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Determination of human urinary organophosphate flame retardant metabolites by solid-phase extraction and gas chromatography–tandem mass spectrometry

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ABSTRACT

Organophosphorus flame retardants (OPFR), phosphorus triesters, are widely used chemicals with a high share of the worldwide flame retardant market. In animal experiments, dialkyl- and diarylphosphates are the main metabolites of OPFR. Therefore we elaborated a GC–MS/MS-method for the detection of OPFR-metabolites in human urine after solid phase extraction and derivatization with pentafluorobenzyl-bromide. The limits of detection range from 0.1 to 1 μg/l. Interday imprecision were 2–8%. The applicability of the method is shown by determination of the internal burden of 30 persons of the German general population. OPFR-metabolite concentrations range from <LOD to 27.5 μg/l for bis-(2-chlorethyl)-phosphate and <LOD to 4.1 μg/l for diphenylphosphate. Di-*m*-cresylphosphate and di-*p*-cresylphosphate cannot be detected in any of the native urine samples.

1. Introduction

The worldwide consumption of flame retardants was 1.5 million tonnes in 2004. With a share of 14% of the global market of the organophosphorus flame retardants (OPFR) were the third important group of flame retardants besides aluminia trihydrate and brominated flame retardants [1].

Due to their broad applicability as flame retardants and plasticizers OPFR are extensively used as additives in plastics. Depending on the particular requirements OPFR are used in the fabrication of furniture, building industry, transportation and many other areas of everyday life [2–4]. Employees of those industries, as well as the general population, can be exposed against OPFR, since they are not chemically bond to the material and leach out easily into the environment.

OPFR consist of the same phosphate base unit. The chemical and physical properties vary intensively depending on the specific moiety of each OPFR. Differences in size and polarity of OPFR have large influence on toxicity, rate of resorption, metabolism and varieties in urinary excretion. Numerous animal experiments were conducted to assume the potential risk of OPFR but the data base is still weak. Organophosphorus triesters of interest for this study are tris-(2-chlorethyl)-phosphate (TCEP), triphenylphosphate (TPP) and the meta and para isomers of tricresylphosphate (TmCP, TpCP). Inhibition of cholinesterase activity was observable in in vitro studies [5]. TCEP is known from animal experiments to be a carcinogen, neurotoxic and reproductive toxicant [6–11]. TmCP and TpCP are proven to be reproductive toxic in several animal studies [12–19]. Only limited information is available on the toxicity of TPP.

With the exception of TPP, OPFR are rapidly resorbed and distributed to the whole body [3,20–24]. The organophosphorus triesters are hydrolysed in blood and urine spontaneously or enzymatically by α-esterases and phosphorylphosphatases [25]. Hence the main metabolites of the trialkyl- and triarylphosphates in animal experiments and in in vitro studies are the corresponding dialkyl- and diarylphosphates [26–28]. Fig. 1 shows the structures of the four OPFR, discussed in this paper and the corresponding metabolites bis-(2-chlorethyl)-phosphate (BCEP), diphenylphosphate (DPP), di-*m*-cresylphosphate (DmCP) and di-*p*-cresylphosphate (DpCP). The selected analytes are metabolites of flame retardants with high production volumes and considerable toxicological relevance. Furthermore they are representatives of two main subgroups of organophosphorus flame retardants, those with aromatic groups and those containing chlorine. Further hydrolysis and oxidation of the alkyl- and aryl-moieties are also possible [26,28].

