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Stir bar sorptive extraction with in situ derivatization and thermal desorption-gas chromatography–mass spectrometry for measurement of phenolic xenoestrogens in human urine samples

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Abstract

A high-sensitivity analytical method that uses stir bar sorptive extraction (SBSE) with in situ derivatization and thermal desorption (TD)-gas chromatography–mass spectrometry (GC–MS) for the simultaneous measurement of trace amounts of phenolic xenoestrogens (PXs), such as 2,4-dichlorophenol (DCP), 4-*tert*-butylphenol (BP), 4-*tert*-octylphenol (OP), 4-nonylphenol technical isomers (NP), pentachlorophenol (PCP) and bisphenol A (BPA), in human urine samples was developed. The urine sample (1 ml) was de-conjugated by adding β-glucuronidase and sulfatase. Then, protein precipitation was performed by the addition of acetonitrile. After centrifugation, the supernatant was diluted with purified water and subjected to SBSE with in situ derivatization and TD-GC–MS. The detection limits of DCP, BP, OP, NP, PCP and BPA in the urine samples were 20, 10, 10, 50, 20 and 20 pg ml^{-1} (ppt), respectively. The calibration curves for PXs were linear and had correlation coefficients higher than 0.99. The average recoveries of those analytes in the urine samples were higher than 95% (RSD: <10%, $n = 6$) with correction using the added surrogate standards. This simple, accurate, sensitive and selective method can be used in the determination of PXs in human urine samples.

Keywords: Phenolic xenoestrogens; Stir bar sorptive extraction (SBSE); Thermal desorption (TD); In situ derivatization

1. Introduction

A number of non-steroidal anthropogenic chemicals are known to mimic the effects of 17β-estradiol, a natural estrogen. Xenoestrogens with markedly different chemical structures have been identified in vitro [1–5] and in some cases, in vivo [6–10]. Many xenoestrogens, including those investigated in this work, possess a phenolic group. Because of their widespread application as industrial chemicals, often in the form of an aqueous solution, phenolic xenoestrogens (PXs) are expected to end up primarily in the aquatic environment via river and sewage, in contrast to phytoestrogens. Recent work has shown that although normally only female fish produce vitellogenin, an increase in plasma vitellogenin levels was detected in wild male fish thriving in rivers polluted by PXs [11–15]. Furthermore, as large amounts of PXs exist in our environment, healthy humans may be exposed to them via a variety of daily activities. Therefore, the risk assessment of PXs is important issue. Previous studies have noted that PXs are released in urine as glucuronides or sulfates, and the extent of conjugation depends on the type of PXs and its concentration in the urine [16–19]. In the present study, we focused on the combined determination of

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such PXs as 2,4-dichlorophenol (DCP), 4-*tert*-butylphenol (BP), 4-*tert*-octylphenol (OP), 4-nonylphenol technical isomers (NP), pentachlorophenol (PCP) and bisphenol A (BPA) in human urine samples.

Analytical methods for the simultaneous measurement of PXs in human biological samples are lacking. On the other hand, the analytical methods for the determination of BPA in human biological samples have been reported, including liquid chromatography (LC) with electrochemical detection (ED), fluorescence detection (FD) and mass spectrometry (MS) [20–23]. However, LC has low resolution and is frequently affected by the sample matrix. Especially, it is reported that the sensitivity is decreased by ion suppression when LC–MS-electrospray ionization (ESI) is used [24]. On the other hand, gas chromatography–mass spectrometry (GC–MS) was initially used for the determination of phenol compounds even though derivatization was required [25–27]. The derivatization leads to sharper peaks and hence to better separation of and higher sensitivity for the phenols. However, the derivatization faces the risk of contamination and hence an overestimation of PXs concentration. In order to overcome this problem, in situ derivatization was developed, which involves the simple addition of a reagent to a liquid sample [28,29].

