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Stir bar sorptive extraction and thermal desorption–gas chromatography–mass spectrometry for the measurement of 4-nonylphenol and 4-*tert*-octylphenol in human biological samples

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Abstract

Alkylphenols, 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP), in human urine and plasma samples were analyzed using stir bar sorptive extraction (SBSE) in combination with thermal desorption–gas chromatography–mass spectrometry (TD–GC–MS). The method involved correction by stable isotopically labeled surrogate standards, 4-(1-methyl)octylphenol-d_5 (m-OP-d_5) and deuterium 4-*tert*-octylphenol (OP-d). A biological sample was extracted for 60 min at room temperature (25 °C) using a stir bar coated with a 500 μm thick polydimethylsiloxane (PDMS) layer. Then, the stir bar was analyzed by TD–GC–MS in the selected ion monitoring (SIM) mode without any derivatization step. The average recoveries in human urine and plasma samples spiked with NP and OP at levels of 0.5 and 10 ng ml^{-1} were between 95.8 and 99.8% with correction using the added surrogate standards. The limits of quantitation were 0.2 ng ml^{-1} for NP and 0.02 ng ml^{-1} for OP. We measured the background levels of NP and OP in five human urine and three human plasma samples from healthy volunteers. NP and OP were not detected in all human urine samples (N.D. $<$ 0.2 ng ml^{-1} for NP, and N.D. $<$ 0.02 ng ml^{-1} for OP). However, 0.2–0.3 ng ml^{-1} for NP and 0.1–0.2 ng ml^{-1} for OP in human plasma samples were observed by this method.

Keywords: Stir bar sorptive extraction; Thermal desorption; 4-Nonylphenol; 4-*tert*-Octylphenol

1. Introduction

Alkylphenolpolyethoxylates (APEOs) comprise the major class of non-ionic surfactants and have a variety of industrial and commercial applications. Alkylphenols, 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP), are the degradation products of APEOs and have been shown to exist in the environment such as seawater, river water and sewage wastewater [1–10]. They have been reported to induce estrogenic responses in male trout [11,12] and a variety of bioassay [13–15]. Recently, the leaching and contamination of NP from food wrapping films, food-contacting plastics, toys and foods have been reported [16–20]. Therefore, healthy humans may be exposed to NP and OP via a variety of daily activities. The risk assessment of NP and OP is an important issue.

In this study, we show that NP and OP can be simultaneously measured in biological samples. Several methods including gas chromatography–mass spectrometry (GC–MS) [21–23], liquid chromatography–mass spectrometry (LC–MS) with online preparation [24], high-performance liquid chromatography with multi-electrode electrochemical coulometric-array detection [25] and capillary electrophoresis [22,26] have been described for the simultaneous determination of NP and OP in biological samples. However, to our knowledge, there are no studies of NP and OP exposure in humans except our studies of human urine and plasma levels [24,25]. In those studies, free NP and OP in human urine samples were not detected ($<$0.3 ng ml^{-1}) and glucuronide NP was detected [24], whereas those in human

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blood samples were lower than the limits of quantifications ($<1.0\,ng\,ml^{-1}$) [25]. The above-mentioned analytical methods may not have sufficient sensitivity for the determination of trace amounts of NP and OP in human biological samples.

Recently, a new sorptive extraction technique using a stir bar coated with polydimethylsiloxane (PDMS) was developed and called stir bar sorptive extraction (SBSE) [27]. Depending upon their octanol/water partition, the compounds are extracted and enriched. Examples of successful applications are the determination of volatiles, semivolatiles, stale-flavored carbonyl compounds, pesticides, mycotoxin, polycyclic aromatic hydrocarbons and dicarboximide fungicides in various samples [28–35]. In addition, SBSE has been applied successfully in biological samples [36–38].

The aim of this study was to determine trace amounts of NP and OP in human urine and plasma samples by SBSE–thermal desorption (TD)–GC–MS.