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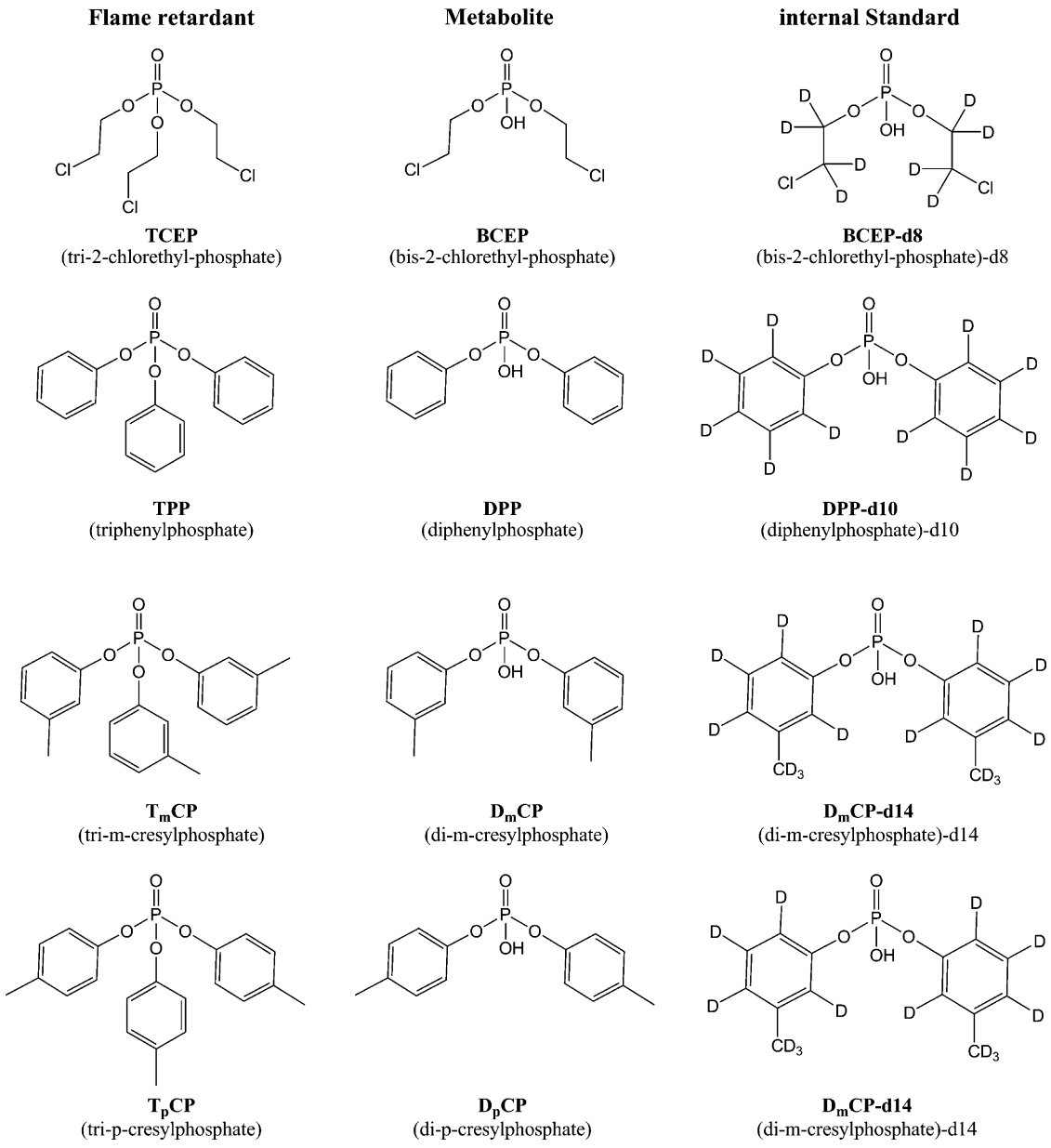

Fig. 1. Flame retardants the corresponding metabolites and the internal standards used.

Previous biomonitoring studies could not determine unmodified OPFR in human blood and plasma samples [29,30]. Unmodified TPP was detected in blood samples of plasma donors in the range of 0.13–0.15 μg/g plasma [31]. Those studies are not really meaningful due to the hydrolysis of the unmodified flame retardants in blood and urine. The metabolite DPP was not detected by Möller et al. in native urine samples, analysed by LC–ESI–MS, due to a high limit of detection (LOD, 25 μg/l) [32].

There is only one previous published method on the determination of OPFR-metabolites in environmental samples by LC–MS/MS after solid phase extraction [33]. Up to now, no appropriate analytical method for human biomonitoring of OPFR-metabolites is published.