Solid-phase extraction (SPE) [20–23,25–27] has been developed for the determination of PXs. However, much time and effort is required for manual SPE. Recently, the simultaneous measurement of BPA and alkylphenols, which include BP, OP and NP, in human urine samples by automated solid-phase extractive derivatization followed by GC–MS has been reported, with quantification limits below 0.1 ng ml^{-1} (BPA), 0.4 ng ml^{-1} (BP), 0.7 ng ml^{-1} (OP) and 5 ng ml^{-1} (NP) [30]. This method is easy to perform and enables automated sample preparation. However, as PXs in urine samples are present in trace amounts, it is thought that the sensitivity of this method is not sufficient. Recently, a new sorptive extraction technique that uses a stir bar coated with polydimethylsiloxane (PDMS) was developed [31] and is known as stir bar sorptive extraction (SBSE). In addition, an analytical method for the determination of endocrine disrupting chemicals (EDCs) has been reported [32,33]. We have reported the determination of OP and NP in tap and river water samples [34] and biological samples [35] by SBSE without derivatization. On the other hand, SBSE with in situ derivatization has been successfully used in the determination of phenolic compounds in various samples [36–39]. In addition, we have performed the determination of BPA in water and body fluid samples [40], chlorophenols in water and body fluid samples [41], estrogens in water samples [42], and PXs in river water samples [43] by SBSE with in situ derivatization. However, to our knowledge, the simultaneous analysis of PXs in human urine samples by SBSE with in situ derivatization has not been reported so far.

The aim of this study was to determine simultaneously trace amounts of PXs in human urine samples by SBSE with in situ derivatization and thermal desorption (TD)-GC–MS.

2. Experimental

2.1. Materials and reagents

DCP, BP, OP, NP, PCP and BPA of environmental analytical grade and acetic acid anhydride for trace analysis were purchased from Kanto Chemical Inc. (Tokyo, Japan). Deuterium BP (a mixture in which the hydrogen of BP was replaced with 11–14 deuterium) (BP-d), deuterium OP (a mixture in which the hydrogen of OP was replaced with 1–12 deuterium) (OP-d), and 4-(1-methyl) octylphenol-d_5 (*m*-OP-d_5) surrogate standards were purchased from Hayashi Pure Chemical Inc. (Osaka, Japan). 2,4-DCP-d_4, PCP-$^{13}C_6$ and BPA-$^{13}C_{12}$ surrogate standards were purchased from Cambridge Isotope Laboratories Inc. (MA, USA). The chemical structures are shown in Fig. 1. *E. coli* β-glucuronidase (25,000 units/0.4 ml, 62,500 units ml^{-1}) and *H. pomatia* sulfatase (3540 units ml^{-1}) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Prior to use, the β-glucuronidase was added to 0.1 M ammonium acetate to make a total concentration of 10,000 units ml^{-1}. Other reagents and solvents were of pesticide or analytical grade and were purchased from Wako Pure Chemical Inc. (Osaka, Japan). The water purification system used was a Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA). The EDS polisher was a new filter purchased from Millipore, Japan.

2.2. Standard solutions

Concentrated solutions (1.0 mg ml^{-1} in acetonitrile) of the compounds were prepared as required by the addition of purified water and a specific amount of surrogate standard. Calibrations (0.1–50 ng ml^{-1} for DCP; 0.05–10 ng ml^{-1} for BP; 0.05–10 ng ml^{-1} for OP; 0.2–10 ng ml^{-1} for NP; 0.1–10 ng ml^{-1} for PCP; and 0.1–10 ng ml^{-1} for BPA) were performed daily for all samples with the surrogate standards.

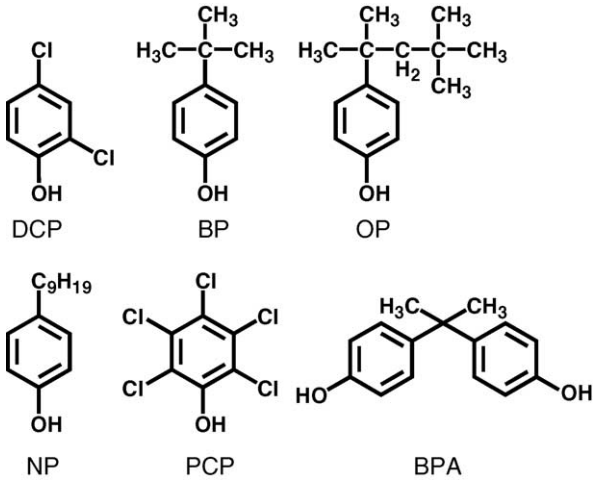

Fig. 1. Chemical structures of PXs.

2.3. Human urine samples

Urine samples were collected from five healthy volunteers (22–25 years old) and stored at −80 °C prior to use.