2. Experimental

2.1. Materials and reagents

4-Nonylphenol (mixture of isomer) and 4-*tert*-octylphenol of environmental analytical grade were purchased from Kanto Chemical Inc., Tokyo, Japan (Fig. 1). 4-(1-Methyl)octylphenol-d_5 (m-OP-d_5) and deuterium 4-*tert*-octylphenol (the mixture by which the hydrogen of OP was replaced by 1–12 deuterium) (OP-d) were purchased from Hayashi Pure Chemical Inc., Osaka, Japan. Methanol of pesticide grade was purchased from Wako Pure Chemical Inc., Osaka, Japan. The water purification system used was Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA). The EDS polisher was a new filter purchased from Millipore, Japan.

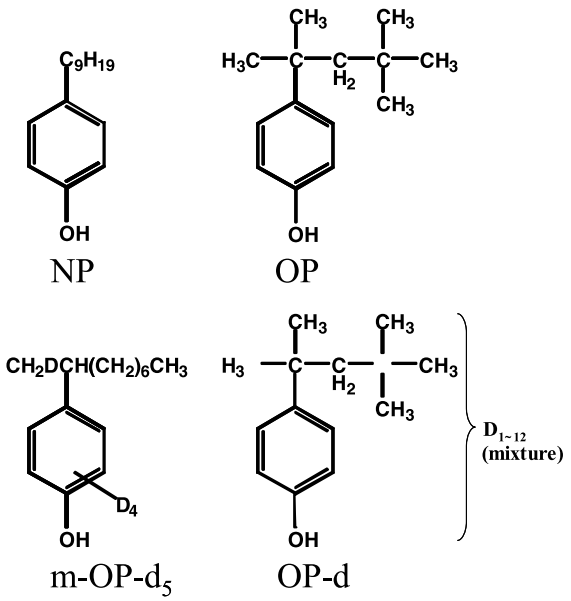

Fig. 1. Chemical structures of NP, OP, m-OP-d_5 and OP-d.

2.2. Standard solutions

Concentrated solutions ($1.0\,mg\,ml^{-1}$ in methanol) of NP and OP were prepared as required by the addition of purified water with specific amounts of surrogate standard. Six-point calibrations ($0.2–20\,ng\,ml^{-1}$ for NP and $0.02–20\,ng\,ml^{-1}$ for OP) were performed daily for all samples with surrogate standards.

2.3. Biological samples

Human urine and plasma samples were, respectively, collected from five and three healthy volunteers (22–25 years old). All samples were stored at −80 °C prior to use.

2.4. Instrumentation

Stir bars coated with a 500 μm thick (24 μl) PDMS (Twister™: the magnetic stirring rod is incorporated in a glass jacket and coated with PDMS) were obtained from Gerstel (Mullheim an der Ruhr, Germany). The stir bars could be used more than 30 times with appropriate reconditioning. For the extraction, 10 ml headspace vials from Agilent Technologies (Palo Alto, CA, USA) and GL Science (Tokyo, Japan) were used. TD–GC–MS analysis was performed using a Gerstel TDS 2 thermodesorption system equipped with a Gerstel TDS-A autosampler and a Gerstel CIS 4 programmable temperature vaporization (PTV) inlet (Gerstel) and an Agilent 6890 gas chromatograph with a 5973 mass-selective detector (Agilent Technologies).

2.5. TD–GC–MS conditions

The temperature of TDS 2 was programmed to increase from 20 °C (held for 1 min) to 280 °C (held for 5 min) at a rate of $60\,°C\,min^{-1}$. The desorbed compounds were cryofocused in the CIS 4 at −150 °C. After desorption, the temperature of CIS 4 was programmed to increase from −150 to 300 °C (held for 10 min) at a rate of $12\,°C\,s^{-1}$ to inject the trapped compounds into the analytical column. Injection was performed in the splitless mode. Separation was accomplished on a DB-5MS fused silica column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies). The oven temperature was programmed to increase from 60 to 280 °C (held for 5 min) at a rate of $15\,°C\,min^{-1}$. Helium was used as the carrier gas at a flow rate of $1.2\,ml\,min^{-1}$. The mass spectrometer was operated in the selected ion monitoring (SIM) mode with electron ionization (ionization voltage: 70 eV). Four ions were monitored for SIM (*m/z* 135, 107 for NP; *m/z* 135, 107 for OP; *m/z* 126 for m-OP-d_5; and *m/z* 140 for OP-d; the underlined number is the *m/z* of the ion used for determination).