Therefore we developed an analytical method for the simultaneous determination of four OPFR metabolites (BCEP, DPP, DmCP, DpCP) in human urine. The applicability of this method is shown by analysis of OPFR levels of 30 persons of the general population of southern Germany.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (lichrosolv, seccosolv), acetone, hexane, toluene, hydrochloric acid (37%) and potassium carbonate were purchased by Merck KGaA (Darmstadt, Germany). 2,3,4,5,6-Pentafluorobenzylbromide was purchased at Aldrich Chemical Co. Inc. (Steinheim, Germany). Deionised water was prepared by a Milli Q Plus System (Millipore, Eschborn, Germany).

As reagent for derivatization, 5 g pentafluorobenzylbromide (PFBBr) was diluted in 6 ml of acetonitrile (seccosolv). Bond Elut PSA (500 mg, 3 ml, PSA = anion exchange material) and Bond Elut FL (500 mg, 3 ml, FL = florisil) cartridges were obtained from Varian Inc. (Middelburg, Netherlands). Isolute ENV+ cartridges (100 mg, 3 ml) were obtained from Biotage (Uppsala, Sweden).

DPP (purity 99%) was purchased at Aldrich Chemical Co. Inc. (Steinheim, Germany). DmCP (95%), DpCP (98%), BCEP (96%) and

the labelled standards DmCP-d14 (>95% total purity, 100% isotopic purity), BCEP-d8 (>99%), DPP-d10 (>99%) were custom synthesised by Kadem Custom Chem (Göttingen, Germany), denoted purities in brackets.

2.2. Instrumentation

The GC–MS/MS system consists of a gas chromatograph (CP-3800, Varian, Darmstadt, Germany) and an autosampler (CP-8400, Varian, Darmstadt, Germany) coupled with a triple quadrupole mass spectrometer (1200L, Varian, Darmstadt, Germany). A J&W DB-35MS (35%-phenyl-methylpolysiloxane) capillary column (60 m, 0.25 mm ID, 0.25 μm; Agilent, Folsom, CA, USA) was used.

2.3. Standard preparation

A stock solution (1 g/l) of each OPFR metabolite was prepared in acetonitrile. The stock solutions (1 g/l) were diluted to a mixed standard working solution with water to a concentration of 10 mg/l each. Calibration standards were prepared in concentrations between 5 and 50 μg/l in pooled urine. Urine samples from multiple donors were pooled for that purpose, frozen, thawed and filtrated before use. The calibration standards were aliquoted to 5 ml portions and stored at −18 °C till analysis. A stock solution (1 g/l) of each deuterium labelled internal standard (BCEP-d8, DPP-d10, DmCP-d14, cf. Fig. 1) was prepared in acetonitrile and diluted to a mixed internal standard working solution (10 mg/l each). All solutions were stored at −18 °C in the dark.

2.4. Sample preparation and solid phase extraction

A flow chart of the sample preparation procedure used to extract OPFR metabolites is summarized in Fig. 2. The urine sample was thawed, shaken and an aliquot of 5 ml was transferred to a 13-ml tube. The urine was spiked with 20 μl internal standard working solution, acidified with 125 μl of 3 M HCl and vortex mixed. The ENV+ cartridges were placed on a Vacmaster SPE station with stopcocks, conditioned with 4 ml acetonitrile and equilibrated with 4 ml 0.1 M HCl. The urine sample was loaded onto the SPE cartridge with a flow-rate of about 0.25 ml/min. The cartridges were dried for 5 min in vacuum, washed with 1 ml 0.1 M HCl afterwards and again dried in vacuum for 10 min to remove remaining water. The analytes were eluted with 7 ml acetonitrile at a rate of about 0.25 ml/min into a 10 ml screw-vial containing 10 mg potassium carbonate to trap remaining acid. The eluate was evaporated to dryness at 50 °C under a gentle stream of nitrogen.

