexane (1+9) for NP, BP and OP and with 40 ml of acetone and hexane (3+7) for BPA,

NP1EO and NP2EO. Each eluate was rotary-vacuum evaporated nearly to dryness at 40°C, transferred to a graduated test-tube (rinsing the flask with methanol) and adjusted to 1.0 ml under a stream of nitrogen at 40°C. A 10-μl volume of the sample solution was injected into the HPLC for analysis. The HPLC measurement was performed, peak areas of the chemicals were integrated and evaluated by the use of calibration curves.

2.6. Calibration curves

A 10-μl volume of each standard mixture of NP, NP1EO, NP2EO, BPA, BP and OP was injected into the HPLC, and calibration curves were constructed by plotting the peak areas against the concentrations for each chemical (0.1–1.0 μg/ml).

3. Results and discussion

3.1. HPLC determination of NP, NP1EO, NP2EO, BPA, BP and OP

Calibration curves of NP, NP1EO, NP2EO, BPA, BP and OP had straight lines as shown in Table 1. Linear range was 0.05–2.0 μg/ml for the six chemicals. Detection limits of the chemicals ($S/N=3$) were 0.01 μg/ml for NP, NP1EO and NP2EO, and 0.005 μg/ml for BPA, BP and OP.

3.2. Florisil column clean-up

For NP and OP, we have already reported Florisil PR column clean-up of biological samples [11]. In this report, the same column clean-up was applied to NP1EO, NP2EO, BP and BPA. BP similarly eluted with the ethyl ether and hexane (1+9) but NP1EO, NP2EO and BPA did not. BPA and NP1EO could elute with ethyl ether and hexane (3+7) and NP2EO with acetone and hexane (3+7). Here, two procedures on clean-up of the six standard chemicals

Table 1
Calibration curves of NP, NP1EO, NP2EO, BPA, BP and OP

Chemical	Equation	Correlation coefficients (r)
NP	$y=383937x-8477$	0.9992
NP1E0	$y=412122x-9014$	0.9988
NP2EO	$y=329851x-7201$	0.9991
BPA	$y=476985x+1313$	0.9998
BP	$y=853579x+1085$	1.0000
OP	$y=565994x-2303$	0.9999