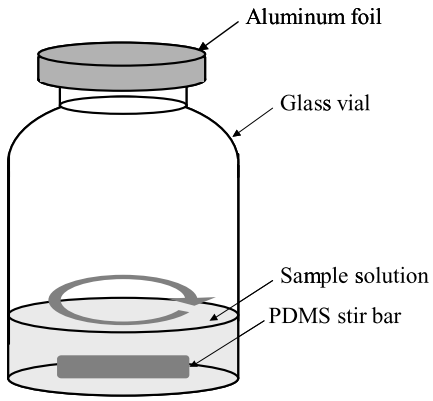

Fig. 2. Schematic of SBSE.

In the quantitative procedure, standard solutions of NP and OP were prepared by dissolving the compounds in water to cover the calibration range. Quantitative analysis was performed in the SIM mode in order to maximize sensitivity. The concentrations were calculated relative to the surrogate standards added to the sample prior to analysis. Six-point calibrations (0.2–20 ng ml^{-1} for NP and 0.02–20 ng ml^{-1} for OP) were performed daily for all samples. Although a blank run of the stir bar was always performed after an analysis, memory effects were never detected.

2.6. SBSE of NP and OP from biological samples

A stir bar was conditioned for 4 h at 300 °C in a flow of helium. One milliliter of biological sample was diluted with distilled water to make a total volume of 2 ml. After the surrogate standards were added, the sample was placed in a glass vial. The stir bar was added and the vial was crimped with aluminum foil. SBSE of the sample was performed at room temperature for 60 min while stirring at 500 rpm (Fig. 2). After the extraction, the stir bar was easily removed with forceps (due to the magnetic attraction effect), rinsed with distilled water, dried with lint-free issue and placed in a glass thermal desorption tube. The thermal desorption tube was then placed in the thermal desorption unit. After thermally desorbing the stir bar in the thermal desorption system, GC–MS was conducted.

3. Results and discussion

3.1. Validation of SBSE–TD–GC–MS

In the mass analysis of standard solutions using electron ionization–MS, *m*/*z* 135 was observed as the main peaks of NP and OP. One surrogate standard, m-OP-d_5, was detected as main fragmentor ion at *m*/*z* 126. The other surrogate standard, OP-d, was detected as many fragmentor ions were observed at the vicinity of *m*/*z* 140. However, since *m*/*z* 135 which is the fragmentor ion of OP was not observed, measurement could be performed satisfactory by using OP-d as surrogate standard (Fig. 3). The mass spectrometer was operated in the SIM mode. For SIM, four ions were monitored (*m*/*z* 135, 107 for NP; *m*/*z* 135, 107 for OP; *m*/*z* 126 for m-OP-d_5; and *m*/*z* 140 for OP-d; the underlined number is the *m*/*z* of the ion used for determination).

Table 1
Validation of NP and OP detection by SBSE–TD–GC–MS

Compound	LOD[a] (S/N = 3; ng ml^{-1})	LOQ[b] (S/N = 10; ng ml^{-1})	Correlation coefficient (r^2)
4-Nonylphenol	0.04	0.2	0.999 (0.2–20)[c]
4-*tert*-Octylphenol	0.004	0.02	0.999 (0.02–20)

[a] LOD: limit of detection.
[b] LOQ: limit of quantitation.
[c] Values in parentheses are the linear ranges of the calibration curves (ng ml^{-1}).

The limit of detection (LOD) was calculated as three times the standard deviation of the analyte concentration determined in the spiked blank sample. Therefore, calculated LODs of NP and OP were 0.04 and 0.004 ng ml^{-1}, respectively (Table 1). On the other hand, the limits of quantitation (LOQs) calculated when the signal-to-noise ratio were higher than 10 was 0.2 for NP and 0.02 ng ml^{-1} for OP in human samples. In addition, the retention time of NP was from 10.05 to 10.45 min and that of OP was 9.30 min. The quantification of NP was performed using the sum of all peaks area. To quantify NP and OP in the sample solutions, the peak ratio of the standards to the stable isotopically labeled surrogate standards was calculated. Calibration curves (0.2–20 ng ml^{-1} for NP and 0.02–20 ng ml^{-1} for OP) were obtained by plotting peak ratios (NP/m-OP-d_5 and OP/OP-d) versus those concentrations using ChemStation software from Agilent Technologies. In the results, the correlation coefficients of the linear calibration curves were higher than 0.999.

