表２. SNPsマーカーの対立遺伝子頻度

No.	dbDBSNP ID	Major allele	Minor allele	Minor Allele Frequency	Gene	Location	Amino acid position
44	**rs3802083**	**T**	**c**	**0.45**	**AHR**	**intron 1**	
45	**rs2282883**	**C**	**t**	**0.45**	**AHR**	**intron 2**	
46	**rs2237297**	**G**	**a**	**0.50**	**AHR**	**intron 2**	
47	NEW18	C	t	0.00	AHR	exon 10	P517S
48	**rs2066853**	**G**	**a**	**0.30**	**AHR**	**exon 10**	**R554K**
49	rs4986826	G	a	0.00	AHR	exon 10	V570I
50	**rs2106728**	**T**	**c**	**0.30**	**AHR**	**intron 10**	
51	rs4987097	A	c	0.00	AHR	3' UTR	
52	rs1557737	G	a	0.00	AHR	3' UTR	
53	rs1803080	C	t	0.00	AHR	3' UTR	
54	rs1803079	C	t	0.00	AHR	3' UTR	
55	NEW19	G	t	0.05	CYP1A1	Promoter	
56	rs2470893	G	a	0.00	CYP1A1	Promoter	
57	rs3809585	C	g	0.05	CYP1A1	Promoter	
58	rs2445619	C	t	0.00	CYP1A1	Promoter	
59	rs2472296	C	t	0.00	CYP1A1	Promoter	
60	rs4646417	C	g	0.00	CYP1A1	Promoter	
61	rs2856831	C	g	0.00	CYP1A1	Promoter	
62	rs2856832	C	g	0.00	CYP1A1	Promoter	
63	rs3826042	G	a	0.15	CYP1A1	Promoter	
64	**rs3826041**	**T**	**g**	**0.35**	**CYP1A1**	**Promoter**	
65	rs4646418	G	a	0.00	CYP1A1	Promoter	
66	rs4646419	A	DEL	0.00	CYP1A1	intron 1	
67	rs4646420	T	c	0.00	CYP1A1	intron 1	
68	**rs4646421**	**C**	**t**	**0.35**	**CYP1A1**	**intron 1**	
69	rs4986885	G	a	0.00	CYP1A1	intron 1	
70	rs4986878	G	a	0.00	CYP1A1	intron 1	
71	rs2606344	A	c	0.00	CYP1A1	intron 1	
72	rs4646422	G	a	0.15	CYP1A1	exon 2	G45D
73	rs2229150	C	t	0.00	CYP1A1	exon 2	R93V
74	rs4987133	T	c	0.00	CYP1A1	exon 3	I286T
75	rs2856833	C	a	0.00	CYP1A1	exon 5	F381L
76	rs1799814	C	a	0.00	CYP1A1	exon 7	T461N
77	**rs1048943**	**A**	**g**	**0.25**	**CYP1A1**	**exon 7**	**I462V**
78	rs2278970	C	g	0.00	CYP1A1	exon 7	A463G
79	rs2606346	G	a	0.00	CYP1A1	3' UTR	
80	rs4986880	C	t	0.00	CYP1A1	3' UTR	
81	rs4986881	C	t	0.00	CYP1A1	3' UTR	
82	rs4986882	A	g	0.00	CYP1A1	3' UTR	
83	rs1800031	T	c	0.00	CYP1A1	3' UTR	
84	rs4986883	A	g	0.00	CYP1A1	3' UTR	
85	rs4986884	T	c	0.00	CYP1A1	3' UTR	
86	**rs5030838**	**T**	**c**	**0.35**	**CYP1A1**	**3' flanking region**	

太字がMinor allele frequency>0.2

表3. 子宮内膜症患者における遺伝学的相関解析

No.	Gene	Gene structure	rs No.	Allele	Patients (2n=86) Allele frequency	Controls (2n118) Allele frequency	OR (90% CI)	χ^2	*P*-value
1	AHR	intron 1	3802083	T/c	0.35	0.33	1.13 (0.63 - 2.02)	0.169	0.681
2	AHR	intron 2	2237297	G/a	0.58	0.54	1.20 (0.68 - 2.12)	0.411	0.521
3	AHR	intron 2	2282883	T/c	0.37	0.32	1.26 (0.69 - 2.31)	0.580	0.446
4	AHR	exon 10	2066853	G/a	0.58	0.56	1.06 (0.60 - 1.89)	0.041	0.839
5	AHR	intron 10	2106728	T/c	0.86	0.75	2.16 (1.04 - 4.46)	4.315	0.038
6	CYP1A1	Promoter	3826041	G/t	0.52	0.34	2.16 (1.24 - 3.78)	7.341	0.007
7	CYP1A1	intron 1	4646421	C/t	0.40	0.28	1.78 (0.98 - 3.23)	3.547	0.060
8	CYP1A1	exon 7	1048943	A/g	0.23	0.20	1.22 (0.61 - 2.42)	0.321	0.571
9	CYP1A1	' flanking regio	5030838	T/c	0.41	0.29	1.67 (0.93 - 2.98)	2.969	0.085
10	ESR1	Promoter	2071454	T/g	0.67	0.67	1.04 (0.57 - 1.87)	0.014	0.907
11	ESR1	intron 1	3778609	C/t	0.33	0.33	-	-	-
12	ESR1	intron 1	2234693	T/c	0.41	0.40	1.03 (0.59 - 1.81)	0.010	0.920
13	ESR1	exon 4	1801132	G/c	0.48	0.46	1.07 (0.62 - 1.87)	0.064	0.801
14	ESR1	intron 4	3798759	A/c	0.81	0.75	1.42 (0.72 - 2.84)	1.014	0.314
15	ESR1	intron 6	273206	G/t	0.30	0.24	1.38 (0.73 - 2.61)	0.968	0.325
16	ESR1	intron 6	2273207	A/g	0.30	0.23	1.44 (0.76 - 2.74)	1.263	0.261
17	ESR1	intron 6	974276	A/g	0.30	0.25	1.32 (0.70 - 2.48)	0.715	0.398
18	ESR1	intron 6	3798575	A/g	0.27	0.23	1.20 (0.63 - 2.27)	0.313	0.576
19	ESR1	intron 6	3798576	T/c	0.25	0.23	1.10 (0.57 - 2.10)	0.075	0.784
20	ESR1	intron 6	NEW4	C/t	0.28	0.23	1.30 (0.69 - 2.47)	0.670	0.413
21	ESR1	intron 6	750686	G/a	0.49	0.36	1.68 (0.96 - 2.96)	3.243	0.072
22	ESR1	intron 6	3778092	A/g	0.28	0.23	1.30 (0.69 - 2.47)	0.670	0.413
23	ESR1	intron 6	3020381	T/a	0.49	0.36	1.67 (0.90 - 3.08)	2.662	0.103
24	ESR1	exon 8	2228480	G/a	0.28	0.19	1.68 (0.82 - 3.43)	2.032	0.154
25	ESR1	3' UTR	3798577	T/c	0.49	0.41	1.37 (0.74 - 2.54)	0.996	0.318
26	ESR1	3' UTR	1062577	T/a	0.77	0.74	1.18 (0.62 - 2.24)	0.241	0.623

