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Quantification of three chlorinated dialkyl phosphates, diphenyl phosphate, 2,3,4,5-tetrabromobenzoic acid, and four other organophosphates in human urine by solid phase extraction-high performance liquid chromatography-tandem mass spectrometry

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Abstract

Polybrominated diphenyl ethers (PBDEs), produced as flame retardants worldwide, have been phased-out in many countries, and chlorinated and non-chlorinated organophosphates and non-PBDE brominated formulations (e.g., Firemaster 550 (FM550)) have entered the consumers' market. Recent studies show that components of organophosphate esters and FM550 are frequently detected in many products common to human environments. Therefore, urinary metabolites of these compounds can be used as human exposure biomarkers. We developed a method to quantify nine compounds in 0.4 mL urine: diphenyl phosphate (DPhP), bis(1,3-dichloro-2-propyl) phosphate (BDCPP), bis-(1-chloro-2-propyl) phosphate, bis-2-chloroethyl phosphate, di-*p*-cresylphosphate, di-*o*-cresylphosphate (DoCP), di-*n*-butyl phosphate, dibenzyl phosphate (DBzP), and 2,3,4,5-tetrabromobenzoic acid. The method relies on an enzymatic hydrolysis of urinary conjugates of the target analytes, automated off-line solid phase extraction, reversed phase high performance liquid chromatography separation, and isotope dilution-electrospray ionization tandem mass spectrometry detection. The method is high-throughput (96 samples/day) with detection limits ranging from 0.05 to 0.16 ng mL^{-1}. Spiked recoveries were 90–113 %, and interday imprecision was 2–8 %. We assessed the suitability of the method by analyzing urine samples collected from a convenience sample of adults ($n = 76$) and from a group of firefighters ($n = 146$). DPhP (median, 0.89; range, 0.26–5.6 ng mL^{-1}) and BDCPP (median, 0.69; range, 0.31–6.8 ng mL^{-1}) were detected in all of the non-occupationally exposed adult samples and all of the firefighter samples (DPhP [median, 2.9; range, 0.24–28 ng mL^{-1}], BDCPP [median, 3.4; range, 0.30–44 ng mL^{-1}]); DBzP and DoCP were not detected in any samples.

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Disclaimer

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Compliance with ethical standards

Conflict of interest

The authors declare they have no competing financial or other conflicts of interest.

Keywords

Flame retardant; Metabolite; Urine; Liquid chromatography; Mass spectrometry

Introduction

Flame retardants (FRs) are either additive or reactive ingredients applied to household and consumer products to reduce the products flammability, and to meet state and federal fire safety standards and regulations. Until recently, the dominant class of FR additives used for household products was polybrominated diphenyl ethers (PBDEs) [1, 2]. Due to their persistence, bioaccumulation, and potential adverse health effects, PentaBDE and OctaBDE formulations were withdrawn from the consumer market in many regions of the world, including Europe and North America [3–5].

To continue to maintain the fire resistance requirements, alternative chemicals, such as chlorinated and non-chlorinated organophosphates and non-PBDE brominated formulations (e.g., Firemaster 550 (FM550)), have been introduced into the commercial flame retardant market [6]. Organophosphate flame retardants (OPFRs) include triphenyl phosphate (TPhP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP), tris(1-chloro-2-propyl) phosphate, tris(2-chloroethyl) phosphate, tricresyl phosphates, tri-*n*-butyl phosphate (TBuP), and tribenzyl phosphate. Of interest, some organophosphates can also be used as plasticizers or lubricants [6–9]. For example, tricresyl phosphates, TPhP, and TBuP are commonly used as plasticizers and lubricants to regulate pore size [9] and as additives for hydraulic fluids [8]. FM550 contains, among other compounds, TPhP and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) [10, 11].

Some OPFRs and chemicals in non-PBDE brominated formulations are frequently detected in a variety of goods such as baby products, children's hand wipes, furniture, as well as in office and house dust [2, 10, 12–16]. Moreover, reported OPFR levels in indoor environments are comparable or higher than those reported for PBDEs [17]. Several OPFRs are potential carcinogens, mutagens, and neurotoxicants [6, 11, 18, 19], with potential adverse health effects [20–22]. Laboratory animal studies show that OPFRs readily metabolize to their dialkyl or diaryl phosphates [23], and EH-TBB metabolizes to TBBA [24]. Therefore, these metabolites can be used as bio-markers of FR exposure. Monitoring urinary metabolites as biomarkers of exposure can be a valuable aid for understanding OPFRs and non-PBDE brominated formulations potential impact on human health.

Recent publications have demonstrated the presence of FR metabolites in human urine by liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry after derivatization [25–29]. Even though these analytical methods are well developed, not all major metabolites are included in one single method and require relatively large sample volumes.