3.2. Optimal conditions and recovery tests for NP and OP in human biological samples subjected to the sample preparation method

SBSE of the compounds from a human biological sample was performed by placing a suitable amount of sample in a headspace vial. The most important parameter affecting SBSE is the extraction time. Therefore, in order to determine the optimum extraction time for the detection of concentrations as low as 10 ng ml^{-1}, the TD–GC–MS signals of NP and OP were investigated. The extraction time was investigated from 10 to 90 min. The extraction time profiles (equilibration curves) of NP and OP in human plasma samples using a stir bar were determined by TD–GC–MS and are shown in Fig. 4. From the extraction efficiencies, NP and OP reached equilibrium after about 90 min. When the extraction time was set at 60 min, the recovery was higher

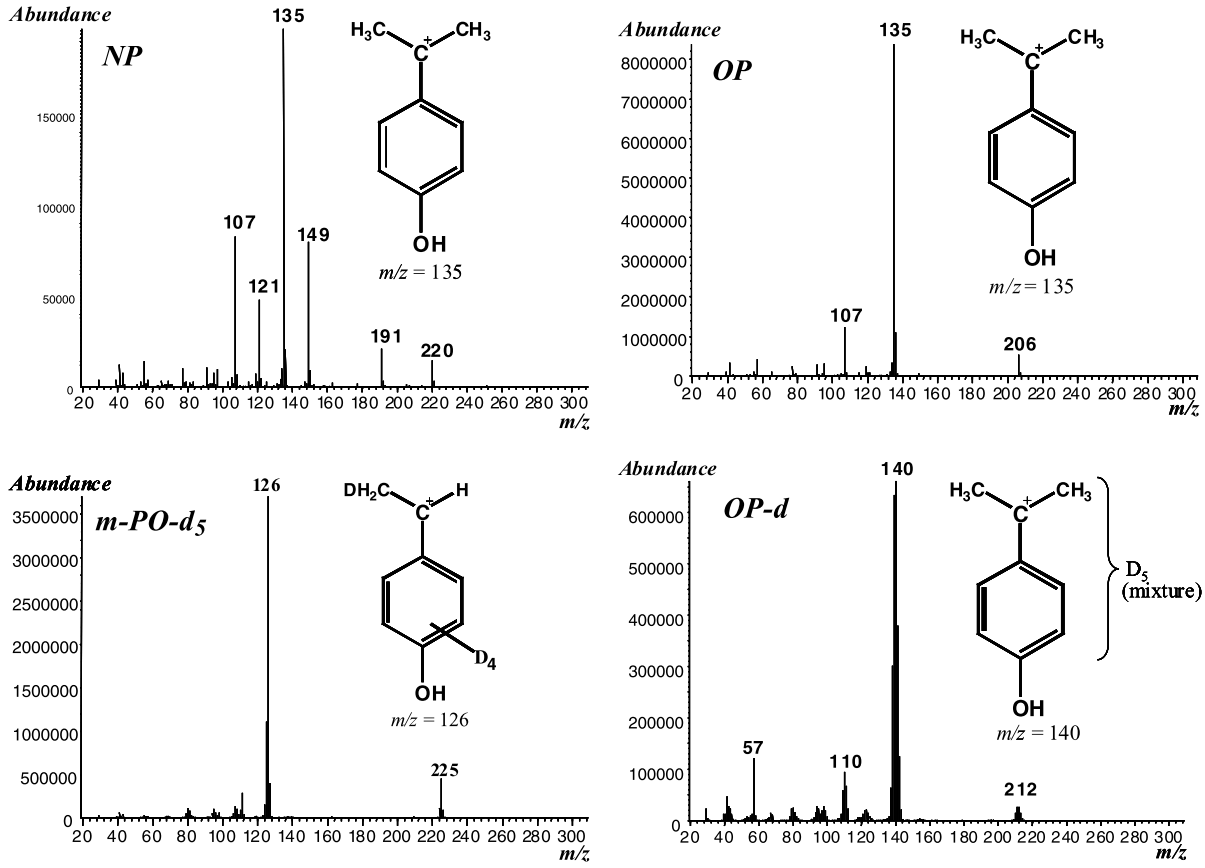

Fig. 3. Mass spectra of NP, OP, m-OP-d_5 and OP-d.

than 90% after 90 min. Therefore, this condition was used for the determination of NP and OP in human urine and plasma samples.