表4. 子宮内膜症状患者における遺伝子型頻度および相関解析

No.	Gene	Gene structure	rs No.	Genotype	Patients (2n=56) Genotype frequency	Controls (2n=98) Genotype frequency	OR (90% CI)	χ^2	*P*-value
1	ESR1	exon 4	rs1801132	C/C	0.33	0.21	1.85 (0.64-5.31)	1.31	0.25
				C/G	0.48	0.47	1.06 (0.41-2.72)	0.01	0.91
				G/G	0.19	0.32	0.48 (0.16-1.51)	1.56	0.21
2	ESR1	exon 8	rs2228480	A/A	0.16	0.02	9.90 (1.49- 65.70)	5.64	0.02
				A/G	0.24	0.21	1.21 (0.39- 3.74)	0.10	0.75
				G/G	0.60	0.77	0.44 (0.16- 1.21)	2.53	0.11
3	AHR	exon 10	rs2066853	A/A	0.18	0.12	1.56 (0.43- 5.62)	0.46	0.50
				A/G	0.57	0.45	1.64 (0.64- 4.16)	1.07	0.30
				G/G	0.25	0.43	0.44 (0.16- 1.23)	2.46	0.12
4	CYP1A1	3' flanking region	rs5030838	C/C	0.19	0.07	3.03 (0.70- 13.19)	2.18	0.14
				C/T	0.48	0.49	0.97 (0.37- 2.55)	0.00	0.96
				T/T	0.33	0.44	0.63 (0.23- 1.71)	0.81	0.37

表5. ESR1 イントロン5におけるマイクロサテライトマーカーの対立遺伝子頻度および相関解析

Allele	PCR product length	repeat No.	PATIENTS (2n=64)	CONTROLS (2n=84)	(OR 90% CI)	χ^2	*P*-value
Allele 1	230	(aaat)n	0.11	0.13	0.81 (0.30 - 2.23)	0.16	0.691
Allele 2	238	(aaat)n+2	0.66	0.74	0.68 (0.33 - 1.37)	1.16	0.280
Allele 3	242	(aaat)n+3	0.06	0.05	1.33 (0.32 - 5.52)	0.16	0.692
Allele 4	258	(aaat)n+7	0.05	0.06	0.78 (0.18 - 3.37)	0.11	0.736
Allele 5	262	(aaat)n+8	0.13	0.02	5.86 (1.41 - 24.37)	5.90	0.015

Ⅲ. 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
K. Inoue, Y. Yoshimura, T. Makino, H. Nakazawa.	Determination of 4-nonylphenol and 4-octylphenol in human blood samples by high-performance liquid chromatography with multi-electrode electrochemical coulometric-array detection.	*Analyst*	125	1959-1961	2000
K. Inoue, S. Kondo, Y. Yoshie, K. Kato, Y. Yoshimura, M. Horie, H. Nakazawa.	Migration of 4-nonylphenol from polyvinyl chloride food packaging film into food simulants and foods.	*Food Additives and Contaminants*	18 (2)	157-164	2001
K. Inoue, M. Kawaguchi, F. Okada, N. Takai, Y. Yoshihiro, M. Horie, S. I. Izumi, T. Makino, H. Nakazawa.	Measurement of 4-nonelphenol and 4-*tert*-octylphenol in human urine by column-switching liquid chromatography-mass spectrometry.	*Analytica Chimica Acta*	486	41-50	2003
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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
J. Narukawa, H. Inoue, S. Kato, H. Yokota.	Glucuronidation of 1-naphthol and excretion into the vein in perfused rat kidney.	*Drug Metabolism And Disposition*	32 (7)	758-761	2004
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The Analyst FULL PAPER

Determination of 4-nonylphenol and 4-octylphenol in human blood samples by high-performance liquid chromatography with multi-electrode electrochemical coulometric-array detection

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Alkylphenols can affect human health because they disrupt the endocrine system. In this study, an analytical method for determining trace amounts of 4-nonylphenol (NP) and 4-octylphenol (OP) in human blood samples was developed. Reversed-phase HPLC with multi-electrode electrochemical coulometric-array detection was used for the determination of NP and OP in plasma and serum samples prepared with a solid-phase extraction method. The separation was achieved using an isocratic mobile phase of 0.7% phosphoric acid–acetonitrile with a C_{18} reversed phase column. The detection limits of NP and OP were 1.0 and 0.5 ng ml^{-1}, respectively. The recoveries of NP and OP added to human plasma samples were above 70.0% with a relative standard deviation of less than 15.5%. The method was found to be applicable to the determination of NP and OP in various human blood samples such as serum and plasma.

1. Introduction

Chemicals that disrupt endocrine function have been found in the environment and linked to adverse effects on the reproductive system in wildlife and humans. Many of these chemicals can bind to the estrogen receptor and initiate the estrogen receptor-mediate *in vitro*. Although environmental exposure to these chemicals has the potential to disrupt reproductive function, their actual impact on reproductive health has not been defined thoroughly. Alkylphenolpolyethoxylates are widely used as non-ionic surfactants in a variety of industrial and commercial applications. Their biodegradation metabolites, 4-nonylphenol (NP) and 4-octylphenol (OP), have been demonstrated to stimulate the growth of estrogen-dependent MCF-7 cells *in vitro* [1,2] and to be associated with estrogenic activity from a recombinant yeast screen.[3]

Several investigations have reported the occurrence and persistence of NP and OP in biological samples and environmental water.[4–7] Therefore, it is highly possible that these compounds leach into the environment, and that humans are exposed to the contamination. Also, NP and OP are used in a wide range of products to which humans are exposed. This is becoming a serious issue, but there are no data on analytical methods for human samples such as blood. Therefore, it was decided to develop a simple and sensitive method for determining NP and OP in various human blood samples.

The pre-treatment of environmental compounds has been performed by liquid–liquid extraction. However, this method is not easy to perform and requires a large amount of organic solvent. In this study, the extraction was carried out by solid-phase extraction (SPE), because the SPE method using extraction cartridges is simple and only requires a small amount of organic solvent.

The determination of alkylphenols using gas chromatography (GC) and high-performance liquid chromatography (HPLC) based methods has been described. Rudel *et al.*[8] and Ahel and Giger[7] used HPLC-UV analysis for aquatic environmental samples. Lower detection limits were possible when using GC-mass spectrometry (MS) after derivatization with reagents such as bis(trimethylsilyl)trifluoroacetamide, phenyltrimethylammonium hydroxide[4] and *N*-methyl-*N*-(*tert*-butyldimethyltrifluoroacetamide).[9] However, there are only a few simple and sensitive methods that do not require derivatization for the determination of trace amounts of alkylphenols in biological samples. Therefore, we developed a simple and sensitive LC method for the determination of alkylphenols (NP and OP). Electrochemical detection offers better selectivity and sensitivity than UV and refractive index detection. Multi-electrode electrochemical detection (ED) has been used for determining phenol compounds in water.[10,11] Our goal was to develop an ED detection method for the determination of NP and OP in human blood samples.