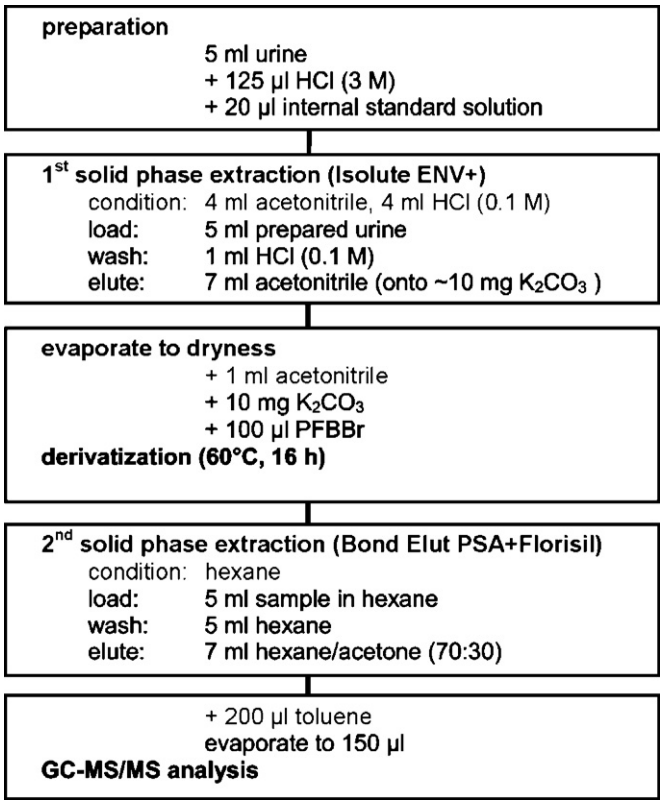

Fig. 2. Flow-chart of the sample preparation of OPFR-metabolites.

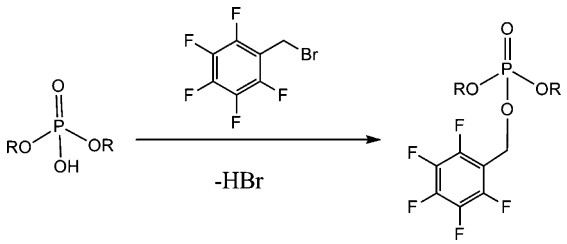

Fig. 3. Formation of pentafluorobenzylesters of OPFR-metabolites during derivatization reaction.

After the residue was resuspended in 1 ml acetonitrile, again 10 mg potassium carbonate and 100 μl pentafluorobenzylbromide derivatization solution were added. The vial was capped and vortex mixed. The derivatization took place at 60 °C within 16 h and formed the pentafluorobenzyl derivates (see Fig. 3). The samples were cooled down to room temperature and vortex mixed. After evaporation to dryness in a gentle stream of nitrogen in a reacti-vap station the residue was reconstituted in 5 ml hexane. After vortex mixing, the samples were transferred to a second SPE-step.

For that purpose two SPE cartridges (Bond Elut PSA, Bond Elut FL) were connected by an adapter, PSA at top, FL at bottom, and placed onto a Vacmaster SPE station. The cartridges were conditioned with 2.5 ml hexane and loaded with the hexane extract, containing the derivatives of the OPFR-metabolites, and the potassium carbonate residue. Gentle vacuum can be attached, if necessary. The cartridges were washed with 5 ml hexane and the analytes were eluted with 7 ml acetone and hexane mixture (30:70). The eluate was concentrated to 1 ml, 200 μl of toluene was added as a keeper. The solution was further evaporated to 150 μl and transferred to a microinsert. Measurement was accomplished by GC–MS/MS.

2.5. Instrumental analysis

Analysis was performed by injecting 1 μl of each sample splitless onto a capillary column. Helium was used as carrier gas at a flow-rate of 1.5 ml/min. The split less injection time was 1.2 min. The injector was set to 260 °C, the transfer line to 250 °C. The temperature of the source was 200 °C. The column temperature was 80 °C initially for 0.5 min. At a rate of 25 °C it is raised to 110 °C and held for 1 min. Subsequent to 270 °C at a rate of 25 °C/min and to 290 °C at a rate of 20 °C/min, hold for 2 min and raised to a final temperature of 300 °C were it is held for 8 min. All analytes eluted in less than 19.5 min. The electron energy for ionisation was 70 eV. The analyte peaks were identified by quantitation and confirmation ions for each pentafluorobenzyl-OPFR metabolite as shown in Table 1 at a scan rate of 5 s^{-1}.

