



Isotope-dilution gas chromatography-mass spectrometry coupled with injection-port butylation for the determination of 4-*t*-octylphenol, 4-nonylphenols and bisphenol A in human urine

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ABSTRACT

An analytical method that utilizes isotope-dilution gas chromatography-mass spectrometry (ID-GC–MS) coupled with injection-port butylation was developed. The method was validated, and confirmed to be able to determine the presence of three commonly detected endocrine-disrupting chemicals (EDCs: 4-*tert*-octylphenol (4-*t*-OP), 4-nonylphenols (4-NPs) and bisphenol A (BPA)) in human urine with high precision and accuracy. After sample preparation by solid-phase extraction, the extract was introduced into GC–MS via injection-port butylation. The butylated target analytes were identified and quantified by using ion-trap mass spectrometry operating in the selected-ion-storage mode, and employing the measurement of peak area ratios of the butylated target analytes and labeled-analogues in the samples and calibration standards. The labeled-analogues were also used to correct the variations associated with the analysis and matrix effect. The limits of quantitation (LOQs) ranged from 0.1 to 0.3 ng/mL. High precisions for both intra- and inter-day analysis ranged from 1 to 6%, and excellent accuracy (mean recovery) ranged from 92 to 105% on two concentration levels. In human urine, the total concentrations of three selected EDCs varied from 1.28 to 7.14 ng/mL. 4-NPs were detected within all collected samples. The developed method allows accurate analysis of trace-level of EDCs in urine, and these target EDCs could act as useful biomarkers to assess exposure in biomonitoring studies and programs.

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1. Introduction

Nowadays, we are exposed to a variety of endocrine disrupting chemicals (EDCs) through industrial pollution, the use of household products, and the ingestion of food. 4-*tert*-octylphenol (4-*t*-OP), 4-nonylphenols (4-NPs), and bisphenol A (BPA) are three well-known EDCs, which have been widely used in both industries and common household application. In particular, these three EDCs are used as surfactants, in the manufacturing of plastics (as plasticizers), in the production of textiles and, in paper and agricultural chemical products [1]. Many studies have observed that they may disrupt the endocrine system and increase the risk of breast cancer in women [2,3]. Moreover, the physical and chemical behaviors of these EDCs are similar to some hydrophobic and semi-volatile organic pollutants, and have been found to accumulate in a variety of aquatic organisms and seafood within the food chain [4–8], as well as have also been found in airborne particles and indoor air [9,10]. These

three EDCs have been detected in human serum, blood, breast milk, hair and urine, indicating human exposure to them through inhalation, direct absorption during dermal contact, or ingestion of food [11–16]. The rise in public concern and interest in investigating the adverse effects from long-term exposure to EDCs spurred us on to make an accurate and reliable method for their detection in human urine samples. This type of high matrix containing sample poses specific challenges for quantitation of target analytes [17]. A valuable method that can be used is isotope dilution mass spectrometric technique (IDMS), since it produces high precision, good accuracy, and definable values. As of now, combination of IDMS with GC or HPLC has been used for the determination of organic chemicals in vegetable oils, infant formula, human urine, and plasma [18–23]. The basic principle of IDMS is that a known amount of labeled-analogue for each target analyte is spiked to the sample and the ratio of the target natural analyte and labeled-analogue is measured using a mass spectrometer. The boon of IDMS is that the concentration of the target analyte can be obtained with high accuracy and is relatively less susceptible to interference than other MS techniques. Moreover, the advantage is that the loss of target analyte during the whole analytical procedure does not influence the analytical

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result, thus making IDMS the best form of internal standardization for quantitation of target analytes in complex matrix.

As a continuation of our work to analyze the presence of EDCs in human specimen, and what their presences might mean for human health, these three EDCs were selected in the development of the method based on SPE coupled with isotope-dilution GC–MS plus injection-port butylation. Moreover, the developed method's precision and trueness were examined, and its applicability in the detection of low to trace-levels of target 4-*t*-OP, 4-NPs, and BPA in the human urine samples was also demonstrated.