2. Experimental

2.1 Materials and reagents

4-Octylphenol (OP) and 4-nonylphenol (NP) standards and methanol of pesticide analysis grade were purchased from Kanto Chemical Industries, Tokyo, Japan. Water purified with a Milli-Q water-purification system from Millipore, Bedford, MA, USA, was used. A 1.0 mg ml^{-1} stock solution of the reference standards was prepared in methanol, and prepared to be 0.5–1000 ng ml^{-1} as required by addition of methanol–water (50 + 50). The SPE cartridges for pre-treatment of NP and OP in samples were Bond-Elut C_{18}, C_2 and Phenyl-base (PH)(500 mg/3 ml) from Varian, Palo Alto, CA, USA.

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2.2 Instrumentation and conditions

The HPLC system consisted of an L-6300 pump from Hitachi (Tokyo, Japan), a 460 Autosampler, ESA (Chelmsford, MA, USA) and a column oven from ESA. The samples were injected in an amount of 50 μl. The column oven was controlled at 40 °C.The reversed-phase column was a Capcell-Pak UG 120 C_{18} (150 × 4.6 mm id) column from Shiseido, Tokyo, Japan. The isocratic mobile phase was phosphoric acid in water–acetonitrile (40 + 60) and the flow rate was 1.0 ml min^{-1}. The detection system consisted of a multi-electrode electrochemical detector containing the cells from a Coul Array Model 6210 instrument (ESA) and the database was Coul Array System Win 32 vol. 1.0. The cell potentials for ED consisted of an increasing array: 100 mV at electrode 1, 200 mV at electrode 2, 350 mV at electrode 3, 500 mV at electrode 4, 650 mV at electrode 5, 700 mV at electrode 6, 750 mV at electrode 7, and 800 mV at electrode 8. Peak confirmation was achieved by comparing the matching retention time and peak ratio (*R*: the dominant channel of electrode 5/sub-dominant channel of electrode 4) between each standard and sample.[11,12]

The within-run reproducibility was evaluated by replicate analyses (n = 10) of the standard solution at a concentration of 100 ng ml^{-1}. The calibration graphs for these standards with the dominant electrode channel were linear throughout the range tested.

2.3 Solid phase extraction of human blood

The NP and OP in plasma samples were pre-treated using three different SPE cartridges: C_{18}, C_2 and PH. In order to prevent the analytes from taking their ionic forms, the plasma samples were acidified to pH 3.0 with 1.0 M HCl. Before extracting the plasma samples, SPE cartridges were conditioned using 5.0 ml of methanol followed by 3.0 ml of water adjusted to pH 3.0 using 1.0 M HCl. A 3.0 ml volume of each plasma sample was acidified and added to 2.0 ml of water. The samples were passed through the SPE cartridges, then washed with 5.0 ml of water. Elution with 3.0 ml of methanol at a low flow rate was used to elute the retained compounds in all the samples. The solutions were evaporated to dryness under a stream of nitrogen at 40 °C. The samples were reconstituted in 150 μl of methanol. When a sample showed a high concentration in the calibration graph range, a larger volume of methanol was used for reconstituting the sample. The obtained samples were analysed by LC-ED.

3. Results and discussion

3.1. LC-ED conditions

Acetonitrile–phosphoric acid in water was used for the mobile phase and the sensitivity of detection was adjusted by varying the concentration of phosphoric acid. Hydrodynamic voltammograms of NP and OP standard elution (concentration: 500 ng ml^{-1}) with various concentrations of phosphoric acid are shown in Fig. 1. According to the results, it was decided to use an isocratic mobile phase of 0.7% phosphoric acid–acetonitrile.

The detection limits were calculated according to IUPAC with $3s_b = A_s - A_b$, Where A_s is the average of the sample signal (area), A_b is the average of the blank signal (area) and s_b is the standard deviation of the blank signal (area). The detection limits were 1.0 ng ml^{-1} for NP and 0.5 ng ml^{-1} for OP. The detection characteristics of these standards are presented in Table 1. Fig. 2 shows the chromatograms for the NP and OP standards.

It was possible to separate and to determine the NP and OP standards in a single run of 25 min by combining reversed-phase chromatography with highly selective detection. The method yields a highly precise determination of standards and might be applied to the detection of trace amounts of NP and OP in blood samples, environmental samples and other biomaterials.

3.2 Pre-treatment of NP and OP using SPE from blood samples

The solid phases, C_{18}, C_2 and PH, were compared for their recoveries, relative standard deviations (RSDs) and cleanness. The results were decisive in determining their preferred application. The recoveries of NP and OP were obtained using 3 ml of plasma spiked with a solution of 500 ng ml^{-1} (NP and OP concentrations of 50 ng ml^{-1} in the plasma samples). The extractions using SPE cartridges were performed according to the above-described method. The recoveries of NP and OP were above 70% with the exception of the C_2 phase (Table 2). The PH phase showed higher recoveries and better cleanness for the plasma samples pre-treated with NP and OP than the C_2 and C_{18} phases. Therefore, the PH phase is more suitable for plasma samples than the other phases. Fig. 3 shows an NP and OP

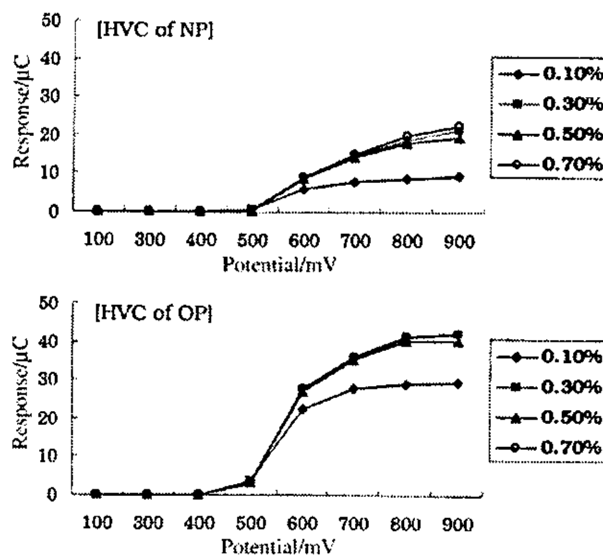

Fig. 1 Hydrodynamic voltammograms (HVCs) of NP and OP standards. Applied voltage: Ch_1 (50 mV) Ch_2 (detector voltage). Standard solution: concentration, 500 ng ml^{-1}.

Table 1 Detection characteristics of NP and OP

Analyte	Retention time/min	RSD_{RT} (%)[a]	RSD_{PA} (%)[b]	Peak ratio[c]	LOD/ ng ml^{-1}	Linear range/ ng ml^{-1}	r
NP	14.6	0.38	1.79	24.2	1.0	10–1000	0.9996
OP	16.2	0.43	1.86	9.2	0.5	5–1000	0.9993

[a] The relative standard deviation within a series of replicate analyses (n = 10) of the retention times. [b] The relative standard deviation within a series of replicate analyses (n = 10) of the peak area. [c] The peak ratio is *R*: the dominant channel of electrode 5/sub-dominant channel of electrode 4.