2.4. Instrumentation

Stir bars coated with a 500-μm-thick (24 μl) PDMS layer were obtained from Gerstel (Mullheim an der Ruhr, Germany). Prior to use, the stir bars were conditioned for 1 h at 300 °C in a flow of helium. Then, the stir bars were kept in new 2 ml vials until immediately prior to use. The stir bars could be used more than 50 times with appropriate re-conditioning. For the extraction, 20 ml headspace vials from Agilent Technologies (Palo Alto, CA, USA) were used. TD-GC–MS 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 6890N gas chromatograph with a 5973N mass-selective detector with an ultra ion source (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 the 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. The injection was performed in the splitless mode. The separation was accomplished on a DB-5MS fused silica column (30 m × 0.25 mm i.d., 0.5 μm film thickness, Agilent Technologies). The oven temperature was programmed to increase from 60 to 300 °C (held for 4 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 impact ionization (ionization voltage: 70 eV). The ions were monitored for SIM (*m/z* 162, 164 for DCP; *m/z* 135, 150 for BP; *m/z* 135, 177 for OP; *m/z* 135, 177 for NP; *m/z* 266, 268 for PCP; *m/z* 213, 228 for BPA; *m/z* 165 for DCP-d_4; *m/z* 145 for BP-d; *m/z* 140 for OP-d; *m/z* 126 for *m*-OP-d_5; *m/z* 276 for PCP-$^{13}C_6$; and *m/z* 225 for BPA-$^{13}C_{12}$. The underlined number is the *m/z* of the ion used for determination.). The monitoring time was programmed from 5 to 10 min for DCP, DCP-d_4, BP and BP-d; from 10 to 14 min for OP, OP-d, NP, *m*-OP-d_5, PCP and PCP-$^{13}C_6$; and from 14 to 17 min for BPA and BPA-$^{13}C_{12}$.

In the quantitative procedure, standard solutions of the compounds were prepared by dissolving the compounds in purified 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. Calibrations (0.1–50 ng ml^{-1} for DCP; 0.05–10 ng ml^{-1} for BP; 0.05–10 ng ml^{-1} for OP; 0.2–10 ng ml^{-1} for NP; 0.1–10 ng ml^{-1} for PCP; and 0.1–10 ng ml^{-1} for BPA) 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 with in situ derivatization of PXs from human urine samples

One ml of human urine sample spiked with surrogate standards was buffered with 1.0 M ammonium acetate solution (50 μl, pH 6.8). After β-glucuronidase (10 μl, 10,000 units ml^{-1}) and sulfatase (10 μl, 3540 units ml^{-1}) were added, the sample was sealed in a glass tube and gently mixed. Quantitative glucuronidase hydrolysis for releasing free chlorophenols was accomplished by incubating at 37 °C for 3 h. This treatment was sufficient to de-conjugate the glucuronidase of glucuronidated BPA [40]. After enzymatic deconjugation, acetonitrile (2.0 ml) was added to the sample. Then, centrifugation was performed (1400 × *g*, 10 min) and the supernatant was transferred to the 20 ml headspace vial and diluted with purified water (15 ml). Potassium carbonate solution (1.0 M, 1.0 ml) for pH adjustment (pH 11.5), and acetic acid anhydride (100 μl) as the derivatization reagent were added to the headspace vial. After holding the sample for 10 min, the stir bar was added and a Teflon-coated silicone septum cap was placed on the vial without crimping. SBSE was performed at room temperature for 150 min while stirring at 1000 rpm. After the extraction, the stir bar was easily removed with forceps (due to magnetic attraction), rinsed with purified water, dried with lint-free tissue and placed inside a glass TD tube. The TD tube was then placed inside the TD system where the stir bar was subjected to TD-GC–MS.

3. Results and discussion

3.1. Derivatization of PXs

In the mass analysis of standard solutions using electron impact ionization (EI)-MS, *m/z* 162, 135, 135, 135, 266 and 213 were observed as the main peaks of acyl derivative of DCP, BP, OP, NP, PCP and BPA, respectively. For the surrogate standards, acyl derivative of DCP-d_4, *m*-OP-d_5, PCP-$^{13}C_6$ and BPA-$^{13}C_{12}$, their main peaks were detected at *m/z* 165, 126, 272 and 225, respectively. However, the fragment ion peaks of the standard compound and the main peak of the surrogate standard were overlapped in the case of PCP. Therefore, the monitoring ion of PCP-$^{13}C_6$ was set at *m/z* 276. On the other hand, many fragment ion peaks were observed in the vicinity of *m/z* 145 and 140, respectively, for BP-d and OP-d surrogate standards. However, because *m/z* 135, which is the monitoring ion of BP and OP, was not observed, measurement could be performed satisfactorily by using BP-d and OP-d as surrogate standards (Fig. 2). The mass spectrometer was op-