2. Experimental

2.1. Chemicals and reagents

Standards: 4-NPs (tech. grade), 4-*t*-OP (purity $\geq 97\%$) and BPA (purity $\geq 99\%$) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Standard stock solutions of each analyte (1000 $\mu\text{g/mL}$) were prepared in methanol and kept at -4°C . A mixed standard solution was prepared at concentration of 50 $\mu\text{g/mL}$ by the dilution with methanol for each analyte, which was used for the preparation of working solutions and calibration curves by further diluted with methanol. Isotope-labeled standards: 4-*n*-nonylphenol ring- $^{13}\text{C}_6$ (4-*n*-NP- ring- $^{13}\text{C}_6$, 100 mg/mL), 4-*t*-octylphenol- ring- $^{13}\text{C}_6$ (4-*t*-OP- ring- $^{13}\text{C}_6$, 10 mg/mL), and bisphenol A- d_{16} (BPA- d_{16} , 100 mg/mL) were also purchased from Sigma-Aldrich. All other solvents and chemicals were supplied by Merck (Darmstadt, Germany), Sigma-Aldrich, and Mallinckrodt Baker (Phillipsburg, NJ, USA) in high purity grade, and were used without further purification. *E. coli* β -glucuronidase (986400 units/g solid) and *H. pomatia* sulfatase (20230 units/g solid) were purchased from Sigma-Aldrich. Prior to use, the β -glucuronidase was added to 0.1 M ammonium acetate to make a total concentration of 10,000 units/mL. Milli-Q water was produced by Millipore Elix[®] 10 RO system plus Millipore Synergy[®] UV system (Millipore SAS, Molsheim, France).

2.2. Sample collection and SPE procedure

The collection of human urine used as samples came from 21 healthy female volunteers within our department, their ages ranging from 20 to 29 years old. Prior to their utilization, all samples were stored at -20°C .

The sample preparation process was as follows: one mL of an aliquot of urine was pipetted into a 2.0 mL conical tube, then mixed with 100 μL of 1.0 M ammonium acetate (pH 6.8) and 10 μL of enzyme solution (containing 10000 units/mL of β -glucuronidase and 2500 units/mL of sulfatase). The mixture was gently mixed and incubated for 2 h at 37°C . After enzymatic de-conjugation, the SPE procedure was performed with Bond Elut pH cartridge (50 mg, 1.0 mL, Agilent, Santa Clara, CA, USA) and operated on a VacMaster vacuum extraction device (IT Sorbent Technology, Cambridge, UK). Before extraction, the cartridge was conditioned using 2 mL of methanol and rinsed by 2 mL of deionized water. Then, 1.0 mL of de-conjugation urine sample spiked with isotope-labeled standard mixture (final concentration 10 ng/mL for each labeled-analogue) was loaded through the cartridge at a rate of about 0.5 mL/min. The cartridge was washed with 2.0 mL of 10% methanolic solution, and subsequently air-dried under vacuum for at least 5 min. All target analytes were then eluted by 1.0 mL of dichloromethane/isopropanol (3:7, v/v) solution. The extract was completely evaporated to dryness under a stream of nitrogen. The residue was then re-dissolved in a solution containing 25 μL of methanol plus 25 μL of 20 mM tetrabutylammonium hydroxide (TBA-OH, as a butylating agent), and subjected to injection-port butylation GC–MS analysis.

2.3. Injection-port butylation GC–MS analysis

The injection-port butylation GC–MS analysis was performed using a Varian 450 GC connected to a Varian 220 ion-trap mass spectrometer (Walnut Creek, CA, USA) operating in the selected ion storage (SIS) mode under electron ionization for quantitation. A ChromatoProbe (Varian) and a temperature-programmed injector were used to introduce 10 μL of sample, as described elsewhere [24]. Temperature of the injection-port was maintained at 100°C for 1.0 min for the reaction of butylation and solvent vaporization. After the vaporization, the temperature was quickly elevated to 250°C (the temperature ramping rate at 200°C/min) to allow the derivatives to be introduced into the GC analytical column. Since the ChromatoProbe device with a disposable micro-vial was used in this study, neither retention effect of the reagent nor thermally degraded components from the sample matrix within the injection-port was detected [24]. Separation was carried out in a DB-5MS capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μm film thickness; Agilent). GC temperature program was set as follows: 100°C holding for 5 min; a temperature ramp of 10°C/min up to 200°C ; and another temperature ramp of 15°C/min up to 300°C (held for 3.3 min), totaling to 25 min. The temperature of the transfer line was set at 280°C . Full-scan EI spectra were acquired under the following conditions: scan time 1 s, mass range 100–500 m/z , ion trap temperature 160°C , solvent delay 14 min, an automatic gain control target set at 20,000, and emission current 20 μA (at 70 eV electron energy).