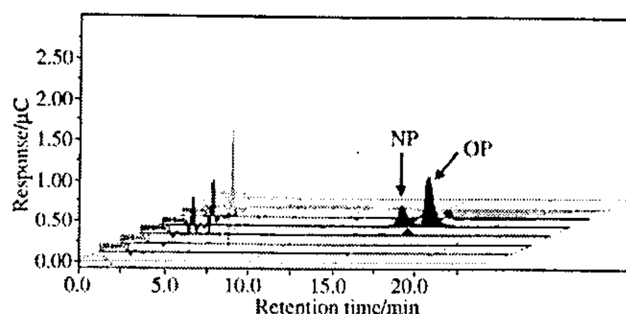

Fig. 2 Chromatogram showing the separation of NP and OP from different classes using an oxidative array. NP and OP standards (1 μg ml^{-1}).

Table 2 Recoveries of NP and OP from plasma samples (n = 6)

Analyte	Solid phase	Conc. added/ ng ml^{-1}	Recovery (%)	RSD (%)
NP	C_2	50	63.3	4.2
OP	C_2	50	52.4	5.4
NP	C_{18}	50	85.9	3.1
OP	C_{18}	50	72.7	4.3
NP	PH	50	83.7	13.1
OP	PH	50	70.2	3.8
NP	PH	5	90.1	15.5
OP	PH	5	80.2	3.9

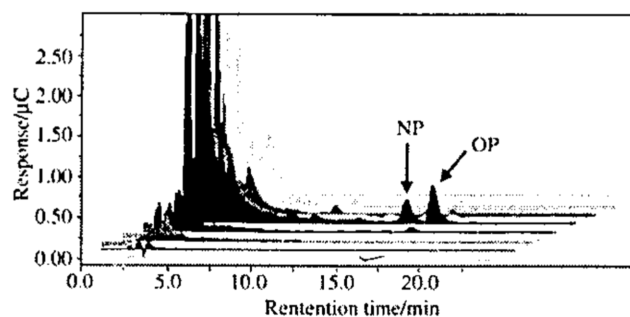

Fig. 3 Chromatogram showing the separation of NP and OP in a human plasma sample. A 3 ml volume of plasma spiked with a solution of 500 ng ml^{-1} (plasma concentration of 50 ng ml^{-1} of sample) was extracted with an SPE cartridge containing PH solid phase.

chromatogram from the recovery test using the PH phase. The recoveries of 5.0 ng ml^{-1} of NP and OP from plasma samples using a PH phase cartridge were obtained. The good recoveries and RSDs for human blood samples pre-treated with NP and OP are shown in Table 2. Thus, it was decided to use a PH cartridge for a simple and selective pre-treatment of NP and OP in plasma samples and to apply the method to determine NP and OP in various human blood samples.

3.3 Application to human blood samples

NP and OP are raw materials used for manufacturing the largest group of non-ionic surfactants and additives for plastics. If these plastics are used for collecting or preparing commercial human blood samples, it is possible that contamination might arise from these materials. Here, NP and OP concentrations in commercial human blood samples were investigated and the results are shown in Table 3. High amounts of NP in commercial human blood samples were detected. This result is probably due to contamination of the commercial samples by

Table 3 NP and OP concentrations of various blood samples (NP: ND <0.5 ng ml^{-1}; OP: ND <0.25 ng ml^{-1})

Sample	Packaging material	Concentration/ng ml^{-1}	
		NP	OP
Commercial human plasma[a]	Glass bottle	13.8	ND[d]
Commercial human serum A[b]	Glass bottle	24.2	ND
Commercial human serum B[b]	Glass bottle	221.7	ND
Commercial human serum C	Plastic bottle	67.9	0.5
Healthy human plasma	Plastic blood bag	Tr[c]	ND

[a] Reconstituted with 1 ml de-ionized water. Total sample volume 1 ml (NP: ND < 1.5 ng ml^{-1}; OP: ND < 0.75 ng ml^{-1}). [b] Reconstituted with de-ionized water. [c] 0.5 < Tr < 1.0 ng ml^{-1} (n = 3). [d] ND = Not detected.

NP from the plastic material used for collection or preparation of the blood samples. The migration of NP and tris(nonylphenoyl)phosphite into food simulants from PVC bottles has been reported.[13] In this study, considerable amounts of NP were found to leach into commercial human blood from PVC tubes or similar products used for collection or preparation of the blood. In contrast, NP and OP concentrations in healthy human plasma samples derived directly from the subjects were at trace levels (range: 0.5–1.0 ng ml of NP and <0.25 ng ml^{-1} of OP) (Table 3).

As regards the pharmacokinetic behavior of NP and OP, a few studies have been reported. OP was found to conjugate with glucuronic acid in rat liver studied *in vitro*.[14] Müller *et al.* investigated the pharmacokinetic behavior of NP in humans and found that the bioavailability after oral application was about 20%.[15] In this study, the very low concentrations of NP and OP in healthy human plasma may be caused by this metabolism of NP and OP.

Acknowledgements

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Migration of 4-nonylphenol from polyvinyl chloride food packaging films into food simulants and foods

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Migration of 4-nonylphenol (NP) from polyvinyl chloride (PVC) films for food packaging into food simulants and foods has been studied in domestic applications such as wrapping of food and reheating in a microwave oven. The migration of NP from the PVC films was determined by high-performance liquid chromatography with electrochemical coulometric-array detection (LC/ED). Twelve PVC films intended for commercial use and ten for domestic applications (total: 22 samples) were analysed. Some of the PVC films (two home-use and ten retail-use) contained NP at concentrations of between 500 and 3300 μg/g. Migration of NP from the films was influenced by the test conditions (n-heptane at 25°C for 60 min, distilled water at 60°C for 30 min and 4% acetic acid at 60°C for 30 min). The amount of NP migrating from the PVC films into n-heptane (0.33–1.6 μg/cm^2) was higher than the amount migrating into distilled water or 4% acetic acid (up to 9.7 ng/cm^2) for the 11 films in which NP was detected. Up to 0.23% of the NP migrated into distilled water and 4% acetic acid and up to 62.5% into n-heptane. In addition, we investigated NP migration into cooked rice samples wrapped in PVC film. Using spiked samples the method gave an average recovery of 83.7% (n = 5) with a standard deviation of 2.5%. Migration of NP ranged from not detectable (< 1.0 ng/g) to 410.0 ng/g by reheating samples in a microwave oven for 1 min and from not detectable to 76.5 ng/g by keeping samples at room temperature for 30 min.