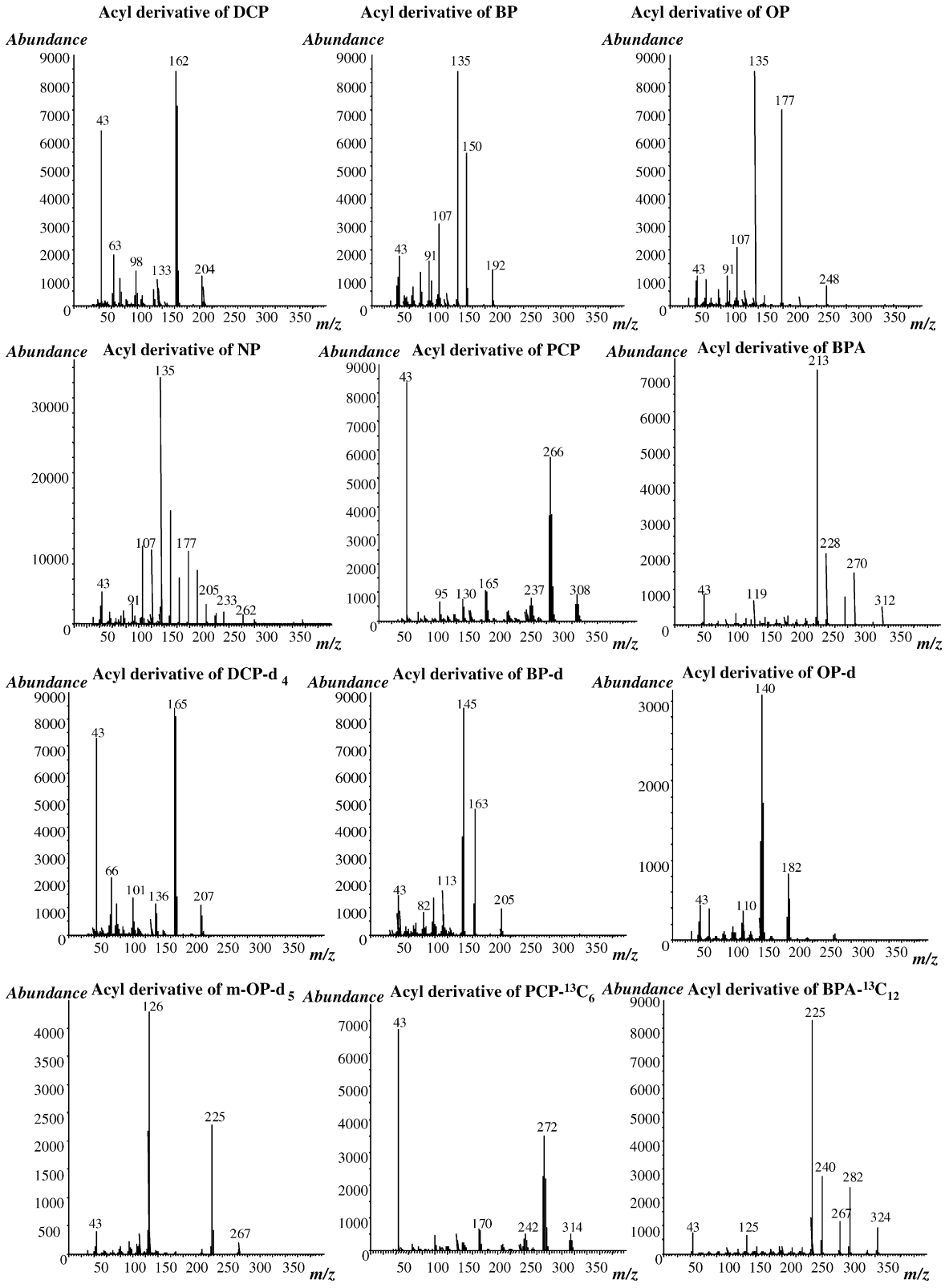

Fig. 2. Mass spectra of acyl derivative of PXs.

erated in the SIM mode. The following ions were monitored (*m/z* 162, 164 for DCP; *m/z* 135, 150 for BP; *m/z* 135, 177 for OP; *m/z* 135, 177 for NP; *m/z* 266, 268 for PCP; *m/z* 213, 228 for BPA; *m/z* 165 for DCP-d_4; *m/z* 145 for BP-d; *m/z* 140 for OP-d; *m/z* 126 for m-OP-d_5; *m/z* 276 for PCP-$^{13}C_6$; and *m/z* 225 for BPA-$^{13}C_{12}$. The underlined number is the *m/z* of the ion used for determination.).