In this work, we present an high performance liquid chromatography (HPLC)-MS/MS method to concurrently quantify biomarkers of eight chlorinated and non-chlorinated organophosphates, and one non-PBDE brominated compound in human urine. We also

assessed the suitability of the method by analyzing 76 randomly collected urine samples from the general population, and 146 urine samples collected from occupationally exposed persons.

Materials and methods

Reagents and standards

Methanol, acetonitrile, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA), and formic acid, acetic acid, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, formic acid, and acetic acid were all HPLC-grade. Deionized water was organically and biologically purified using a NANOpure Infinity ultrapure water system (Barnstead/Thermolyne, IA). Diphenyl phosphate (DPhP), DPhP-d_{10}, di-*o*-cresylphosphate (DoCP), DoCP-d_{14}, di-*p*-cresylphosphate (DpCP), DpCP-d_{14}, bis-(1-chloro-2-propyl) phosphate (BCPP), BCPP-d_{12}, bis-2-chloroethyl phosphate (BCEtP), and BCEtP-d_8 were purchased from Toronto Research Chemicals, TRC (Toronto, Canada). Bis-(1,3-dichloro-2-propyl) phosphate (BDCPP), BDCPP-d_{10}, 2,3,4,5-tetrabromobenzoic acid (TBBA), and TBBA-$^{13}C_6$ were purchased from Wellington Laboratories (Guelph, Canada). Di-*n*-butyl phosphate (DBuP), DBuP-d_{18}, di-benzyl-phosphate (DBzP), DBzP-d_{14}, β-glucuronidase Type H-1 from Helix Pomatia, and 4-methylumbelliferyl β-D-glucuronidase hydrate (UMB) were purchased from Sigma-Aldrich. UMB-$^{13}C_4$ was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All chemicals and standard materials were used without further purification. The analytes chemical structures, abbreviations, and the names of parent compounds are shown in Fig. 1.

Individual stock solutions of standards and labeled internal standards were prepared by dissolving measured amounts of the solid compound or by diluting in appropriate solvent. Using these individual stock solutions, two intermediate stock solutions with all target analytes were prepared in 1:1 (*v*/*v*) methanol/water giving a concentration of individual compounds of 1000 and 500 ng mL^{-1}. Ten calibration standard solutions containing all target analytes were prepared by diluting appropriate amounts from intermediate stock solutions in 1:1 (*v*/*v*) methanol/water. A 100-μL spike from these calibration standards to 400 μL of urine covers a final concentration range of 0.05 to 40 ng mL^{-1}. 4-Methylumbelliferyl glucuronide and $^{13}C_4$-4-methylumbelliferone were used as deconjugation standards to monitor the extent of the enzymatic reaction. The individual stock solutions were prepared by dissolving measured amounts of 4-methylumbelliferyl glucuronide and $^{13}C_4$-4-methylumbelliferone in methanol. By mixing appropriate amounts from isotope-labeled standards and deconjugation standards in 1:1 (*v*/*v*) methanol/water, the spiking solution of isotope-labeled standards and deconjugation standards mixture was prepared, so that a 100-μL spike to 400 μL of urine would result in 10 ng mL^{-1} concentration of the individual labeled compounds, 750 ng mL^{-1} of 4-methylumbelliferyl glucuronide, and 150 ng mL^{-1} of $^{13}C_4$-4-methylumbelliferone. All stock solutions and standards were stored at or below −10 °C in amber glass vials to prevent photo degradation.

Human urine collection for method development and validation

Urine samples were collected anonymously in Atlanta, GA, in 2015 from a diverse group of adult volunteers with no documented occupational exposure to the target flame retardants. The Centers for Disease Control and Prevention Human Subjects Institutional Review Board (IRB) reviewed and approved the study protocol. A waiver of informed consent was requested under 45 CFR 46.116(d). We did not have access to any personal or demographic data.

The individual urine samples with the overall lowest concentrations ($N = 52$) of endogenous target analytes were combined to form a blank pool. The blank pool was stored at or below −20 °C in glass vials. Quality control (QC) materials were prepared by spiking portions of blank urine with native target compounds. The approximate concentrations of the target analytes were 4 ng mL^{-1} (low concentration QC (QCL)) and 15 ng mL^{-1} (high concentration QC (QCH)). The spiked QC materials were refrigerated, mixed for over 24 h, then dispensed in 1 mL aliquots into polypropylene vials, and stored at or below −20 °C until use.

We also analyzed urine samples from firefighters collected in 2010–2011 for a US National Institute for Occupational Safety and Health (NIOSH) study to evaluate firefighters' exposures to potential toxic chemicals during structural firefighting while wearing fireproof clothing and self-contained breathing apparatus (SCBA) [30, 31]. Samples were collected ~20 min after or 3 h after structural firefighting performed while wearing full protective clothing and SCBA respirators. All participants gave consent to have their residual urine stored without identifiers for future research purposes, and the study protocol was approved by the NIOSH IRB. The analysis of these de-identified specimens for urinary flame retardants biomarkers was determined not to constitute engagement in human subjects research.