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Keywords: 4-nonylphenol, polyvinyl chloride films, HPLC, electrochemical detector, food simulants

Introduction

Plastic food packaging films are used in domestic applications such as wrapping of hot foods and reheating in a microwave oven. As a consequence, the plastics film may release a variety of chemicals such as additives and residual solvents into foods. The migration of plasticizers from plastics into food has been studied in several surveys, in particular the migration of some phthalates and adipates from polyvinyl chloride (PVC) films. Surveys carried out by Castle *et al.* (1987) and Startin *et al.* (1987) found di-(2-ethylhexyl)adipate (DEHA) in retail foods wrapped in plasticized PVC film. Petersen and Breindahl (1998) exposed PVC films to the official EU food simulant, olive oil, and determined DEHA by GC/MS. However, the migration of other chemicals with the exception of the phthalates and adipates in PVC films for food-wrapping has not been investigated.

4-Nonylphenol (NP) is used in a variety of industrial applications, such as in the manufacturing of the surfactants and plastics. It was found that NP stimulates the growth of oestrogen-dependent MCF-7 cells *in vitro* (Soto *et al.* 1991) and production of vitellogenin in cultured trout hepatocytes (White *et al.* 1994). Surveys of NP in water from rivers, sewage effluents and estuaries (Marcomini *et al.* 1987, Ding and Tzing 1998) and biological samples (Tsuda *et al.* 1999) have been carried out in many countries. These results indicate the common occurrence of NP in the environment. However, only a few reports have been published on the presence of NP in food or plastic films for food packing (Kawamura *et al.* 1999a, Sasaki *et al.* 1999).

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In this paper, food simulants and food after exposure to PVC films have been analysed for NP by high-performance liquid chromatography with electrochemical coulometric-array detection (LC/ED).

Materials and methods

Samples

PVC films of types used in the home and for retail-use were obtained from various supermarkets and corner shops in Japan. A total of 22 PVC films were obtained in 1999–2000 (see table 2). The films for home-use (10 samples) and commercial-use (12 samples) were stored in their original packaging at room temperature. In addition, we were supplied with two samples of a new type of PVC film (samples 8 and 11).

Chemicals

4-Nonylphenol (NP) was purchased from Kanto Chemical Co. Inc. (Japan). The pesticide analytical grade of cyclohexane, *n*-hexane, acetone and methanol and the HPLC analytical grade of *n*-heptane and 2-propanol were purchased from Wako Pure Chemical Industries Ltd (Japan). Acetic acid and phosphoric acid were reagent grade and were also obtained from Wako Pure Chemical Industries Ltd (Japan). The water was purified with a Milli-Q water-purification system from Millipore (USA). All glassware was washed with acetone, followed by heating at 200°C for 4 h prior to use.

Standard solution

A stock solution of 1.0 mg/ml of NP was prepared in methanol, and diluted to concentrations of 1.0–1000 ng/ml as required by addition of methanol–water (50:50). The calibration graphs for these standards of the dominant channel of electrode were linear within the range tested. The quantification was based on external standards.

Analytical method

The HPLC system comprising a pump, an auto injector, and a column oven was model L-6300 from Hitachi Co. (Japan). The autosampler 460 was from Kontron Co. (USA) and the column oven from ESA Co. (USA). After injection of each sample (50 µl), methanol was injected as a blank. The HPLC column was a Capcell Pak UG 120 C_{18} (4.6 × 150 mm) column from Shiseido Co. (Japan) and was controlled at 40°C. The isocratic mobile phase was 0.5% phosphoric acid in aqueous–acetonitrile (40:60) at a flow-rate of 1.0 ml/min. The detection system consisted of a multi-electrode electrochemical detector containing the cells from Coul Array Model 6210 (ESA Co., USA) and the database was Coul Array System Win 32 vol. 1.0. The cell potentials of ED consisted of an increasing array: 200 mV at electrode 1, 300 mV at electrode 2, 450 mV at electrode 3, 550 mV at electrode 4, 670 mV at electrode 5 and 750 mV at electrode 6. The indicated potentials are referred to the solid-state palladium reference electrode built in the coulometric cells; their absolute value is about 250 mV lower than the corresponding potential measured by using an Ag/AgCl reference electrode (Achilli *et al.* 1996). The peak confirmation was achieved by comparing the matching retention time and the peak ratio (*R*: the dominant channel of electrode 5/subdominant channel of electrode 4) between each standard and sample (Risso *et al.* 1991, Achilli *et al.* 1995).

Testing condition

The analytical procedure used for this survey was as reported by Kawamura *et al.* (1999b) for determining additives in PVC products. The NP in the PVC film sample (0.5 g) was extracted with 10 ml of mixed solution of cyclohexane and 2-propanol (1:1 (v:v), 10 ml) by soaking overnight at room temperature. One ml of solution was concentrated to dryness and redissolved in 5 ml of methanol, then analysed by LC/ED.

Migration test conditions

The solvents selected for the migration of NP from the PVC films were distilled water, 4% acetic acid and *n*-heptane. The migration into distilled water and 4%

acetic acid was carried out for 30 min at 60°C. The solutions of distilled water and 4% acetic acid were analysed by LC/ED. In contrast, the migration test into *n*-heptane was carried out for 60 min at 25°C and 5 ml of the *n*-heptane solution obtained was evaporated to dryness using a rotary evaporator. The samples were redissolved in 5 ml of methanol, and determined by LC/ED. Migration levels of NP were calculated by the ratio of volume to film area. A 100 cm^2 piece of the film was cut using a template and immersed in 10.0 ml of pre-heated solvents. These food simulant solvents and the following conditions are as stipulated in Japanese regulations. This condition was adapted for this purpose because this is simple and useful.

Exposure of PVC films to food

The issue of whether NP contained in the PVC films is released into food, requires migration levels of NP into food to be determined. It is reasonable to test this potential for migration using rice as a model food.

Recovery tests of NP added to cooked rice samples were performed by extraction with acetonitrile using only glassware. A rice sample (10 mg) was spiked with a solution of 100 µg/ml (sample concentration of 100 ng/g). Extraction of NP was carried out with acetonitrile (30 ml) added to 10 mg of the rice sample and sonicated for 10 min. The acetonitrile solution was then collected in a flask. The rice sample was re-extracted three times with acetonitrile (30 ml) in the same manner. *n*-Hexane saturated with acetonitrile (30 ml) was added to the mixture, followed by vigorous shaking in a separating funnel. The acetonitrile phase was collected in a 200 ml round-bottomed flask and evaporated to dryness with a rotary evaporator. The samples were redissolved in 10 ml of methanol.

Determination of NP levels in food (cooked rice) wrapped in PVC film was carried out as above. The cooked rice was allowed to cool to room temperature for 10 min. The rice sample (10 g) was wrapped completely in PVC film. The mass of the PVC film was 4 g and 90 cm^2 of film was in contact with the 10 g rice sample. This sample wrapped in PVC film was exposed in two ways. In the first the sample was reheated in a microwave oven (500 W, 2450 MHz) for 1 min. In the second, the sample was kept for 30 min at room temperature. Subsequent extraction employed acetonitrile, as described above. Samples (10 mg) were taken from the outside, which was in contact with the PVC film, and the inside, where there was no direct contact with PVC film. The migration of NP in sample is indicated as 'outside of rice' according to the outward of samples and total migration levels per 10 g by the calculation according to the measurement of the inward and outward of sample. Furthermore, based on our residue test, we investigated only these four films from same residue range levels.