2.4. Method evaluation

The above mentioned method, after development, was evaluated using spiked deionized water and urine sample #7. The standard solution was spiked with both deionized water and urine samples (final concentration 10 ng/mL for each target analyte). The limits of detection (LODs) and limits of quantitation (LOQs), defined as signal/noise (S/N) ratios of 3 and 10, respectively, were determined through SPE coupled with injection-port butylation GC–MS procedures of the spiked real samples, as described in previous reports [25]. Precision and trueness were evaluated by intra- and inter-day analyses of the spiked urine samples. The value of intra-day precision was found by analyzing five urine samples spiked with the standard mixture on the same day ($n=5$), while the value of inter-day precision was found on five consecutive days ($n=5$). Trueness was calculated as the mean recovery of these spiked samples.

3. Results and discussion

3.1. Mass spectra of butylated target analytes

Firstly, suitable GC separation and stable mass spectra of butylated 4-*t*-OP, 4-*t*-OP- ring- $^{13}\text{C}_6$, 4-NPs, 4-*n*-NP- ring- $^{13}\text{C}_6$, as well as dibutylated BPA and BPA- d_{16} were obtained to provide the optimal detection sensitivity and specificity. Fig. 1 displays the SIS chromatogram of butylated target analytes with their corresponding labeled-analogues, and the mass spectra of butylated (a) 4-*t*-OP, (b) 4-NPs, and (c) dibutylated BPA detected in a spiked urine sample #7. No molecular ions were observed in these butylated target analytes under electron-impact ionization. The base peak was observed at $[\text{M}-71]^+$ for butylated alkylphenols (including 4-*t*-OP- ring- $^{13}\text{C}_6$), such as m/z 191 for 4-*t*-OP, m/z 197 for 4-*t*-OP- ring- $^{13}\text{C}_6$, and m/z 205 for 4-NPs, which can be attributed to the losses of C_5H_{11} in the alkyl moiety from the molecular ion. Since 4-NPs containing a mixture of branched nonyl groups, intensive characteristic ions at $[\text{M}-71-(\text{CH}_2)_n]^+$ were also observed, assigned to the loss of