3.2. Theoretical recovery of SBSE

Table 1 shows $\log K_{o/w}$ and the theoretical recoveries of the compounds investigated in this work. The $K_{o/w}$ values were calculated from the log P predictor, which is available from Interactive Analysis Inc. (Bedford, MA, USA), and the KowWin program, which is available from Syracuse Research Corporation (SRC, USA). Theoretical recovery was calculated as follows:

$$\text{theoretical recovery} = \frac{K_{o/w}/\beta}{1 + K_{o/w}/\beta} = \frac{1}{\beta/K_{o/w} + 1}$$

where $\beta = V_w/V_{PDMS}$, V_{PDMS} is the volume of PDMS and V_w is the volume of water. The theoretical recoveries of SBSE were calculated based on an 18 ml sample volume and a stir bar with a phase thickness of 500 μm (24 μl of PDMS). The results revealed that the theoretical recoveries of PXs were increased by the derivatization. Especially, the increase of theoretical recoveries of compounds which have small $\log K_{o/w}$ was remarkably observed. The chromatogram of human urine sample spiked with PXs standard solution (10 ng ml^{-1}) subjected to SBSE with in situ derivatization was compared with that subjected to SBSE without derivatization, and an increase in sensitivity was observed in the former (Fig. 3). The in situ derivatization method exhibited approximately 5–100-fold higher sensitivity than the method without derivatization.

Table 1
$\log K_{o/w}$ and theoretical recoveries of PXs and their acyl derivatives by SBSE

Analyte	$\log K_{o/w}$ [a]	Theoretical recovery (%)
DCP	2.80	45.7
DCP acetate	2.88	50.3
BP	3.28	77.8
BP acetate	3.74	80.8
OP	4.41	99.6
OP acetate	5.53	99.7
NP	5.38	99.9
NP acetate	6.28	99.9
PCP	4.74	98.7
PCP acetate	4.81	98.9
BPA	3.50	85.3
BPA diacetate	4.48	89.4

[a] $\log K_{o/w}$ values for all compounds as calculated from "the log P predictor" and "SRC KowWin" as well as calculated recoveries.

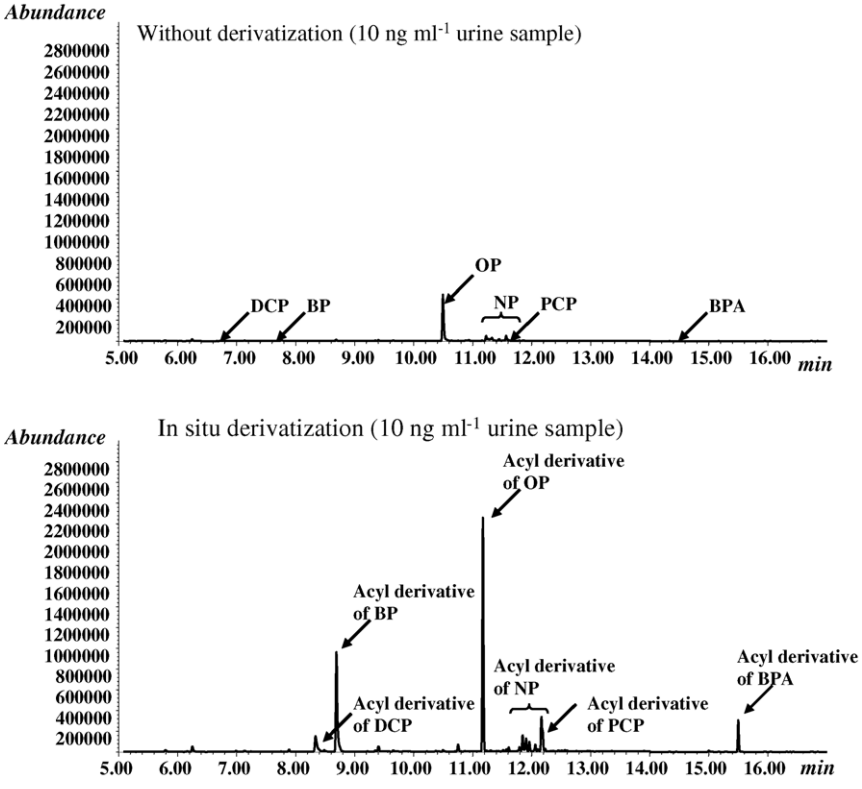

Fig. 3. Comparison of chromatogram of PXs in human urine sample subjected to SBSE with in situ derivatization with that subjected to SBSE without derivatization. For SBSE with in situ derivatization: After protein precipitation and centrifugation of the human urine sample (1 ml) spiked with 10 ng ml^{-1} PXs standard solution, the supernatant was diluted with purified water (15 ml). A PDMS-coated stir bar and derivatization reagents were added to the sample and stirring was commenced for 150 min at room temperature (25 °C) in a glass vial. The extract was then subjected to TD-GC–MS. For SBSE without derivatization: The same procedure was performed except that no derivatization reagents were added.