Results and discussion

LC/ED analytical performance

ED was employed in order to utilize the well-known electro-activity of the phenolic group present in the NP molecule. Phenolic oxidation potentials generally shift to more negative values with an increase in pH, therefore we chose to include phosphoric acid in the mobile phase. Thus, acetonitrile–phosphoric acid in water was used for the mobile phase and the sensitivity of detection was optimized by altering the concentration of phosphoric acid. Hydrodynamic voltammograms of authentic NP standard at various concentrations of phosphoric acid are shown in figure 1. The 0.5% phosphoric acid showed good sensitivity, with a detection limit of 1.0 ng/ml with S/N = 3. The calibration curve for the NP standard constructed by plotting the concentration versus the peak area showed a good linearity in the range of 10–1000 ng/ml ($r^2 = 0.998$). When the NP standard and the residue sample was consecutively measured, the retention times were 9.6 min with good separation (figure 2). Confirmation of results was carried out by comparing the ratio (R) between a standard and the actual sample. The R value of the authentic NP standard was 40.1 and that of the residue sample (from PVC film No.1) was 39.3, confirming that the peak in the residue sample is due to NP.

Residues of NP in PVC films

The levels of NP in the PVC films intended for food-packaging are shown in table 1 and were in the range of not detected (< 500 µg/g) to 3300 µg/g. The highest amount was in one of the films intended for home-use

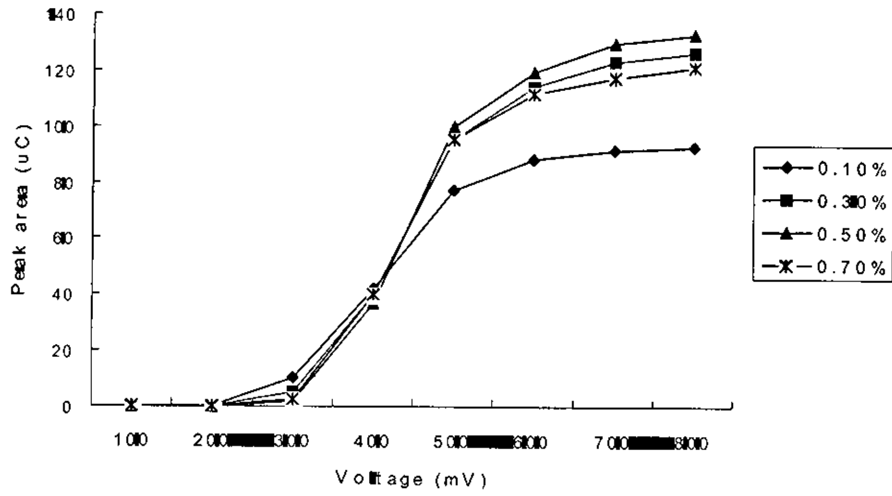

Figure 1. Hydrodynamic voltammograms of NP (1000 ng/ml) for mobile phase. Applied voltage; Ch_1 (50 mV) Ch_2 (detector voltage). Mobile phase: acetonitrile and 0.1–0.7% phosphoric acid in water.

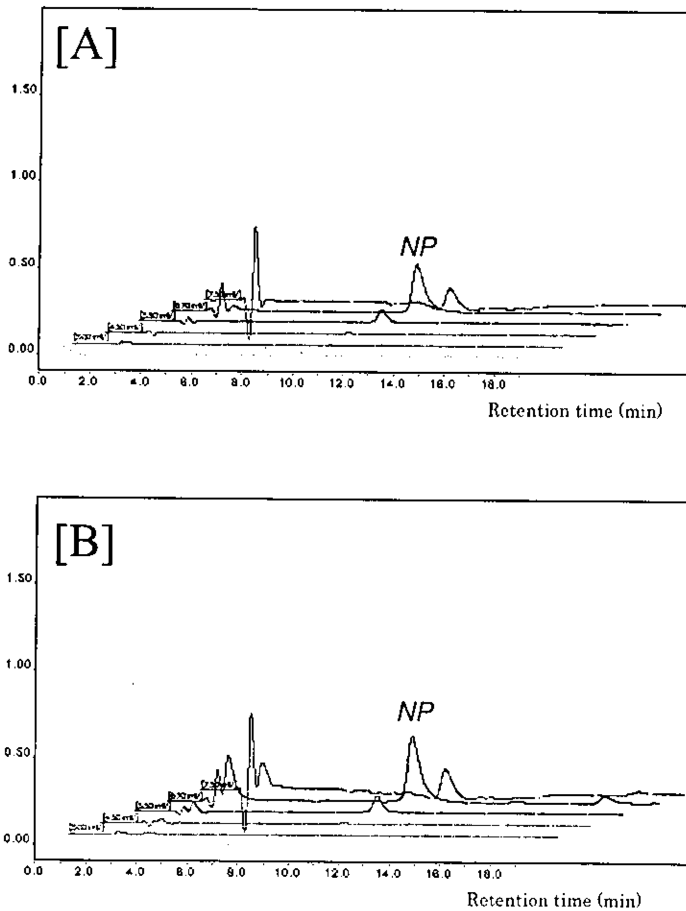

Figure 2. LC/ED chromatograms of standard and residue sample from PVC film. [A]: Standard of NP (500 ng/ml) R = 40.1. [B]: Residue sample (PVC film of sample No.1) R = 39.3.

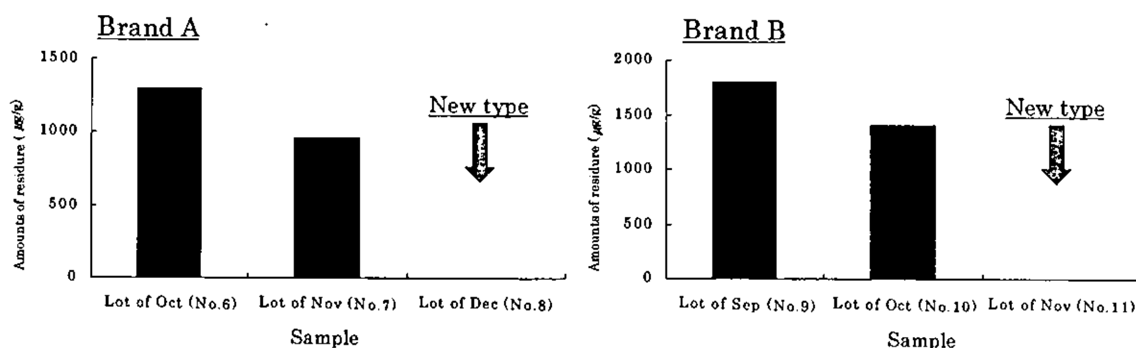

Figure 3. Comparison of residue in PVC films from different productive lots. The comparison of residue NP from old versus new batches of PVC films.