Sample preparation and automated off-line solid phase extraction (SPE)

Each analytical run, prepared in a 96-well plate (2 mL square well, Varian, Lake Forest, CA, USA), included a solvent blank, ten calibration standards, two QCL, two QCH, and the study samples. A 96-well format spreadsheet with sample locations was used to guide the spiking. One hundred microliters from the labeled/deconjugation standard spiking mixture was aliquoted to each well. Then 100 μL of calibration standard solutions were spiked to the wells assigned for each calibration level. Subsequently, 400 μL of deionized water was added to solvent blank and calibration standards, and 400 μL of QCs or study urines was added to the corresponding wells. After that, 400 μL of enzyme solution was dispensed to each well. The enzyme solution (pH ~5) was prepared immediately before every analytical run by adding β-glucuronidase/sulfatase with a specific activity of about 500 units mg^{-1} to 0.2 M sodium acetate buffer to produce a solution with a minimum of 1000 units of enzyme activity per sample. Then the 96-well plate was covered with a cover mat and the samples were incubated at 37 °C for at least 6 h (typically overnight).

After the enzymatic hydrolysis, 800 μL of 2 % (*v/v*) formic acid in deionized water was added to each sample. Then the 96-well plate was placed on a TOMTEC Quadra4

semiautomated SPE station (Hamden, CT, USA). Urine samples, reagent blank, and calibration standards were pipette-mixed twice right before loading onto a 96-well format SPE cartridge (60 mg Strata XAW polymeric SPE packing with 1.5 mL liquid space, Phenomenex, Torrance, CA) which was previously conditioned with 2 × 430 μL of HPLC-grade 2 % (*v/v*) formic acid in methanol followed by 2 × 430 μL of 2 % (*v/v*) formic acid in deionized water. After sample loading (6 × 310 μL), the wells were washed with 2 × 430 μL of 2 % (*v/v*) formic acid in deionized water followed by 2 % (*v/v*) formic acid in methanol and dried under vacuum. The target analytes were eluted with 3 × 400 μL of 2 % (*v/v*) NH_4OH in methanol. The eluates were evaporated to dryness under a stream of dry nitrogen (UHP grade) at 40 °C in a Turbovap 96 concentration workstation (Caliper Life Sciences, Hopkinton, MA, USA). The evaporated extracts were reconstituted with 50 μL of 1:1 (*v/v*) acetonitrile/water mixture.

Chromatographic separation and detection

HPLC was performed on an Agilent 1290 (Agilent Technologies, Santa Clara, CA, USA) system equipped with a binary pump, an autosampler with a cooling thermostat module, and a temperature-controlled column compartment. Chromatographic separation was performed on a ZORBAX Eclipse XDB-C8 column (4.6 × 150 mm, 5 μm) from Agilent Technologies kept at 45 °C during analysis and operated at a flow rate of 0.7 mL min^{-1}. The reconstituted SPE extracts were kept in the autosampler at 4 °C and an injection volume of 10 μL was used for the analysis. Analytes were separated with the gradient shown in Table 1 using 20 mM ammonium acetate in deionized water as mobile phase A and acetonitrile as mobile phase B. All analytes eluted within 8 min.

Mass spectrometry analysis was performed on an AB Sciex 5500 Qtrap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbo ion spray (ESI) ionization probe. The parameters were set as follows: curtain gas 20 psi, collision gas medium option, ionspray voltage −4500 V, temperature 450 °C, and ion source gases 45 psi. The mass spectrometer was operated in scheduled multiple reaction monitoring (s-MRM) mode using negative polarity. Table 2 shows the transitions and collision energies used for each analyte.

Analyst software version 1.6.2 (Applied Biosystems) was used to control all system components, the data collection, and analysis. To evaluate the difference between the urinary concentrations of the target analytes with and without enzymatic treatment, we performed a parametric analysis of variance (ANOVA) test. Statistical significance was set at $p < 0.05$.

Results and discussion

Selection of ionization mode

We tested negative ESI and negative atmospheric pressure chemical ionization (APCI) to evaluate the best ionization strategy. All compounds ionized under both ionization modes; however, some analytes, especially the chlorinated compounds and TBBA, were more sensitive in ESI than they were in APCI. Therefore, considering overall efficiency, we selected negative ESI as the ionization method.