However, as the improvement of the theoretical recovery was small, it was suggested that the increase in sensitivity also had participation of facilitation vaporization in GC–MS analysis besides the improvement of the theoretical recovery.

3.3. Optimization of protein precipitation

We have previously reported the determination of BPA or chlorophenols in human urine sample by SBSE with in situ derivatization without protein precipitation [40,41]. However, when this method was employed in the determination of OP and NP in human urine sample, the prevention of derivatization was observed. Then, the human urine sample was subjected to protein precipitation prior to SBSE with in situ derivatization. The volume of acetonitrile for protein precipitation was also examined because in theory, the recovery is decreased as the volume of the organic solvent is increased. After enzymatic de-conjugation of the human urine sample (1 ml) spiked with 10 ng ml^{-1} PXs standard solution, protein precipitation was performed by adding 0–3.0 ml of acetonitrile. Then, after centrifugation (1400 × *g*, 10 min), the supernatant was diluted with purified water (15 ml) and subjected to SBSE with in situ derivatization and TD-GC–MS. The results are shown Fig. 4. When 2 ml of acetonitrile was used, the relative responses of the PXs other than DCP were the highest. A previous study has indicated that the concentration of DCP in human urine sample is high level [41]. Therefore, this condition was chosen for protein precipitation.

3.4. Optimum time for SBSE with in situ derivatization

One important parameter affecting SBSE was the extraction time. To determine the optimum extraction time, human urine samples spiked with PXs standard solutions (10 ng ml^{-1}) were used. The extraction time profiles (equilibration curves) of the analytes using SBSE with in situ derivatization are shown in Fig. 5. All compounds reached equilibrium after approximately 150 min. Therefore, this condition was used for the determination of PXs in human urine samples. Because surrogate standards were used, the extraction time need not be strictly made constant.

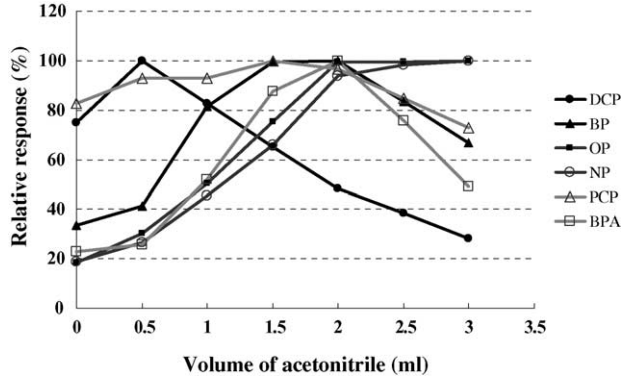

Fig. 4. Determination of volume of acetonitrile for optimization of protein precipitation.

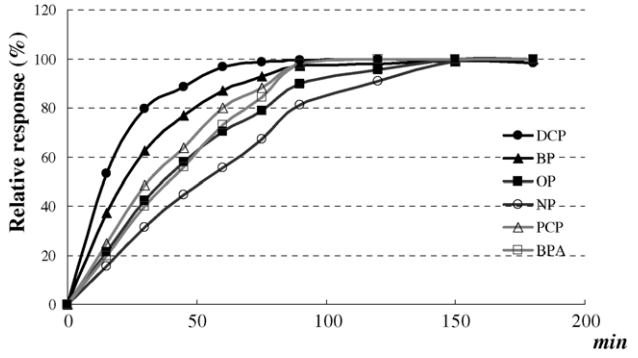

Fig. 5. Optimum time for SBSE with in situ derivatization. After enzymatic de-conjugation, protein precipitation and centrifugation of the human urine sample spiked with 10 ng ml^{-1} PXs standard solution, the supernatant was diluted with purified water (15 ml). A PDMS-coated stir bar and derivatization reagents were added to the sample and stirring was commenced for 0–180 min at room temperature (25 °C) in a glass vial. The extract was then subjected to TD-GC–MS.