Table 1. Residue of NP in PVC films for food-wrapping.

Residue range (µg/g)	Detection of sample No. (µg/g)
> 2000	No. 14(3300)
1900–1500	*No. 9(1800)*
1400–1000	*No. 1(1400), No. 4 (1200), No. 6(1200)*
	No. 7 (1000), No. 10(1400), No. 13 (1200)
900–500	*No. 2(650), No. 3(830), No. 12(500)*
ND (< 500)	*No. 5, No. 8, No. 11,* No. 15–22

Home-use type: normal font.
Retail-use type: Italic font.

but many of the films intended for retail-use also contained NP. On investigation of NP levels in the different of PVC films, it was found that NP was not detected in the new type of PVC films. It was thought that PVC film manufacturers had changed to the new type of films for safety reasons.

Non-ionic surfactants based on nonylphenol polyethoxylates are widely used by the textile and pulp and paper industries. Polyethylene glycol ethers of nonylphenol are frequently used as emulsifiers. However, it is not likely that the presence of NP is due to its presence as a contaminant as in the worst case the PVC contains only 0.33% of NP. The migration of NP (and NP as a degradation product) from tris(nonylphenoyl) phosphite into food simulants from PVC materials is not an unexpected finding.

Migration test of NP from PVC films

Table 2 shows that up to 1.6 µg/cm^2 of NP migrated from the PVC films into the *n*-heptane. This level was higher than that migrating into distilled water and 4% acetic acid. The amount of NP migrating into distilled water and 4% acetic acid was in the range not detected—9.7 ng/cm^2 (not detected = < 1.0 ng/cm^2).

The relationship between the migration of NP from the PVC films into *n*-heptane (60 min, 25°C) and the residue of NP in the PVC films is shown in figure 4. A correlation of $R^2 = 0.8798$ was observed, therefore the residue of NP in the PVC films (µg/g) was related to the NP migration levels from the PVC films. All film samples were of similar thickness, about 0.7 mg/cm^2. Migration into distilled water and 4% acetic acid was at levels up to 0.23% (No. 0, 4% acetic acid), and into *n*-heptane at levels up to 62.5% (No. 2).

Exposure of PVC films to food

The method used for the extraction of NP from the rice was chosen for its convenience. The rice samples for recovery tests and for exposure to NP were cooked. The chromatographic resolution achieved

Table 2. Migration test of NP in PVC films for food-wrapping.

Sample (No.)	Type	*n*-Heptane[a] (μg/cm²)	Water[b] (ng/cm²)	AcOH[c] (ng/cm²)
1	Retail	0.89	2.3	3.5
2	Retail	0.58	ND	ND
3	Retail	0.64	ND	ND
4	Retail	0.75	2.2	1.5
5	Retail	ND	ND	ND
6	Retail	0.92	1.5	3.2
7	Retail	0.69	ND	ND
8	Retail	ND	ND	ND
9	Retail	1.13	3.5	1.5
10	Retail	1.01	3.2	4.5
11	Retail	ND	ND	ND
12	Retail	0.33	ND	ND
13	Home	0.53	ND	ND
14	Home	1.6	9.7	7.2
15	Home	ND	ND	ND
16	Home	ND	ND	ND
17	Home	ND	ND	ND
18	Home	ND	ND	ND
19	Home	ND	ND	ND
20	Home	ND	ND	ND
21	Home	ND	ND	ND
22	Home	ND	ND	ND

[a] *n*-Heptane (25°C, 60 min); unit: μg/cm²; ND < 1.0 μg/cm².
[b] Water (60°C, 30 min; unit: ng/cm²; ND < 1.0 ng/cm².
[c] 4% AcOH (60°C, 30 min; unit: ng/cm²; ND < 1.0 ng/cm².

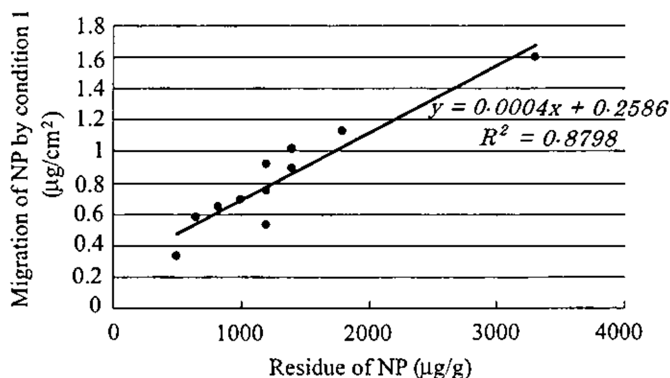

Figure 4. Correlation between results from the residue tests and migration test (n-heptane, 25 °C, 60 min).

was quite adequate, as shown in figure 5, and replicate recoveries were 86.3%, 86.2%, 83.3%, 81.4% and 81.2%. The average recovery was 83.7% with a standard deviation of 2.5%. The result of recovery tests suggested that the method could be successfully applied for the monitoring of NP in the rice samples.

The migration of NP from PVC films to rice samples is shown in table 3, for PVC films No. 14, 9, 1 and 16. These results indicate a maximum migration of NP into cooked rice of 171.8 ng/g. If a person with a 50 kg body weight takes 200 g of rice per day, the total amount of NP ingested per day is 0.7 μg/kg bw/day (171.8 ng/g × 200 g/50 kg). This estimated intake indicates a safety margin of about 70 000 taking into account the highest intake of NP and a NOAEL of 50 mg/kg bw/day (Chapin *et al.* 1999 and Cunny *et al.* 1997). Other reports and this

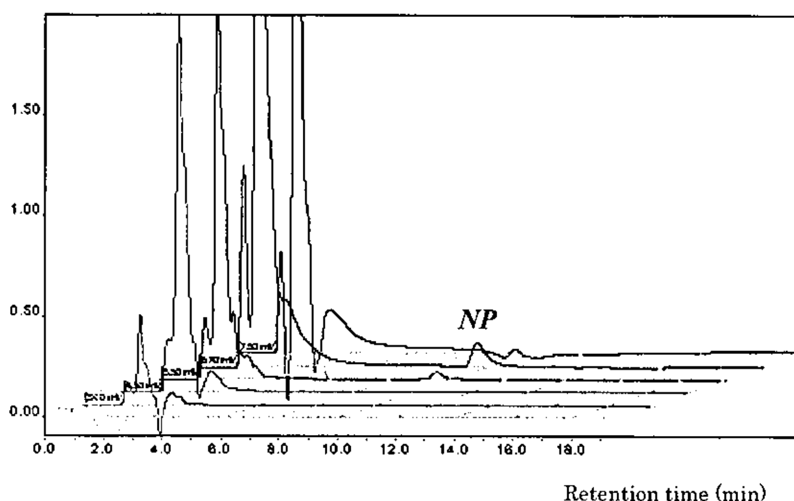

Figure 5. LC/ED chromatograms of rice sample wrapped in PVC film. The rice sample wrapped in PVC film is sample No. 2 taken from the outside.