Selection of the analytical column

We tested several reversed phase C8 and C18 columns with different dimensions and particle sizes. One of our main targets was to have a short analytical run time (in order to analyze all 96 samples within a 24-h period). Some columns we examined could not completely separate DoCP and DpCP, and some needed comparably longer run times to elute all analytes and completely resolve cresol isomers peaks. We selected ZORBAX Eclipse XDB-C8 column (4.6 × 150 mm, 5 μm) because it eluted all nine analytes in less than 8 min, and completely resolved DoCP and DpCP. The ion chromatograms for native and labeled BCPP appeared as a cluster of three peaks, and the full cluster was integrated for quantitation purposes. Figure 2 shows a typical ion chromatograms for all target analytes in urine.

Optimization of SPE conditions

Previous studies have shown better recoveries for some of the flame retardant biomarkers using weak anion exchange sorbents than other sorbents [25, 27]. Therefore, we chose Strata-X-AW to evaluate recoveries of all nine target analytes. Recoveries were evaluated by using pre- and post-extraction spiked aliquots from the blank urine pool. Portions from breakthrough and wash steps were also tested to evaluate potential losses during sample loading and washing steps. Samples were eluted with 1–5 % of NH_4OH in 80–100 % methanol and in 80–100 % acetonitrile. Care was taken to control the sample pass through flow rate to as low as possible by regulating the negative vacuum supply to the SPE plate between no vacuum and −2 in Hg. As expected, drying time of the eluents increased when the solvent composition was less than 100 %. Significant improvement of recoveries was not observed with the increase of NH_4OH above 2 %. Elution with methanol improved recovery compared with acetonitrile (data not shown). Because Strata-X-AW extracts eluted with 2 % NH_4OH in methanol provided satisfying recoveries for all target analytes, we did not evaluate the performance of other weak anion exchange sorbents.

Enzymatic deconjugation

Many metabolites eliminated in urine are present in their conjugated form [32]. A commonly used approach to quantify the urinary concentrations of these compounds is to first hydrolyze the conjugates to report total (conjugated plus unconjugated) concentrations [33, 34]. Also, while many authors have examined the use of enzymatic hydrolysis as the OPFR biomarkers may be eliminated as conjugates in urine [35], only a few recently published methods utilized enzymatic deconjugation [36, 37]. To evaluate the optimal deconjugation conditions, we treated 12 urine samples (in triplicate) separately with three types of enzymes, β-glucuronidase (*Escherichia coli* K-12), β-glucuronidase (*E. coli*, recombinant), and β-glucuronidase/sulfatase (Helix Pomatia, type H-1), and compared the concentrations of the target analytes to those obtained without enzymatic treatment. We observed no noticeable differences regardless of the type of enzyme used (data not shown), and selected β-glucuronidase/sulfatase (Helix Pomatia, type H-1) to hydrolyze the conjugates. In Table 3, we present the concentrations detected with and without β-glucuronidase/sulfatase (Helix Pomatia, type H-1) treatment. None of the samples tested had detectable concentrations of DBzP, DoCP, DpCP, or TBBA. For the other analytes, the concentrations in enzymatically

treated and non-treated samples differed significantly for DPhP ($p = 0.002$) and DBuP ($p = 0.004$), but not for BDCPP, BCPP, or BCEtP (all $p > 0.05$).

Next, we evaluated the optimal conditions for the enzymatic treatment with β-glucuronidase/sulfatase (Helix Pomatia, type H-1) by changing the amount of enzyme and by changing the incubation time and analyzing in duplicate four samples with detectable concentrations of DBuP, DPhP, BDCPP, and BCEtP. For all analytes, the concentration increased with increasing amounts of enzyme with a maximum at 400 μL. Only DBuP concentrations increased for at least 6 h while concentrations of the other analytes did not increase noticeably after 5 h. For convenience, we chose overnight incubation with 400 μL of enzyme (about 1000 units of enzyme activity per sample).

Matrix effects

The composition of urine varies considerably from person to person, and even within a person, with regard to types and concentrations of solutes. This complexity may cause matrix-dependent ion enhancement or ion suppression [38, 39]. Matrix effects can be accounted for, at least in part, by utilizing stable isotope labeled internal standards or by preparing calibration standards in the same matrix as study samples. Each target compound in this method is quantified with its own deuterium or ^{13}C labeled internal standard. However, even with such provisions, matrix effects may exist. To evaluate matrix effects, we analyzed ten sets of calibration curves constructed in ten different urines or deionized water. The mean slope ± standard deviation in urine and in water for each analyte, and the percent difference between the slopes are shown in Table 4. Within our experimental conditions, none of the urines tested was free of all target analytes, but mean slopes in urine for every analyte were not considerably different from their mean slopes in water; therefore, a water-based calibration curve was chosen for quantification.