3.5. Figures of merit of SBSE with in situ derivatization and TD-GC–MS

The calculated detection limits (LODs) of DCP, BP, OP, NP, PCP and BPA in human urine samples subjected to SBSE with in situ derivatization and TD-GC–MS were 20, 10, 10, 50, 20 and 20 pg ml^{-1}, respectively, when the ratio of the compound's signal to the background signal (S/N) was 3. In addition, the limits of quantification (LOQs) calculated when S/N > 10 were 100, 50, 50, 200, 100 and 100 pg ml^{-1}, respectively. The peak area ratio with respect to each surrogate standard was plotted and the response was found to be linear over the calibration range with correlation coefficients (*r*) of 0.99. The results are summarized in Table 2. When the SBSE method was compared with the SPE method [30], the SBSE method was found to be superior in terms of sensitivity.

The recovery and precision of the method were assessed by replicate analysis ($n = 6$) of human urine samples spiked with surrogate standards at the 0.5 and 5.0 ng ml^{-1} levels. The precision was evaluated by calculating relative standard deviation (RSD). In the sample preparation of urine sample, since the concentrations of PXs in blank urine were high levels after de-conjugation process. Then, protein precipitation

Table 2
Figures of merit of SBSE with in situ derivatization and TD-GC–MS

Analyte	LOD (pg ml^{-1})[a]	LOQ (pg ml^{-1})[b]	Correlation coefficient (*r*)
DCP	20	100	0.99 (0.1–50)[c]
BP	10	50	0.99 (0.05–10)
OP	10	50	0.99 (0.05–10)
NP	50	200	0.99 (0.2–10)
PCP	20	100	0.99 (0.1–10)
BPA	20	100	0.99 (0.1–10)

[a] LOD: limit of detection (S/N = 3).
[b] LOQ: limit of quantification (S/N > 10).
[c] Values in parentheses are the linear ranges of the calibration curves (ng ml^{-1}).

was performed immediately after adding the enzymes. Non-spiked and spiked samples were subjected to SBSE with in situ derivatization and TD-GC–MS. The recovery was calculated by subtracting the results for the non-spiked samples from those for the spiked samples. The results were obtained by using calibration curves of the standard solutions with surrogate standards. The average recovery was higher than 95 % (RSD: <10%) for all human urine samples (Table 3). Therefore, the method is applicable to the precise determination of trace amounts of PXs in human urine samples.