2.6. Study subject and sample collection

30 Spot urine samples, randomly selected form the general population of southern Germany were analysed to show the applicability of the elaborated method. All study subjects were

Table 1
Pentafluorobenzyl derivates of OPFR analytes and their labelled analogues, quantitation and confirmation ions, collision energies (CE) and retention times (RT).

Metabolite	Quantitation ion			Confirmation ion			RT (min)
	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	CE (eV)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	CE (eV)	
BCEP	367	305	10	367	207	10	14.65
BCEP-d8	375	310	10	410	375	10	14.59
DPP	430	249	10	430	317	10	17.61
DPP-d10	440	259	10	440	178	10	17.56
DmCP	458	277	10	458	255	15	18.82
DmCP-d14	472	291	10	472	338	10	18.68
DpCP	458	277	10	458	255	15	19.33

occupationally not exposed to OPFR. The demographic characteristics are as follows: 16 females and 14 males, aged between 11 and 68 years, with a median of 39.5 years. All but one were non-smokers. Creatinine levels ranged from 0.08 to 1.45 g/l with a median of 0.67 g/l. The samples were stored without any pre-treatment in polyethylene-containers till analysis.

2.7. Daily operation and quality control procedure

Quality control (QC) material was prepared based on urine. The urine was pooled from multiple donors, frozen, thawed and filtrated before use. Two concentrations of QC-material were produced by adding standard solution to pooled urine. The lower concentration was 10 μg/l and the higher concentration was 30 μg/l. QC material at both concentrations was analysed during each run. Additionally one blank water sample and a full calibration of 5 points were extracted and analysed along with the unknown samples in each run. For calibration the ratio of the peak area of standard to internal standard was plotted versus the concentration by Varian Workstation 6.41 software with a regression weighting of $1/x$.

2.8. Limit of detection and limit of quantification

Limits of detection were defined as a signal-to-noise ratio of three for the quantifier ion trace. Limits of quantification (LOQ) were defined as three times the limit of detection or a signal-to-noise ratio of nine.

2.9. Relative recoveries in different urinary matrices

Ten different urine samples with creatinine-levels of 0.28–2.95 g/l were spiked with standard solution at two different concentrations, 20 (c_{low}) and 40 μg/l (c_{high}). The samples were extracted as described in Section 2.4. Relative recovery was determined from the ratio of the area of the analyte and internal standard in reference to the calibration graphs.

2.10. Imprecision

Urine spiked at two concentration levels, 10 and 30 μg/l was analysed. Relative standard deviations (R.S.D.) were determined for inter-day and intra-day variations based on a set of six samples in each case.

2.11. Confirmation of detection

Confirmatory ions were used to confirm a detected peak as target analyte. The ratio of quantitation ion to confirmatory ion had to be consistent with the ratio determined from standards. Variations of less than 20% of the ratio quantitation ion to confirmation ion were accepted. DmCP-d14 was used as internal standard for the determination of DpCP. The exact differences in retention time of standards and internal standards are given in Table 1.

3. Results and discussion

Based on an existing method for analysis of organophosphorus (OP) pesticides metabolites by Hemakanthi De Alwis and Needham [34] we developed a robust and reliable method. OPFR are structural similar to OP pesticides. The first step in analysing OPFR metabolites was the extraction of the polar components from polar urine. Solid phase extraction was selected because we received better separation from matrix components and lower LODs compared to other methods of preparation of OPs like lyophilization and liquid-liquid extraction [35,36]. Anion exchange solid phase extraction of OPFR metabolites worked even worse.