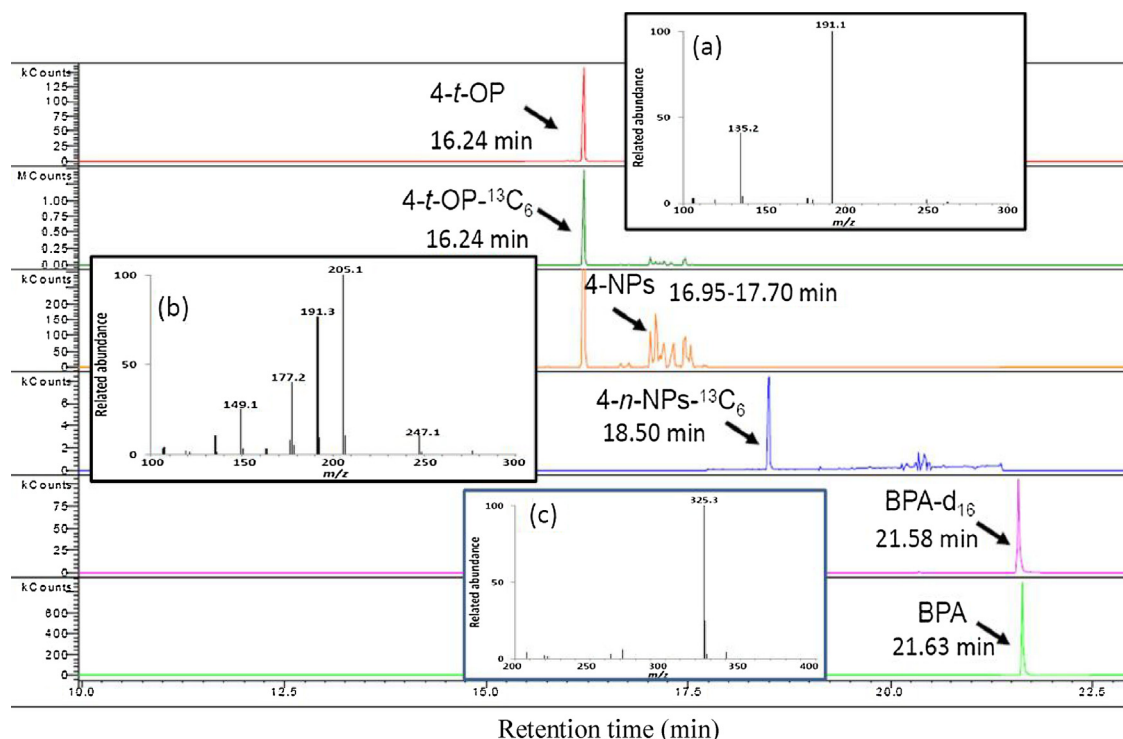


Fig. 1. SIS chromatogram of butylated target analytes with their corresponding labeled-analogues, and the mass spectra of butylated (a) 4-t-OP, (b) 4-NPs, and (c) dibutylated BPA detected in a spiked urine sample #7.

Table 1
Detection characteristics, linear range, linearity, limits of detection and quantitation.

Analyte	Retention time (min)	Quantitation Ion (m/z) ^a	Linear range (ng/mL)	Regression equation	r^2	Mandel test F-test ^b	LOD (ng/mL)	LOQ (ng/mL)
4-t-OP	16.24	135, 191	0.5–50	$y = 0.182x + 0.01$	0.9999	2.215	0.03	0.1
4-t-OP- ¹³ C ₆	16.24	141, 197						
4-NPs	16.95–17.70	149, 177, 191, 205 ,	2.0–200	$y = 0.371x + 0.03$	0.9999	0.462	0.1	0.3
4-n-NP- ¹³ C ₆	18.50	267						
BPA	21.63	325	0.5–50	$y = 0.113x + 0.01$	0.9998	−0.528	0.03	0.1
BPA-d ₁₆	21.58	337						

^a Ions in **bold** are the base peaks in EI mass spectra.

^b $F_{\text{critical}} = 10.128$ (95% confidence level).

various numbers of CH₂ groups from the [M–71]⁺ ions, such as the ions at m/z 191 and 177. Moreover, characteristic ions were observed at m/z 135, 141 and 149, which were attributed to the loss of C₄H₈ (butyl moiety) from the [M–71]⁺ ions of butylated 4-t-OP, 4-t-OP- ring-¹³C₆ and 4-NPs, respectively. However, the base peak [M–15]⁺ (m/z 267) was observed for 4-n-NP- ring-¹³C₆, assigned to the losses of CH₃ in the butyl moiety from the molecular ion. For dibutylated BPA and BPA-d₁₆, only the base peak was observed at [M–15]⁺, such as m/z 325 for BPA and m/z 337 for BPA-d₁₆, which were also attributed to the losses of CH₃ in the butyl moiety from the molecular ion. Table 1 summarizes the retention times and the major ions acted as quantitation ions for the ID-GC-MS-SIS analysis. In this study, sum of the total peak areas of the ions m/z 149, 177, 191 and 205, from the retention times 16.95–17.70 min of the SIS chromatogram, were integrated for 4-NPs quantitation. These total peak areas was also assessed manually after integration in order to increase the replicability of identification and quantitation. Among the different injections analyzed, this step provided the best precision for peak area integration.