Recoveries

Recoveries were evaluated at four concentrations (2, 8, 16, 30 ng mL^{-1}) by using pre- and post-extraction spiked aliquots from the blank urine pool. Each level was prepared and analyzed in triplicate for five different days. Relative recoveries, calculated as the ratio of response ratios (native/label) for pre- and post-spiked extractions, are shown in Table 5. Recoveries of 90–113 % were obtained for all analytes at all concentrations considered.

Precision and accuracy

The precision [40] was calculated as the coefficient of variation (%CV) of repeat measurements ($N = 40$) of the QCL and QCH materials prepared in duplicate. Samples were prepared by two analysts and analyzed in two instruments over the course of 1 month. CVs ranged from 2.7 to 7.5 % (Table 6) with both interday and intraday imprecisions <7 %. Accuracy was calculated at three different concentrations with 20 repeat measurements. Accuracy, expressed as percent error of measured value to its nominal value, ranged from 94 to 108 % (Table 6).

Analytical sensitivity

The limits of detection (LODs) were assessed by 20 repeated measurements of low concentration standards and by plotting the standard deviation of the measured concentration versus the concentration of the standard. The expected standard deviation at the zero concentration, S_0, was determined by the y-intercept of a linear regression analysis of the above plot. The LODs were calculated as three times S_0 [41] and are shown in Table 6.

Method application

The applicability of the method was tested by analyzing 76 urine samples collected anonymously in 2015 from Atlanta adult residents with no known occupational exposure to flame retardants or their metabolites. All of the samples had detectable concentrations of DPhP and BDCPP, and none had detectable concentrations of DBzP, DoCP, or TBBA (Table 7).

The method was also tested by analyzing 146 urine samples collected from firefighters after structural firefighting performed while wearing full protective clothing and SCBA respirators. Again, all of the urine samples had detectable concentrations of DPhP and BDCPP. None of the samples tested had detectable concentrations of DBzP or DoCP (Table 7).

Median concentrations of BDCPP and DPhP in the firefighters' samples were approximately five and three times higher, respectively, than the median from the general population samples suggesting that occupational exposures may be higher than background exposures.

Together, these results show that the current method is sensitive enough to detect urinary concentrations of flame retardant biomarkers from background exposures in the general population as well as higher concentrations encountered in occupationally exposed populations.

Conclusions

We developed a sensitive HPLC-isotope dilution tandem mass spectrometry method with a semiautomated SPE sample cleanup for the quantification of biomarkers of eight chlorinated and non-chlorinated organophosphates, and one non-PBDE brominated compound in urine. Two major advantages of this method are the use of a relatively small sample volume and the high sample throughput. Our preliminary data suggest that the method is sensitive, precise, and accurate enough to detect trace-level concentrations of these compounds in urine. Potential applications may include obtaining reference range concentrations of these biomarkers for large-scale general population studies such as the National Health and Nutrition Examination Survey. Nevertheless, additional considerations, such as adequate collection protocols, handling and storage of the samples, and data on the temporal stability of the analytes in urine, are needed to demonstrate the utility of these measures for exposure and risk assessment purposes.

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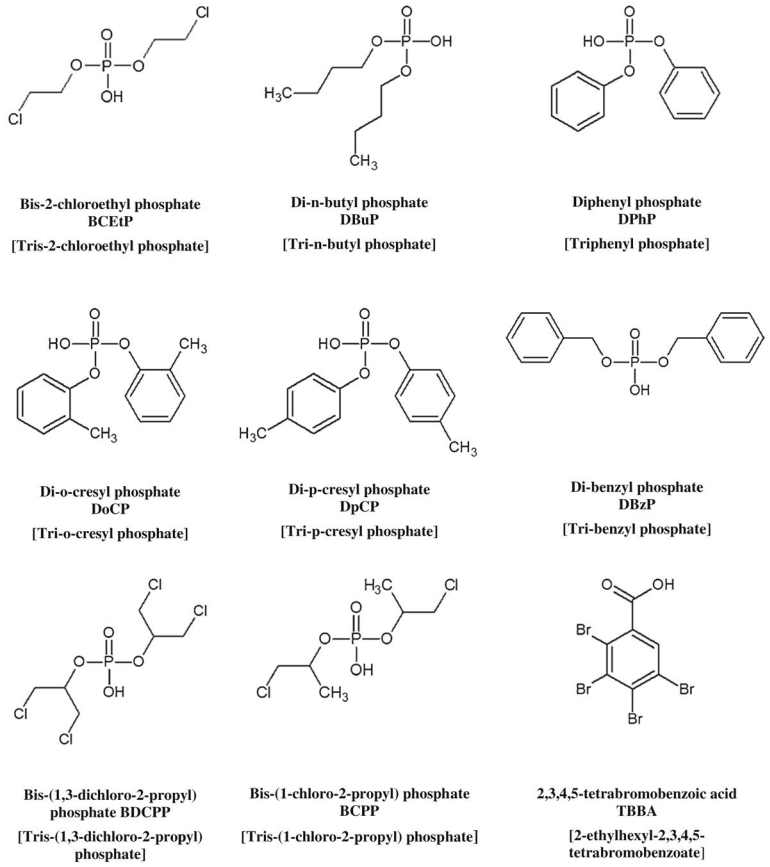

Fig. 1.