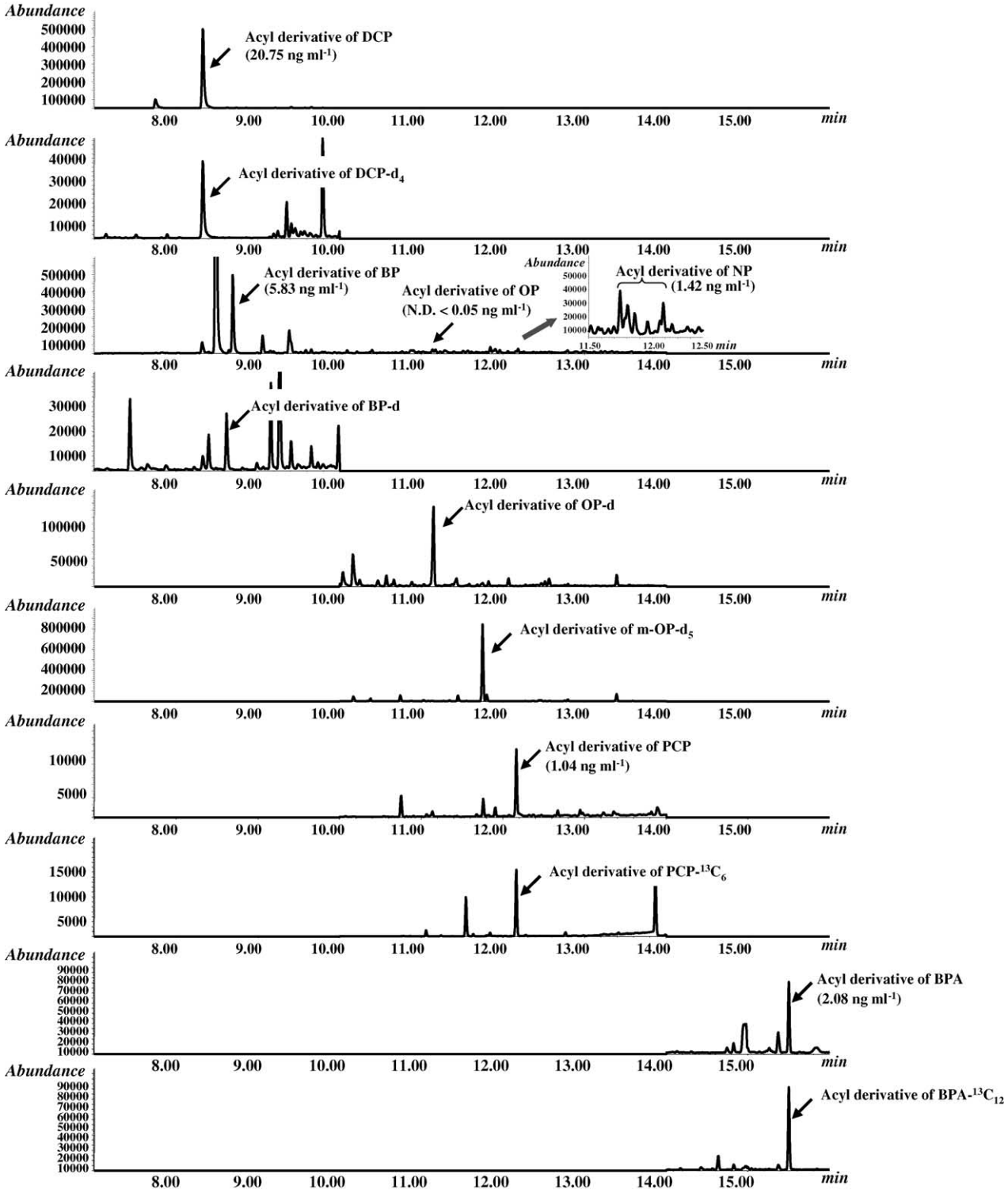

Fig. 6. Chromatograms of acyl derivative of PXs and surrogate standards in human urine sample (Volunteer A).

Table 3
Recoveries of PXs in spiked human urine samples

Analyte	Amount spiked			
	0.5 ng ml^{-1}		5.0 ng ml^{-1}	
	Recovery (%)	RSD (%)[a]	Recovery (%)	RSD (%)[a]
DCP	99.1	4.2	99.6	2.7
BP	99.0	5.3	99.5	2.7
OP	98.9	4.5	97.8	3.7
NP	101.7	8.6	101.8	5.1
PCP	95.0	4.4	99.8	2.5
BPA	95.2	4.8	98.9	4.2

[a] The recoveries and precision were also examined by replicate analysis ($n = 6$) of human urine samples.

Table 4
Concentrations of PXs in human urine samples

Analyte	Human urine sample (ng ml^{-1})				
	A	B	C	D	E
DCP	20.75	41.86	30.47	14.06	20.02
BP	5.83	0.82	0.93	0.10	3.53
OP	N.D.	N.D.	N.D.	0.05	N.D.
NP	1.42	1.04	1.24	2.00	2.00
PCP	1.04	0.46	0.46	N.D.	0.28
BPA	2.08	5.41	N.D.	0.93	1.84

N.D. indicates DCP, BP, OP, NP, PCP and BPA concentrations lower than 0.1, 0.05, 0.05, 0.2, 0.1 and 0.1 ng ml^{-1}, respectively.

3.6. Determination of PXs in human urine sample

Five urine samples collected from healthy human volunteers were measured by present method, and the results are shown in Table 4. Typical chromatograms of the urine samples (Volunteer A) are shown in Fig. 6. DCP, BP, OP, NP, PCP and BPA were detected in the urine samples. In particular, DCP in the urine samples was detected at high concentrations. The concentrations of the other PXs in the urine samples were very low and could not be quantified by the SPE method [30]. In addition, to our knowledge, the method which has detected trace level of technical NP in human urine sample from healthy volunteer is not reported. However, SBSE with in situ derivatization and TD-GC–MS enabled the successful determination of trace amounts of PXs in the urine samples and was therefore useful for evaluating human exposure to PXs.

4. Conclusions

The determination of trace amounts of DCP, BP, OP, NP, PCP and BPA in human urine samples using SBSE with in situ derivatization and TD-GC–MS was investigated. The proposed method has many practical advantages, including a small sample volume (1.0 ml) and simplicity of extraction. The LODs of DCP, BP, OP, NP, PCP and BPA were 20, 10, 10, 50, 20 and 20 pg ml^{-1}, respectively, and the LOQs were 100, 50, 50, 200, 100 and 100 pg ml^{-1}, respectively. The average recoveries were higher than 95% with acceptable precision (RSD: <10%, $n = 6$) for the human urine samples spiked with these compounds at 0.5 and 5.0 ng ml^{-1} levels and corrected by adding surrogate standards. This simple, accurate and highly sensitive method is expected to have potential applications in the evaluation of human exposure to PXs.

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