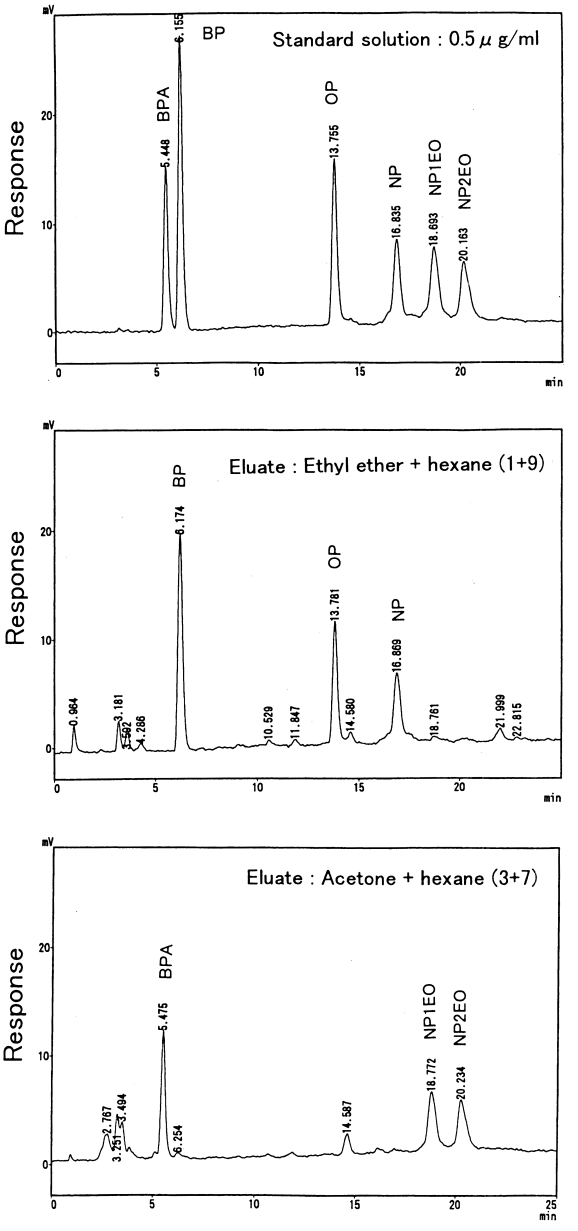

Fig. 1. HPLC chromatograms of standard and recovered NP, NP1EO, NP2E0, BPA, BP and OP from Japanese smelt after Florisil PR clean-up.

were selected. One was ethyl ether and hexane (1+9)→ethyl ether and hexane (3+7)→acetone and hexane (3+7) and the other was ethyl ether and hexane (1+9)→acetone and hexane (3+7). The two procedures were applied to the extract of Japanese smelt (whole body). There were little differences between the results of the two procedures of clean-up. Therefore, the latter rapid clean-up (Fig. 1) was selected in the present study.

3.3. Recoveries of NP, NP1EO, NP2EO, BPA, BP and OP from fish and shellfish

Recovery tests were performed by adding 0.5 ml of the standard mixture (1.0 μg/ml) of NP, BP, OP, BPA, NP1EO and NP2EO to 5 g of the homogenized tissue sample. The recoveries by the proposed method are shown in Table 2. Good recoveries were obtained for NP (81.8–84.3%), NP1EO (83.5–84.3%) and NP2EO (90.5–96.2%), but not for BP (71.0–73.4%) and BPA (70.7–72.9%). The slightly low recoveries of BP and BPA was probably due to their evaporation in the rotary-vacuum evaporation procedure in the sample preparation. It was important not to prolong the step of the rotary-vacuum evaporation to prevent the low recoveries of BP and BPA. On the other hand, Good reproducibility of determination was obtained as shown in the RSD values of the six chemicals (NP 2.5–5.5%, NP1EO 1.9–4.7%, NP2EO 0.2–3.9%, BPA 1.8–4.8%, BP 3.0–7.2%, OP 3.8–5.7%). Detection limits ($S/N=3$) were 2 ng/g for NP, NP1EO and NP2EO, and 1 ng/g for BPA, BP and OP.

3.4. Comparison of the proposed method with the other methods

The GC–MS method by Wahlberg et al. [8] and the HPLC method by Ahel et al. [7] are compared with the proposed HPLC method for the determination of NP, NP1EO and NP2EO in biological samples. For the recoveries of NP, NP1EO and NP2EO, the proposed HPLC method (81.8–84.3, 84.3–84.8 and 90.5–96.2%) is not widely different from the HPLC method (100, 96 and 82%) by Ahel et al. [7], but better than the GC–MS method (93, 34 and 65%) by Wahlberg et al. [8]. Next the detection limits of the proposed method (each 2 ng/g wet weight) are lower than those of the method of Ahel et al. (each 10 ng/g wet weight) and comparable with those of the method of Wahlberg et al. (NP 1 ng/g, NP1EO and NP2EO 6 ng/g wet weight). Finally the time consumed in the operations of the three methods is presumed to be Ahel et al. [7]<the proposed method<Wahlberg et al. [8]. The time needed for the sample preparation is approximately 1.5, 2.5 and 3 h per sample, respectively. The proposed method is simple, highly sensitive, not so time-consuming and there is no need for special apparatus.