Chemical structures, abbreviations, and [parent compound] for target flame retardant metabolites

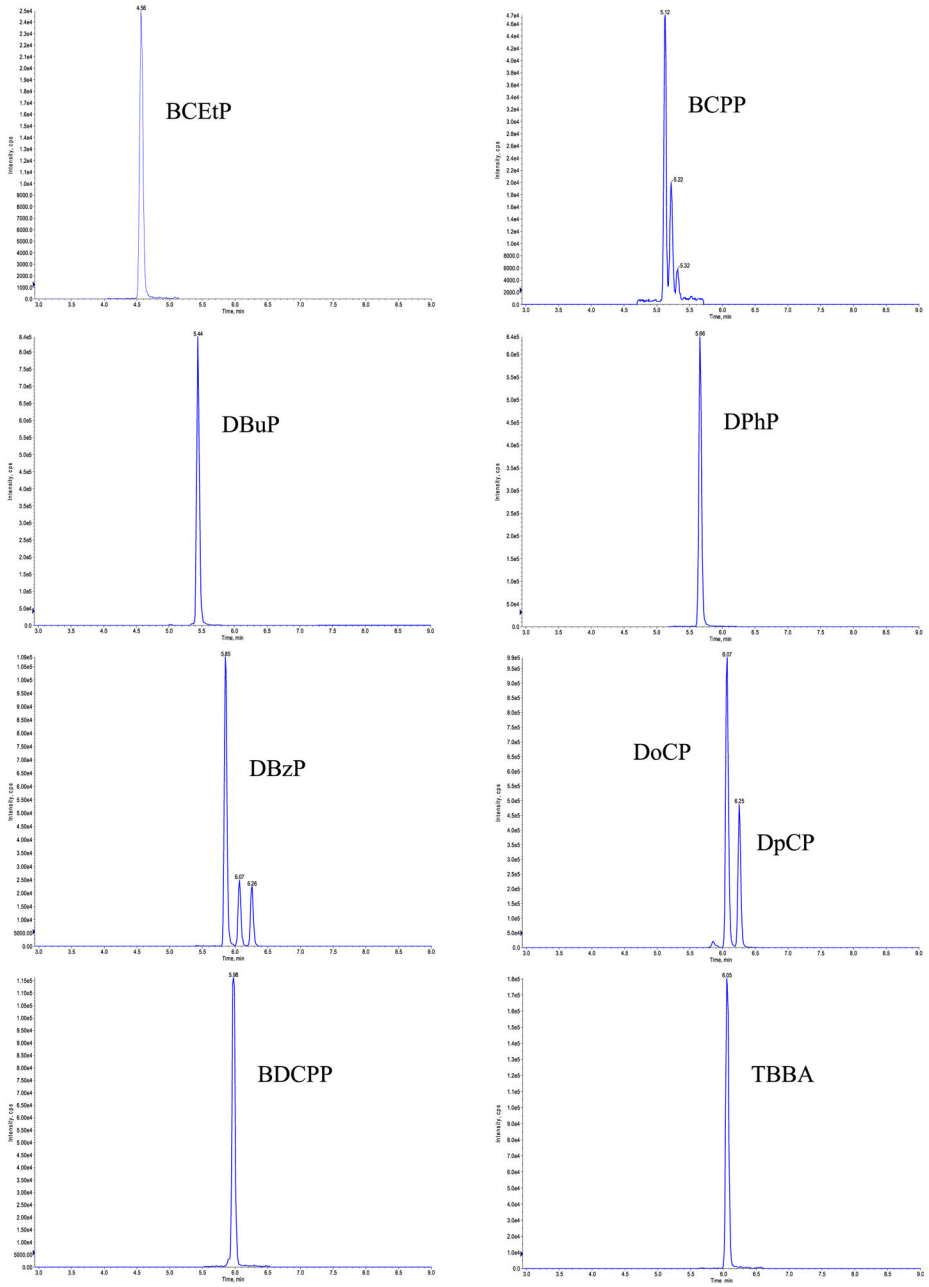

Fig. 2.
Typical chromatograms of the target analytes in urine (0. 3 ng on column)

Table 1

HPLC gradient program

Time (min)	A%	B%
0.0	95	5
0.5	95	5
7.0	25	75
8.5	0	100
9.3	0	100
10.3	95	5
14.0	95	5