The thermal desorption conditions were investigated. It is important that the analyte is completely isolated from PDMS stir bar on thermal desorption conditions. The temperature of TDS 2 was programmed to increase from 20 °C (held for 1 min) to 280 °C (held for 5 min) at a rate of 60 °C min^{-1}. The desorbed compounds were cryofocused in the CIS 4 at −150 °C. After desorption, the temperature of CIS 4 was programmed to increase from −150 to 300 °C (held for 10 min) at a rate of 12 °C s^{-1} to inject the trapped compounds into the analytical column. NP and OP were analyzed using SBSE based on these conditions. The analyte were not detected as a result of analyzing again that PDMS stir bar analyzed at once. The peaks by memory effects were never detected. Therefore, it was possible to have analyzed satisfactory on this TD conditions.

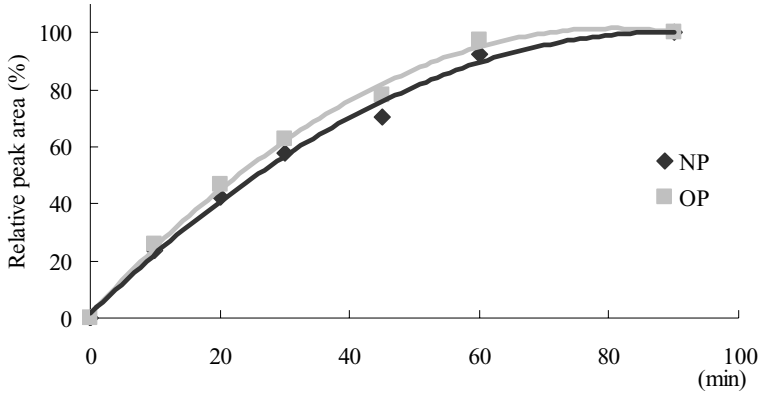

Fig. 4. Extraction time profiles of NP and OP in water samples using stir bar. A stir bar coated with polydimethylsiloxane is added to a human plasma sample and stirring is commenced for 10–90 min at room temperature (25 °C) in a glass vial. The extract is then analyzed by TD–GC–MS.

We investigated whether the recoveries of NP and OP (0.5 and 10 ng ml^{-1}) from human urine and plasma samples could be calculated by using SBSE. The average recoveries of NP and OP ranged from 95.8 to 99.8% with R.S.D. < 5% (Table 2). The results show that the method enables the precise determination of NP and OP, and can be applied to the detection of those compounds in urine and plasma samples.

3.3. Application of the assay

We measured the concentrations of NP and OP in five urine and three plasma samples obtained from healthy volunteers. Using this method, NP and OP could not be detected in all the urine samples, whereas they could be detected in all the plasma samples (Table 3). The detection ranges of NP and OP were from 0.2 to 0.3 ng ml^{-1} and from 0.1 to