Isolute ENV$^+$ cartridges proved to be most suitable to extract both, the less polar diarylphosphates as well as the much more polar BCEP from the polar urine matrix. Other cartridges like Bond Elut PPL, Oasis HLB and Isolute 101 turned out to be less effective in separation of the analytes and produced variable recoveries. Especially the highly polar and highly water soluble BCEP showed considerable higher losses on those cartridges.

The ENV+ material is based on cross-linked styrene–divinylbenzene copolymer, which is functionalised by hydroxy-groups at the aromatic rings. The retention mechanism of the SPE is both, polar and non-polar. The polar hydroxy-groups of the material interact with the polar phosphate moiety and the non-polar aromatic systems of the material provide non-polar interaction with the aromatic moieties of the OPFR.

Protonation plays an important role in analytes' retention behaviour. Deprotonated OPFR, due to the higher polarity, showed almost no retention. So it was necessary to acidify the urine sample to achieve reproducible results. Experiments showed that a lower pH did not increase the recoveries. Additionally, low flow rates were necessary to allow interaction between analytes and solid phase material and result in a higher value of reproducibility and better recoveries. Furthermore, SPE clean up is also a preconcentration step. Large volumes (5 ml) of urine were indispensable to achieve the required LODs.

We accomplished the derivatization of the analytes by pentafluorobenzylbromide usually used for derivatization of phenols, thiols and carboxylic acid. Compared to other derivatizating agents (silylating reagents, diazomethane, diazotoluene and chloriodopropane), pentafluorobenzylbromide showed the best performance in our experiments in matters of resulting peak area, response of the derivates and fragmentation in MSMS-experiments.

A post-derivatization procedure was necessary for removal of unreacted PFBBr and additional sample clean-up. Without this second clean up step the performance of the instrument declined during one run, resulting in very high LODs and damage of the GC-column.

The combination of Bond Elut PSA and Bond Elut FL provided both, excellent matrix separation and removal of unreacted PFBBr.

Table 2 summarizes the validation data of the developed method. We obtained linear calibration curves from 5 to 50 μg/l with correlation coefficients greater then 0.999 for all analytes.

Table 2
Validation data for the determination of OPFR metabolites (c = concentration, Q = quality control material).

Analyte	LOD (μg/l)	Pearson coefficient of correlation	Mean relative recovery (%)		Range of relative recovery (%)		Relative recovery R.S.D. (%)		Intra-day imprecision R.S.D. (%)		Inter-day imprecision R.S.D. (%)	
			c_{low} (N = 10)	c_{high} (N = 10)	c_{low} (N = 10)	c_{high} (N = 10)	c_{low} (N = 10)	c_{high} (N = 10)	Q_{low} (N = 6)	Q_{high} (N = 6)	Q_{low} (N = 6)	Q_{high} (N = 6)
BCEP	0.1	0.9997	98.3	98.7	79–113	93–104	7.4	4.2	6.3	1.9	5.1	2.0
DPP	0.5	0.9995	99.3	102.7	89–109	95–114	6.6	7.3	3.3	1.7	8.1	2.2
DmCP	1.0	0.9997	99.5	99.4	85–104	94–109	3.3	5.1	2.0	3.3	4.3	5.3
DpCP	1.0	0.9990	100.7	101.6	89–112	93–113	9.6	6.0	7.9	0.8	6.0	5.9