A—20 mM ammonium acetate in water; B—acetonitrile

Table 2

Flame retardant metabolites and their isotope labeled analogs, quantitation and confirmation ions, and collision energies (CE)

Metabolite	Quantitation ion			Confirmation ion		
	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (eV)	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (eV)
BCEtP	221	35	25	223	37	31
BCEtP-d_8	229	35	27			
BCPP	249	35	33	251	37	27
BCPP-d_{12}	261	35	33			
BDCPP	319	35	40	319	37	39
BDCPP-d_{10}	329	35	40			
DBuP	209	79	28	209	153	19
DBuP-d_{18}	227	79	30			
DBzP	277	79	33	277	63	30
DBzP-d_{14}	291	79	36			
DPhP	249	93	33	249	155	28
DPhP-d_{10}	259	98	33			
DoCP	277	107	34	277	169	31
DoCP-d_{14}	291	114	34			
DpCP	277	107	35	277	169	30
DpCP-d_{14}	291	114	35			
TBBA	436.7	392.7	14	434.7	390.7	13
TBBA-$^{13}C_6$	442.7	398.7	14			

Table 3

Concentrations of select analytes with and without β-glucuronidase/sulfatase (Helix Pomatia, type H-1) treatment

Sample	Mean concentration ± standard deviation (ng mL^{-1})[a]									
	BCEtP		BCPP		BDCPP		DBuP		DPhP	
	Without enzyme	With enzyme	Without enzyme	With enzyme	Without enzyme	With enzyme	Without enzyme	With enzyme	Without enzyme	With enzyme
1	2.37 ± 0.02	2.40 ± 0.01	0.54 ± 0.03	0.49 ± 0.03	5.69 ± 0.18	5.85 ± 0.23	1.04 ± 0.03	1.11 ± 0.07	16.5 ± 0.15	20.5 ± 0.21
2	0.83 ± 0.01	0.94 ± 0.05	0.23 ± 0.02	0.28 ± 0.02	4.26 ± 0.04	4.50 ± 0.17	0.68 ± 0.06	0.95 ± 0.03	9.10 ± 0.25	10.4 ± 0.34
3	7.47 ± 0.14	7.80 ± 0.02	0.24 ± 0.02	0.21 ± 0.01	1.45 ± 0.14	1.75 ± 0.54	0.29 ± 0.02	0.49 ± 0.04	6.79 ± 0.20	7.86 ± 0.15
4	0.73 ± 0.01	0.74 ± 0	2.20 ± 0.02	2.24 ± 0.04	3.61 ± 0.01	3.68 ± 0.09	0.23 ± 0.02	0.29 ± 0.02	4.64 ± 0.05	4.90 ± 0.18
5	1.20 ± 0.01	1.28 ± 0.01	1.98 ± 0.03	1.96 ± 0.08	0.85 ± 0.03	0.91 ± 0.02	**	**	1.32 ± 0.03	1.49 ± 0.05
6	4.67 ± 0.10	4.81 ± 0.10	0.07 ± 0 *	0.07 ± 0 *	2.73 ± 0.15	2.76 ± 0.14	1.76 ± 0.04	1.98 ± 0.01	19.4 ± 0.17	21.3 ± 0.15
7	1.93 ± 0.02	1.95 ± 0.02	0.46 ± 0.02	0.44 ± 0.04	6.89 ± 0.46	7.50 ± 0.20	1.09 ± 0.05	1.33 ± 0.04	18.3 ± 0.46	22.4 ± 0.40
8	1.79 ± 0.03	1.84 ± 0.03	1.99 ± 0.01	1.98 ± 0.04	4.01 ± 0.08	3.97 ± 0.19	0.34 ± 0.02	0.39 ± 0.02	7.33 ± 0.27	8.22 ± 0.22
9	1.10 ± 0.01	1.18 ± 0.03	0.17 ± 0.01	0.22 ± 0.01	20.3 ± 0.06	20.8 ± 0.32	0.40 ± 0	0.45 ± 0.02	8.68 ± 0.12	10.7 ± 0.35
10	1.48 ± 0.03	1.51 ± 0.06	0.38 ± 0.06	0.33 ± 0.04	7.29 ± 0.11	7.48 ± 0.20	0.88 ± 0.07	1.02 ± 0.04	13.7 ± 0.38	16.4 ± 0.15
11	1.30 ± 0.05	1.31 ± 0.01	0.07 ± 0 *	0.13 ± 0	2.77 ± 0.02	2.84 ± 0.03	0.38 ± 0.01	0.45 ± 0.01	7.43 ± 0.57	8.07 ± 0.06
12	4.04 ± 0.14	4.45 ± 0.07	1.34 ± 0.08	1.36 ± 0.04	**	**	0.54 ± 0.02	0.55 ± 0.01	11.1 ± 0	11.7 ± 0.23
13	1.21 ± 0.05	1.25 ± 0.04	0.85 ± 0.03	0.91 ± 0.01	6.65 ± 0.06	7.00 ± 0.25	0.16 ± 0.01	0.20 ± 0.01	3.21 ± 0.04	3.31 ± 0.03
14	0.99 ± 0.01	0.99 ± 0.02	0.66 ± 0.04	0.71 ± 0.03	5.73 ± 0.08	5.88 ± 0.17	0.39 ± 0.03	0.58 ± 0	6.54 ± 0.37	6.83 ± 0.12