The precisions of isotope dilution-MS-SIS (ID-MS-SIS) and MS-SIS quantitation were further evaluated via intra-day and inter-day analysis on two concentration levels. As shown in Table 2, excellent precisions were obtained for ID-MS-SIS, with RSD% ranging between 1 and 6%, whereas the precisions for MS-SIS were 4–10%.

Moreover, a t -test (with confidence intervals at 95%) was used to evaluate the precisions of the two methods. The t -calculated values were 2.977 and 3.002 for 2 and 10 ng/mL spiked concentration levels, respectively, which are both higher than the t -tabulated value ($t_{(95\%, df=5)} = 2.571$). The results indicate that the precisions of the ID-MS-SIS quantitation were significantly better than those obtained from the MS-SIS results.

3.2. Method performance

The feasibility and efficiency of the method's usage to analyze three selected EDCs in gathered urine samples were also evaluated using the method's linear response range, LODs and LOQs, as well as the precision and trueness. Linearity of calibration curve was evaluated at five-point calibration levels for each target analyte ranging from 0.5 to 50 ng/mL for 4-t-OP and BPA, and 2.0–200 ng/mL for 4-NPs. Satisfactory linearity was obtained with coefficient of determination (r^2) for three target EDCs greater than 0.9998 (Table 1). The calculated linearity was further confirmed using Mandel's fitting test [24]. The calculated F -values, which ranged from −0.528 to 2.215, were lower than the tabulated limit ($F_{\text{tab}} = 10.13$) at the 95% confidence level (Table 1), demonstrated that linear regression (instead of quadratic) fit the experimental data the best. For LODs, defined as S/N ratio of 3, ranged from 0.03 to 0.1 ng/mL, and the

Table 2

Accuracy and precision of ID-MS-SIS and MS-SIS on two concentration levels.

Analytes	ID-MS-SIS				MS-SIS			
	Intra-day (n = 5)		Inter-day (n = 5)		Intra-day (n = 5)		Inter-day (n = 5)	
	2 ng/mL	10 ng/mL	2 ng/mL	10 ng/mL	2 ng/mL	10 ng/mL	2 ng/mL	10 ng/mL
4-t-OP	101 ^a (3) ^b	105 ^a (1) ^b	99 ^a (3) ^b	98 ^a (2) ^b	84 ^a (10) ^b	89 ^a (8) ^b	85 ^a (8) ^b	89 ^a (6) ^b
4-NPs	97(5)	92(5)	97(6)	92(3)	97(5)	92(5)	97(6)	92(3)
BPA	101(4)	101(2)	95(2)	97(2)	83(9)	81(8)	81(6)	84(8)

ID-MS-SIS: isotope-dilution selected-ion-storage quantitation; MS-SIS: selected-ion-storage quantitation (n-NP-¹³C₆ was used as the internal standard).^a Mean spiked recovery (%; accuracy (or known as trueness); n = 5).^b Relative standard deviation (% RSD, precision; n = 5) of spiked recovery.**Table 3**

Concentrations (ng/mL) and spiked recovery (%) of target analysis detected in female urine samples using SPE plus ID-GC-MS.

Sample	Analytes		
	4-t-OP	4-NPs	BPA
1	N.D.	4.78	N.D.
2	0.56	3.58	N.D.
3	N.D.	6.69	N.D.
4	1.08	2.73	0.70
5	0.25	5.18	N.D.
6	1.20	1.41	0.29
7	N.D.	0.96	0.32
Recovery (S-7)	92% ^a (5) ^b	95% ^a (5) ^b	91% ^a (4) ^b
8	0.11	3.53	N.D.
9	0.51	1.08	0.26
10	0.19	4.26	N.D.
11	N.D.	5.09	0.70
12	N.D.	2.38	2.04
13	1.26	3.29	0.98
14	0.50	1.52	N.D.
Recovery (S-14)	95% ^a (3) ^b	99% ^a (5) ^b	98% ^a (5) ^b
15	N.D.	5.10	2.04
16	N.D.	3.47	1.64
17	0.14	0.65	1.48
18	1.57	1.76	0.53
19	N.D.	2.96	1.85
20	0.41	1.47	0.63
21	1.6	1.32	1.06
Recovery (S-21)	99% ^a (6) ^b	94% ^a (2) ^b	91% ^a (2) ^b

N.D.: Not detected at the LOQ listed in Table 1.