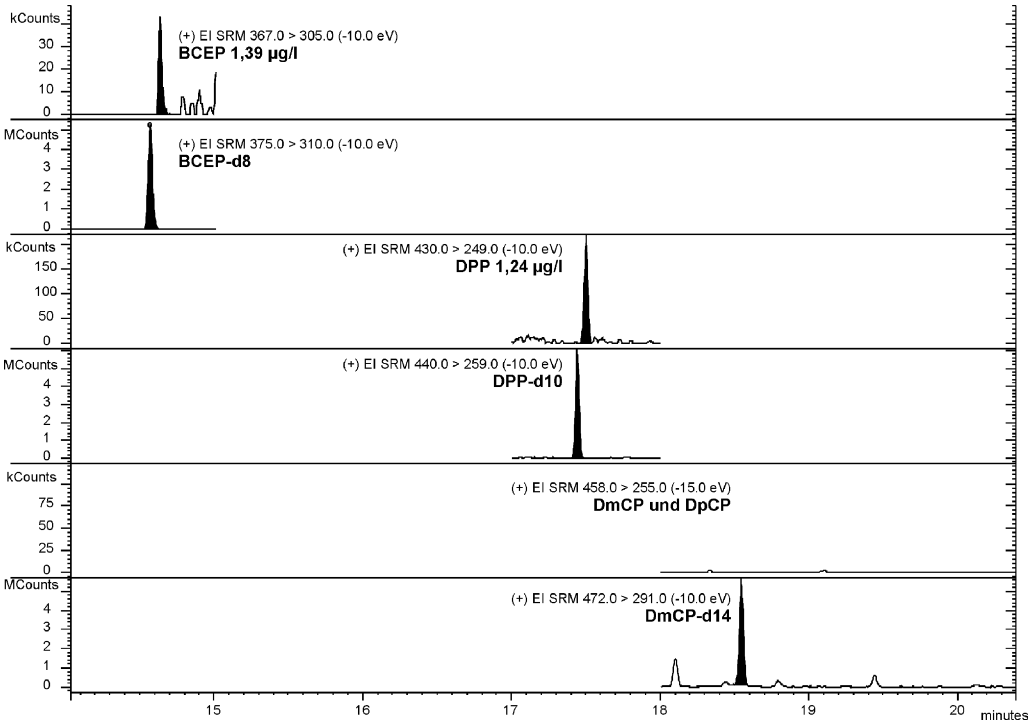

Fig. 4. Chromatogram of a native urine sample containing 1.40 μg/l BCEP and 1.24 μg/l DPP. DmCP and DpCP <LOD.

LODs ranging from 0.1 to 1.0 μg/l and LOQs ranging from 0.3 to 3.0 μg/l indicate the high sensitivity of the method. The chromatogram of a native urine sample, containing 1.40 μg/l BCEP and 1.24 μg/l DPP (Fig. 4) shows the high sensitivity and selectivity down to low concentrations.

Relative recoveries determined for 10 different urine samples spiked with 20 and 40 μg/l were obtained to reflect the robustness of the method in terms of different biological matrices. Mean relative recoveries were 98–101% for samples spiked with 20 μg/l and 99–103% for samples spiked with 40 μg/l. The relative recoveries ranged from 79 to 113% for the lower concentration and 93–114% for the higher concentration, depending on the analyte. The relative standard deviations of the relative recoveries were 3–10% for samples spiked with 20 μg/l and 5–7% for samples spiked with 40 μg/l. That means, even for extremely varying compositions of urine, the method proofed to be robust and evidenced a good accuracy. Native concentrations were found to be <LOD to 5.3 μg/l (BCEP), <LOD to 1.9 μg/l (DPP) and <LOD (DmCP, DpCP).

Fig. 5 shows a urine sample spiked with standard solution (25 μg/l) and internal standard solution (40 μg/l). As it can be seen it is also possible to determine DmCP and DpCP in spiked urine samples.

Within series imprecision and day to day imprecision were determined for two concentrations 10 μg/l (Q_{low}) and 30 μg/l (Q_{high}). Relative standard deviations of the day to day imprecision (N = 6) were 5–8% for Q_{low} and 2–6% for Q_{high}. Within series imprecision was ascertained to be 2–8% for Q_{low} and 1–3% for Q_{high} (N = 6). The determined values for imprecision point out that this method delivers highly reproducibility results. None of the analysed blank water samples contained any traces of OPFR-metabolites. Therefore related problems and artefacts from the labelled internal standards can be excluded.

This is the first method published that enables the determination of four OPFR metabolites in native human urine samples. There is only one previously published method by Möller et al. [32] to determine DPP in human urine samples by LC–ESI–MS. Due to the high limits of detection (25 μg/l) DPP could only be determined in spiked urine samples and not in native samples in that study.