Table 3. Migration of NP into PVC films to food (rice sample).

	Content in rice (ng/g)			
	Outside of rice		Total	
Sample No.	Case 1	Case 2	Case 1	Case 2
Control	ND	ND	ND	ND
14	410.0	76.5	171.8	35.0
9	80.6	19.0	34.3	7.6
1	57.3	13.1	24.4	5.2
16	ND	ND	ND	ND

Not detected (ND) < 1.0 ng/g.

result lead to the conclusion that there is little need to be concerned about NP exposure from the diet at present (Toppari *et al.* 1996).

Conclusions

An HPLC method has successfully been applied to the analysis of NP in PVC films and to study migration into food simulants and rice. Some PVC films contained NP at concentrations of between 500 and 3300 µg/g. The migration of NP from the PVC films was influenced by the test conditions (*n*-heptane at 25°C for 60 min, distilled water at 60°C for 30 min and 4% acetic acid at 60°C for 30 min). The amounts of NP (0.33–1.6 µg/cm^2) migrating from PVC films into *n*-heptane were higher than those migrating into distilled water and 4% acetic acid (not detected—9.7 ng/cm^2).

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Measurement of 4-nonylphenol and 4-*tert*-octylphenol in human urine by column-switching liquid chromatography–mass spectrometry

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Abstract

We report a method for determining 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP) levels in human urine samples by column-switching liquid chromatography–electrospray mass spectrometry after enzymatic deglucuronidation. The method involves enzymatic deconjugation by β-glucuronidase and correction by the stable isotopically labeled internal standard, 4-(1-methyl)octylphenol-d_5. The compounds were separated by reversed-phase chromatography with a C_{18} column, and detected by selected ion monitoring in the negative mode. After adding an internal standard to urine samples, a direct analysis was carried out. The average recoveries of OP and NP were above 85.0% with correction using the added internal standard. The quantitation limit in the urine samples was 0.3 ng ml^{-1}. The method enables the precise determination of standards and may be applied to the detection of trace amounts of OP and NP in human urine samples.

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1. Introduction

The alkylphenols 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP) exist mainly as intermediates in the manufacturing industry; NP and OP are also degradation products of non-ionic surfactants alkylphenols ethoxylate used in industrial and institutional formulations. Alkylphenols, OP and NP, have been shown to be exist in the environment such as river water and sewage sludge, and in fish tissue [1–5]. In addition, the estrogenic activity of OP and NP has been extensively evaluated in a variety of assays [6–8].

Recently, many novel analytical techniques have been used for the determination of OP and NP in environmental and water samples. The reliable methods for NP analysis are direct solid-phase microextraction (SPME)–gas chromatography–mass spectrometry (GC–MS) [1,9], liquid chromatography (LC) with on-line solid-phase extraction (SPE) [10], and LC with coulometric-array detection [11]. In addition, there are

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many reports on the use of LC with mass spectrometry (LC–MS) for the determination of NP in environmental samples [12–15]. LC–MS is very useful for the determination of trace levels of alkylphenols such as OP and NP because the MS detector has higher sensitivity, selectivity and reliability than other commonly used detectors. In contrast, few studies are available regarding the use of the high-throughput and on-line sample preparation LC–MS method for the identification and quantitative analysis of OP and NP in human biological samples.

The importance of knowing the sources of human exposure is poorly understood. Recently, leaching and contamination of NP from food wrapping films, food-contacting plastics, toys and foods have been reported [16–20]. Therefore, it is possible that healthy humans are exposed to OP and NP via a variety of daily activities. However, to our knowledge, there have been no studies on OP and NP exposure in humans except for our study of human blood levels [21]. In at study, trace levels of OP and NP ranging from ND (<0.5) to 1.0 ng ml^{-1} were found in human blood samples [21]. However, blood is thought to be unsuitable for exposure assessment of healthy people. Additionally, compared to urine collection, blood collection is thought to be unsuitable for exposure assessment of healthy people because of its invasiveness. Equally important in measuring OP and NP in human biological samples are the following: their contamination in the laboratory and during pretreatment, their accurate and sensitive measurement, and the monitoring of their free forms and metabolites. In the present study, we took necessary precautions to prevent contamination during sampling and pretreatment. Moreover, an accurate, sensitive, selective and high-throughput analytical method was developed for the quantification of OP and NP by using internal standards. The use of β-glucuronidase to hydrolyze glucuronide metabolites enables the quantification of both free and glucuronidated forms of OP and NP. We evaluated the method for screening these compounds in human urine samples with creatinine correction.

In the present study, we employed column-switching LC with an electrospray MS detector coupled with on-line extraction, enzymatic deconjugation, and creatinine correction for the sensitive, selective and accurate determination of OP and NP. This novel method was successfully used to determinate OP and NP in urine samples from healthy humans, and the main source of human exposure was clarify.

2. Experimental

2.1. Materials and reagents

4-Nonylphenol (mixture) (NP), [2,3,5,6-$^2H_4$]4-*n*-nonylphenol (NP-d_4) and 4-*tert*-octylphenol (OP) of environmental analytical grade were purchased from Kanto Chemical Inc., Tokyo, Japan. [2,3,5,6-$^2H_4$]4-*tert*-Octylphenol (OP-d_4) and 4-(1-methyl)octylphenol-d_5 (*m*-OP-d_5) were from Hayashi, Osaka, Japan. The structures of these stable isotopically labeled internal standards are shown in Fig. 1. *E. coli* β-glucuronidase (15 μl, 89 U ml^{-1}) was used for enzymatic deconjugation (Fluka, Buchs, Switzerland). Other reagents and solvents were of pesticide or HPLC grade and purchased from Wako, Osaka, Japan. The distilled water purification system was Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA). Creatinine determination was carried out using creatinine test wako (Law of Jaffé) from Wako.

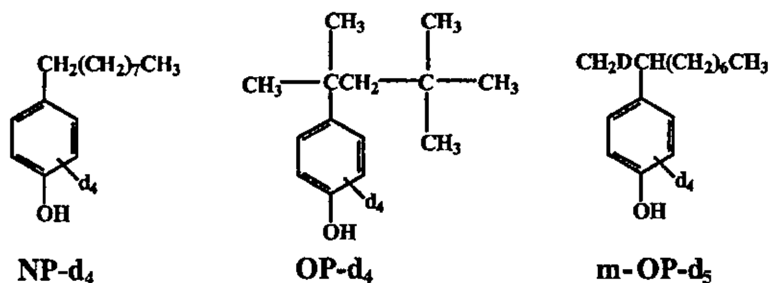

Fig. 1. Structures of stable isotopically labeled internal standards: NP-d_4, [2,3,5,6-$^2H_4$]4-*n*-nonylphenol; OP-d_4, [2,3,5,6-$^2H_4$]4-*tert*-octylphenol; *m*-OP-d_5, 4-(1-methyl)octylphenol-d_5.