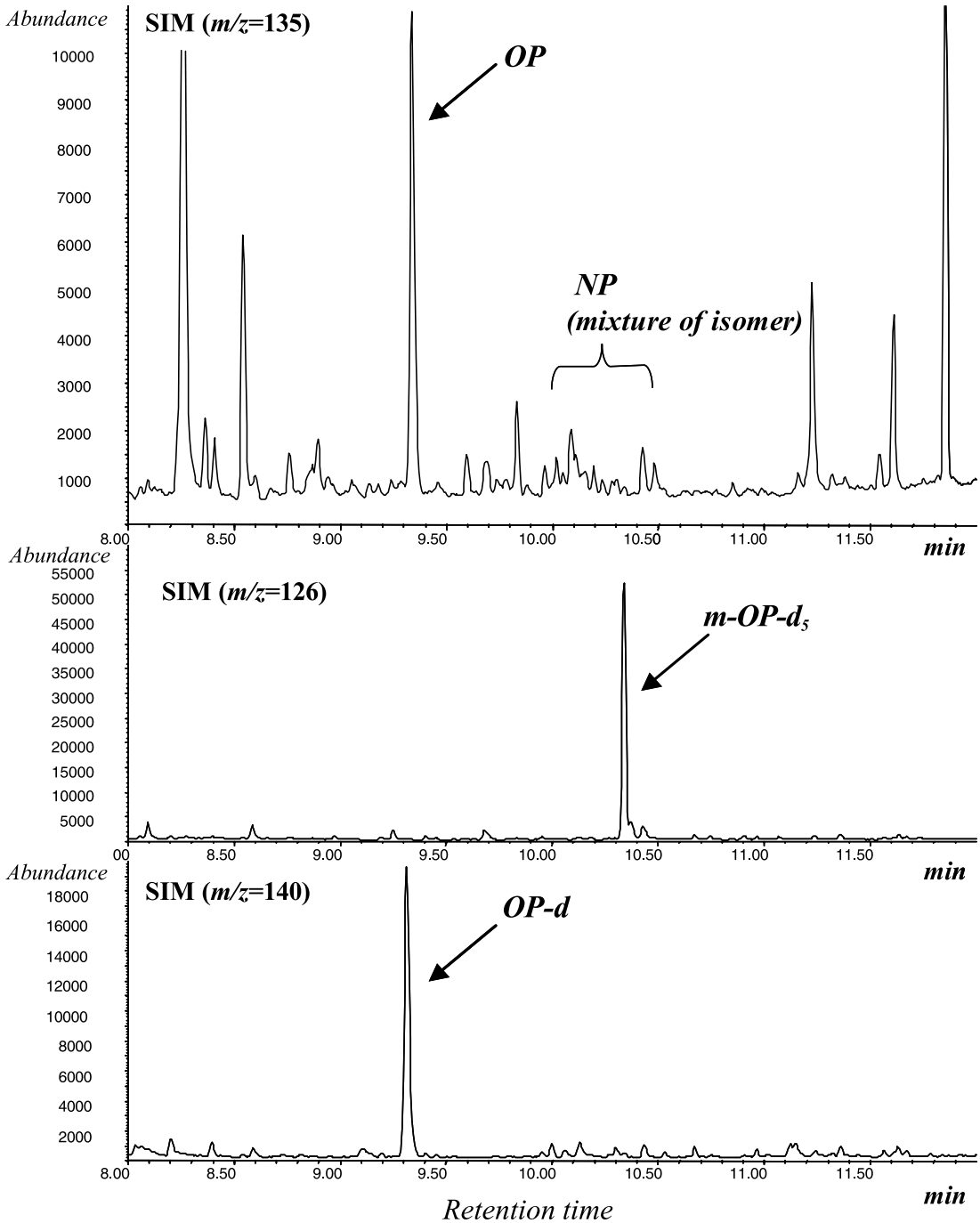

Fig. 5. Chromatograms of NP, OP and surrogate standards (m-OP-d_5 and OP-d) in human plasma sample (volunteer F). Detection levels: NP, 0.3 ng ml^{-1}; OP, 0.2 ng ml^{-1}.

Table 2
Percentage recovery of NP and OP in human biological samples

Compound	Spiked amount ($ng\,ml^{-1}$)	Urine		Plasma	
		Recovery (%)	R.S.D. (%)[a]	Recovery (%)	R.S.D. (%)[a]
4-Nonylphenol	0.5	98.5	1.6	98.1	2.5
	10	99.8	2.6	99.2	3.2
4-*tert*-Octylphenol	0.5	98.1	1.5	95.8	3.0
	10	99.1	3.9	96.5	2.9

[a] Recoveries and precision were also examined by replicate analysis ($n = 6$) of human biological samples fortified with NP and OP at concentrations of 0.5 and 10 $ng\,ml^{-1}$.

Table 3
Concentrations of NP and OP in human urine and plasma samples

Volunteer	Age (years)	Sample type	NP[a] ($ng\,ml^{-1}$)	OP[b] ($ng\,ml^{-1}$)
A	24	Urine	N.D.	N.D.
B	22	Urine	N.D.	N.D.
C	23	Urine	N.D.	N.D.
D	22	Urine	N.D.	N.D.
E	22	Urine	N.D.	N.D.
F	24	Plasma	0.3	0.2
G	25	Plasma	0.2	0.1
H	24	Plasma	0.2	0.2

[a] N.D. indicates NP concentrations lower than 0.2 $ng\,ml^{-1}$.
[b] N.D. indicates OP concentrations lower than 0.02 $ng\,ml^{-1}$.