^a Mean spiked recovery (n = 3) at final concentration of 10 ng/mL for each analyte.^b Relative standard deviation (%RSD) of recovery is given in parentheses (n = 3).

LOQs, defined as S/N ratio of 10, ranged from 0.1 to 0.3 ng/mL for 1 mL of the acquired urine samples (Table 1). The satisfactory precision (%RSD) for ID-MS-SIS quantitation on two concentration levels for three selected EDCs was 6% or less, whereas the trueness (mean recoveries) varied from 92 to 105%, as displayed in Table 2. Collectively, these data indicate that SPE plus ID-GC-MS can produce high reproducibility, linearity, and sensitivity. All of these factors contribute to the determination of the presence of the desired target EDCs in urine samples.

3.3. Method applications

Urine samples were collected from young female adults in our department, and these samples were utilized to appraise the developed method's usefulness. Table 3 lists the concentrations (ng/mL) of the three commonly detected EDCs in human urine samples, and their total concentrations ranged from 1.28 to 7.14 ng/mL. The recoveries of these target analytes from spiked urine samples varied from 91 to 99%, and the precision was 6% or less (Table 3). The chemical that causes the most concern, 4-NPs, and commonly found in seafood samples and human specimen [4–8,11–13,15], was also detected in all the urine samples collected in this study.

Preliminary results show that the average concentrations of 4-t-OP, 4-NPs and BPA were 0.72 ± 0.55 ng/mL (ranging from N.D. to 1.60 ng/mL), 3.01 ± 1.70 ng/mL (ranging from 0.65 to 6.69 ng/mL), and 1.04 ± 0.65 ng/mL (ranging from N.D. to 2.04 ng/mL), respectively. Our results are similar to those reported by Zhang et al. from samples collected in China [26], and Azzouz et al. in Spain [15]. Zhang et al. found 4-t-OP and BPA in children and adults urine samples at concentrations ranging from N.D. to 0.34 ng/mL, and from 0.13 to 5.65 ng/mL, respectively [26]. Azzouz et al. found 4-t-OP, 4-NPs and BPA in adult urine samples from Jaén (Spain) with mean concentrations varying from N.D. to 3.0 ± 0.2 ng/mL, from N.D. to 1.8 ± 0.1 ng/mL, and from N.D. to 8.9 ± 0.5 ng/mL, respectively [15]. However, our results are much lower than the values reported by Zhou et al. from the samples collected from uterine leiomyoma women in China [14], and Choi, et al. from the samples collected from young, obese girls in Korea [27]. Zhou et al. found 4-t-OP, 4-NPs, and BPA in the urine samples of women with uterine leiomyoma in China, with mean concentrations of 2.77 ± 2.22 ng/mL, 4.09 ± 5.51 ng/mL, and 13.9 ± 12.7 ng/mL, respectively, whereas only 4-NPs and BPA were found in the control group, with mean concentrations of 3.84 ± 3.90 ng/mL and 8.50 ± 12.2 ng/mL, respectively [14]. Choi et al. found 4-t-OP, 4-NPs, and BPA in the urine samples of young, obese girls in Korea, with mean concentrations of 7.48 ± 7.44 ng/mL, 10.77 ± 14.52 ng/mL, and 2.72 ± 1.82 ng/mL, respectively, whereas 4-t-OP, 4-NPs, and BPA were found in the control group, with mean concentrations of 9.04 ± 10.10 ng/mL, 10.87 ± 17.01 ng/mL, and 3.66 ± 3.23 ng/mL, respectively [27].

4. Conclusions

A highly precise and accurate isotope-dilution GC-MS with injection-port butylation method for the determination of three commonly EDCs in human urine samples was described. This technique demonstrated its practical applicability to perform quality detection, and produced highly precise and accurate recovery rates when investigating real urine samples. Overall, this technique proved to be a reliable and precision detection method that can be utilized to examine the presence of EDCs in urine samples. The developed method can certainly be adapted with ease by routine analytical procedures that may desire to track and examine EDCs for human biomonitoring program.

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