3.5. Application to fish and shellfish

Fish and shellfish samples were analyzed by the proposed method. Typical HPLC chromatograms of NP, NP1EO, NP2EO and other alkylphenols in shellfish from River A are shown in Fig. 2. An interfering peak was observed near the peak of NP

Table 2
Recovery of NP, NP1EO, and other alkylphenols from fish and shellfish samples

Sample	Added amount (μg)	Recovery (%)[a]					
		NP	NP1EO	NP2EO	BPA	BP	OP
Fish 1[b]	0.5	81.9±4.5	84.3±4.0	91.3±3.5	70.7±3.4	72.4±5.2	77.1±4.4
Fish 2[c]	0.5	84.3±1.9	84.8±1.8	96.2±0.2	72.8±1.7	73.4±2.6	80.8±3.5
Shellfish[d]	0.5	81.2±2.2	83.5±1.6	90.5±3.5	72.9±1.3	71.0±2.1	83.2±3.2

[a] Mean±SD, $n=5$.
[b] Carp (body length 50 cm) muscle, 5 g.
[c] Japanese smelt (body length 5–6 cm) whole body, 5 g.
[d] Corbicula (body length 1–1.5 cm) whole body except shell, 5 g.

Table 3
Concentrations of NP,NP1EO, NP2EO and other alkylphenols in fish and shellfish samples

Samples	Part	Date[a]	Locations	Concentrations (ng/g, wet weight)					
				NP	NP1EO	NP2EO	BPA	BP	OP
River snail	Meat and viscera	1999.7.16	River A	19	21	6.2	<1	<1	<1
River snail	Meat and viscera	1999.9.10	River A	11	15	3	<1	<1	<1
Melanian snail	Meat and viscera	1999.7.16	River B	3	4	5	<1	<1	<1
Melanian snail	Meat and viscera	1999.9.10	River B	2	2	<2	<1	<1	<1
Corbicula	Meat and viscera	2000.1.16	Lake A	3	4	<2	<1	<1	<1
Japanese smelt	Whole body	2000.1.19	Lake B	6	3	<2	8	<1	<1
Oyster	Meat and viscera	2000.1.16	Inland Sea	<2	<2	<2	<1	<1	<1
Cod	Muscle	2000.1.19	Unknown	<2	<2	<2	<1	<1	<1

[a] Sampling date for river snail and melanian snail and purchasing date for corbicula, Japanese smelt, oyster and cod.

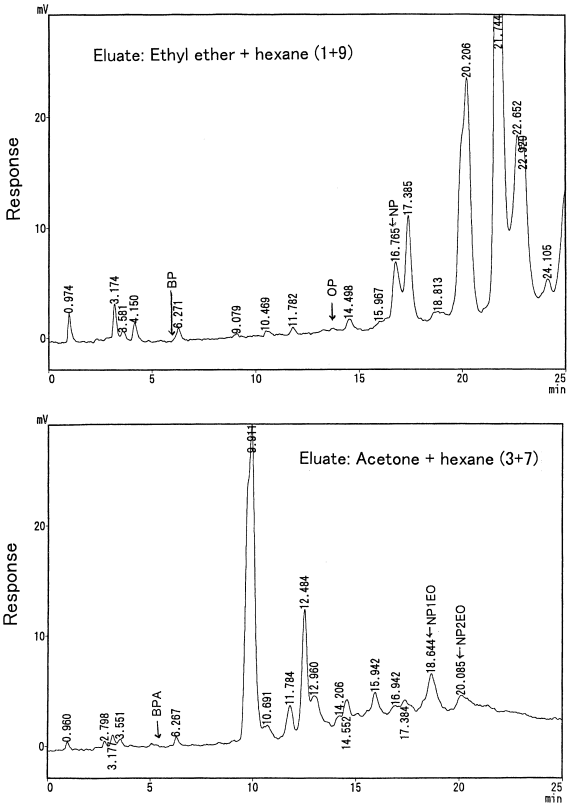

Fig. 2. HPLC chromatograms of NP, NP1EO, NP2E0, BPA, BP and OP in river snail from River A.

but little influence was given on the determination of NP. For fish samples, no interfering peaks were observed near the peaks of the six chemicals, as shown in Fig. 1. The application data for eight different samples were calculated and are summarized in Table 3. For example, NP, NP1EO and NP2EO were detected in 19, 21 and 6 ng/g in river snail from River A and 6, 3 and <2 ng/g in Japanese smelt from Lake B, respectively.

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