0.2 $ng\,ml^{-1}$, respectively. The typical chromatograms of human plasma sample (volunteer F) are shown in Fig. 5.

4. Conclusions

The determination of trace amounts of NP and OP in human urine and plasma samples using the SBSE method followed by TD–GC–MS without any derivatization steps was investigated. The average recoveries were between 95.8 and 99.8% in the urine and plasma samples spiked with NP and OP at concentrations of 0.5 and 10 $ng\,ml^{-1}$ with correction using the added isotopically labeled surrogate standards. This simple, accurate and highly sensitive method is expected to have potential applications in various biological samples. For instance, the LOD and LOQ in presented method were compared with the published analytical techniques such as LC–MS with online preparation and LC–electrochemical detection with solid phase extraction [24,25]. In the results, this presented method is most sensitively LOD and LOQ than others.

In all human urine samples, NP and OP were not detected (<0.2 and $<0.02\,ng\,ml^{-1}$) by this method. In a previous study where the pharmacokinetic behavior NP was investigated, the elimination half-life from blood and the bioavailability (determined from oral and intravenous AUCs) were found to be 2–3 h and 20% [39]. The low bioavailability was due to extensive metabolism as glucuronide during the first passage in the liver and excretion via the urine. Therefore, there are reasons to think that NP and OP exposure levels are very trace or non-amounts, or that NP and OP are glucuronidated in liver microsomes and excreted of the formed glucuronide.

On the other hand, although bioavailability of alkylphenols is low levels, free NP and OP in blood was observed. The following could be considered as this reason. In an estimation of the daily intake of alkylphenols, the oral intake of NP by non-occupationally exposed human was found to be less than 0.16 mg per day [40]. However, alkylphenols was not only exposed by the meal, but existing in air sample was reported [41]. From these reasons, it was consider that alkylphenols are always exposed and exist as a free object in blood. This study showed that free NP and OP existed in human plasma.

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References

[1] M.H.I. Comber, T.D. Williams, K.M. Stewart, Water Res. 27 (1993) 273.
[2] P. Braun, M. Moeder, St. Schrader, P. Popp, P. Kuschk, W. Engewald, J. Chromatogr. A 988 (2003) 41.
[3] R. Espejo, K. Valter, M. Simona, Y. Janin, P. Arrizabalaga, J. Chromatogr. A 976 (2002) 335.
[4] R. Jeannot, H. Sabik, E. Sauvard, T. Dagnac, K. Dohrendorf, J. Chromatogr. A 974 (2002) 143.
[5] M. Petrovic, S. Tavazzi, D. Barcelo, J. Chromatogr. A 971 (2002) 37.
[6] M. Petrovic, S. Lacorte, P. Viana, D. Barcelo, J. Chromatogr. A 959 (2002) 15.
[7] M. Petrovic, A. Diaz, F. Ventura, D. Barcelo, Anal. Chem. 73 (2001) 5886.
[8] P.L. Ferguson, C.R. Iden, B.J. Brownawell, Anal. Chem. 72 (2000) 4322.
[9] A. Diaz, F. Ventrura, M.T. Galceran, Anal. Chem. 74 (2002) 3869.
[10] R.J.W. Meesters, H.Fr. Schroeder, Anal. Chem. 74 (2002) 3566.
[11] S. Jobling, J.P. Sumpter, Aquat. Toxicol. 27 (1993) 361.
[12] C.E. Purdom, P.A. Hardiman, V.J. Bye, N.C. Eno, C.R. Tyler, J.P. Sumpter, Chem. Ecol. 8 (1994) 275.
[13] A.M. Soto, H. Justicia, J.W. Wray, C. Sonnenschein, Environ. Health Perspect. 92 (1991) 167.
[14] D.H. Han, M.S. Denison, H. Tachibana, K. Yamada, Biosci. Biotechnol. Biochem. 66 (2002) 1479.
[15] J. Schwaiger, U. Mallow, H. Ferling, S. Knoerr, Th. Braunbeck, W. Kalbfus, R.D. Negele, Aquat. Toxicol. 59 (2002) 177.
[16] K. Inoue, S. Kondo, Y. Yoshie, K. Kato, Y. Yoshimura, M. Horie, H. Nakazawa, Food Addit. Contam. 18 (2001) 157.
[17] K. Inoue, N. Kobayashi, Y. Yoshimura, M. Horie, H. Nakazawa, Jpn. J. Food Chem. 9 (2002) 46.
[18] T. Isobe, N. Nakada, Y. Mato, H. Nishiyama, H. Kumata, H. Takada, Jpn. J. Environ. Chem. 12 (2002) 621.