*Concentrations below limit of detection (LOD) were replaced with LOD/√2;

**unable to quantify chromatographic interference

[a]Calculated from three replicate measurements

Table 4

The mean slope of calibration curve ± standard deviation in water and in urine for each flame retardant metabolite, and % difference between the slopes

Analyte	Slope ± standard deviation[a]		% Difference
	Water curve	Urine curve	
BCEtP	0.1048 ± 0.0034	0.1058 ± 0.0022	1.0
BCPP	0.0921 ± 0.0005	0.0894 ± 0.0025	2.9
BDCPP	0.1001 ± 0.0013	0.0956 ± 0.0027	4.6
DBuP	0.1130 ± 0.0008	0.1173 ± 0.0073	3.7
DBzP	0.1065 ± 0.0026	0.1048 ± 0.0040	1.7
DPhP	0.1185 ± 0.0019	0.1198 ± 0.0097	1.0
DoCP	0.0912 ± 0.0008	0.0870 ± 0.0018	4.7
DpCP	0.0986 ± 0.0031	0.0942 ± 0.0021	4.6
TBBA	0.0806 ± 0.0016	0.0780 ± 0.0017	3.2

[a] $N = 10$

Table 5

Relative recoveries (recovery [%]) of off-line SPE and relative standard deviations of recovery (RSD [%]) for flame retardant metabolites at four different concentration levels (2, 8, 16, and 30 ng mL^{-1})

Analyte	2 ng mL^{-1}		8 ng mL^{-1}		16 ng mL^{-1}		30 ng mL^{-1}	
	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD
BCEtP	94	14	105	14	92	23	97	10
BCPP	96	14	100	7	104	4	100	8
BDCPP	99	14	95	9	101	13	102	17
DBuP	97	7	103	8	99	11	98	9
DBzP	99	9	98	10	113	21	105	8
DPhP	102	9	106	15	108	8	98	11
DoCP	96	14	95	14	102	13	93	9
DpCP	93	9	103	10	98	14	90	14
TBBA	101	17	102	17	103	20	103	14

Table 6

Method validation data for flame retardants metabolites

Analyte	LOD (ng mL^{-1})	Accuracy (%)[a]			Precision (%)[b]	
		0.5 (ng mL^{-1})	5 (ng mL^{-1})	25 (ng mL^{-1})	QCL 4 (ng mL^{-1})	QCH 15 (ng mL^{-1})
BCEtP	0.08	96.5	100.3	100.5	3.4	3.4
BCPP	0.1	96.9	98.7	100.7	3.4	3.0
BDCPP	0.11	96.3	106.3	99.0	4.3	3.0
DBuP	0.05	98.1	102.4	102.0	7.5	5.7
DBzP	0.05	101.6	101.0	99.0	3.4	3.9
DPhP	0.16	105.0	101.9	97.9	3.5	2.9
DoCP	0.05	96.2	98.4	105.1	3.4	3.6
DpCP	0.05	97.2	93.5	107.9	4.2	3.9
TBBA	0.05	99.1	99.8	100.0	2.7	3.0

[a] $N = 20$

[b] $N = 40$, two instruments used by two analysts over 1 month

Table 7

Method application data for flame retardants metabolites in urine from anonymous adult volunteers (general population) and from firefighters (exposed population)

Analyte	General population (*N* = 76)			Exposed population (*N* = 146)		
	Detection frequency (%)	Median (ng mL^{-1})	Range (ng mL^{-1})	Detection frequency (%)	Median (ng mL^{-1})	Range (ng mL^{-1})
BCEtP	10	<LOD	<LOD–4.1	90	0.86	<LOD–10
BCPP	5	<LOD	<LOD–0.98	63	0.24	<LOD–2.9
BDCPP	100	0.69	0.31–6.8	100	3.4	0.30–44
DBuP	5	<LOD	<LOD–0.26	92	0.18	<LOD–2.4
DPhP	100	0.89	0.26–5.6	100	2.9	0.24–28
DpCP	0	<LOD		34	<LOD	<LOD–0.31
TBBA	0	<LOD		5	<LOD	<LOD–0.21

DBzP and DoCP were not detected in any of the samples tested