[19] Y. Kawamura, T. Maehara, H. Iijima, T. Yamada, Jpn. J. Food Hyg. Soc. 41 (2000) 212.
[20] K. Guenther, V. Heinke, B. Thiele, E. Kleist, H. Prast, T. Raecker, Environ. Sci. Technol. 36 (2002) 1676.
[21] T. Tsuda, A. Takino, M. Kojima, H. Harada, K. Muraki, J. Chromatogr. B 723 (1999) 273.
[22] F. Regan, A. Moran, B. Fogarty, E. Dempsey, J. Chromatogr. B 770 (2002) 243.
[23] U. Bolz, W. Korner, H. Hagenmaier, Chemosphere 40 (2000) 929.
[24] K. Inoue, M. Kawaguchi, F. Okada, N. Takai, Y. Yoshimura, M. Horie, S. Izumi, T. Makino, H. Nakazawa, Anal. Chim. Acta 486 (2003) 41.
[25] K. Inoue, Y. Yoshimura, T. Makino, H. Nakazawa, Analyst 125 (2000) 1959.
[26] M. Katayama, Y. Matsuda, T. Sasaki, K. Shimokawa, S. Kaneko, T. Iwamoto, Biomed. Chromatogr. 15 (2001) 437.
[27] E. Baltussen, P. Sandra, F. David, C. Cramers, J. Microcolumn Sep. 11 (1999) 737.
[28] P. Sandra, B. Tienpont, F. David, J. Chromatogr. A 1000 (2003) 299.
[29] V.M. León, B. Álvarez, M.A. Cobollo, S. Muñoz, I. Valor, J. Chromatogr. A 999 (2003) 91.
[30] J.C.R. Demyttenaere, J.I.S. Martinez, R. Verhé, P. Sandra, N.D. Kimpe, J. Chromatogr. A 985 (2003) 221.
[31] J.C.R. Demyttenaere, R.M. Moriña, P. Sandra, J. Chromatogr. A 985 (2003) 127.
[32] N. Ochiai, K. Sasamoto, S. Daishima, A.C. Heiden, A. Hoffmann, J. Chromatogr. A 986 (2003) 101.
[33] C. Blasco, G. Font, Y. Pico, J. Chromatogr. A 970 (2002) 201.
[34] B. Kolahgar, A. Hoffmann, A.C. Heiden, J. Chromatogr. A 963 (2002) 225.
[35] P. Sandra, B. Tienpont, J. Vercammen, A. Tredoux, T. Sandra, F. David, J. Chromatogr. A 928 (2001) 117.
[36] T. Benijts, J. Vercammen, R. Dams, H.P. Tuan, W. Lambert, P. Sandra, J. Chromatogr. B 755 (2001) 137.
[37] B. Tienpont, F. David, K. Desmet, P. Sandra, Anal. Bioanal. Chem. 373 (2002) 46.
[38] T. Kumazawa, X.P. Lee, M. Takano, H. Seno, T. Arinobu, A. Ishii, O. Suzuki, K. Sato, Jpn. J. Forensic Toxicol. 20 (2002) 295.
[39] S. Müller, P. Schmid, C. Schlatter, Environ. Toxicol. Pharm. 5 (1998) 257.
[40] S. Müller, P. Schmid, C. Schlatter, Environ. Toxicol. Pharm. 6 (1998) 27.
[41] G.G. Ying, B. Williams, R. Kookana, Environ. Int. 28 (2002) 215.