We examined 30 native urine samples of the German general population. The results of the biomonitoring data are summarized in Table 3. BCEP was found in 50% and DPP in 30% of all urine samples. DmCP and DpCP could not be determined

Table 3
Results of biomonitoring study (N = 30) (>LOD = number of samples > limit of detection).

Analyte	>LOD (%)	Median (μg/l)	Range (μg/l)
BCEP	50	<LOD	<LOD–27.5
DPP	30	<LOD	<LOD–4.1
DmCP	0	<LOD	
DpCP	0	<LOD	

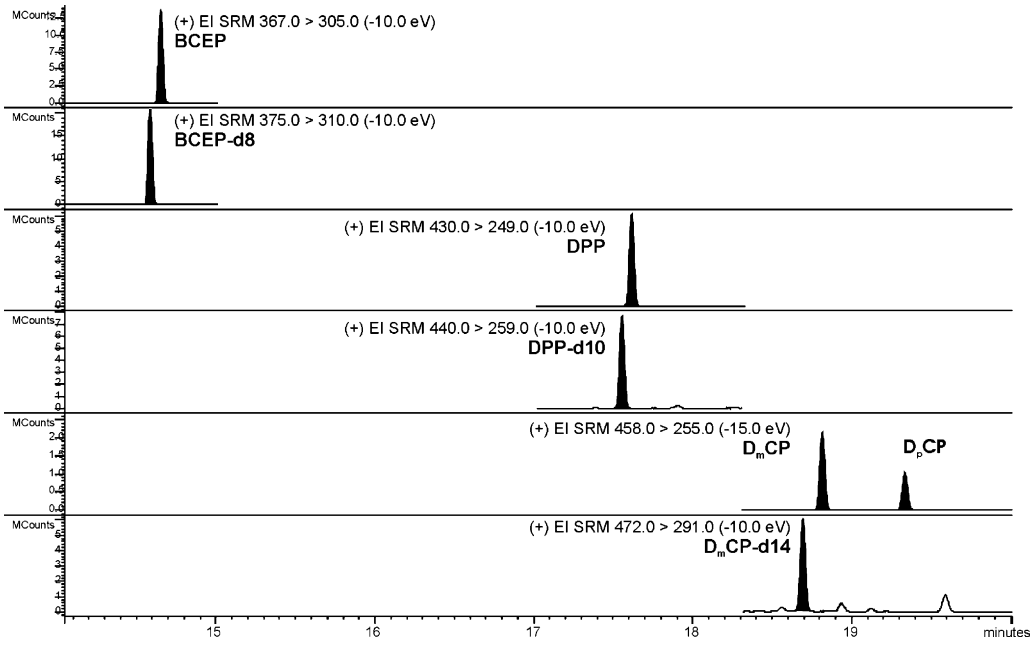

Fig. 5. GC–MS/MS-chromatogram of a native urine sample spiked with standards (25 μg/l) and labelled internal standards (40 μg/l).

in any of the urine samples. Urine samples contained BCEP and DPP in concentrations from <LOD to 27.5 μg/l and <LOD to 4.1 μg/l, respectively. The median levels were <LOD. The results show that this method is sensitive enough to detect the background exposure levels in the general population as well as higher concentrations like in monitoring of occupationally exposed workers.

The four OPFR investigated in this study are metabolites of ubiquitously occurring environmental pollutants [37–44]. Several methods have been published on the determination of unmodified OPFR in environmental samples [45]. For TCP in house dust samples of private homes in Germany median levels of 2.2 mg/kg have been detected [42]. The other two OPFR are occurring in the same order of concentrations. In air samples the level of TCP is much lower than that of TCEP [46,47]. Depending on the uptake route the low levels of TCP in air might be one of the reasons why we were not able to detect the metabolites of TmCP and TpCP. Another reason may be due to their much lower urinary excretion rates in animal experiments compared to that of TCEP [19,21,28].

4. Conclusions

We developed a robust and reliable method to determine four OPFR metabolites in human urine. Determination of OPFR metabolites in trace level concentrations is possible after solid phase extraction, derivatization via pentafluorobenzylbromide and purification in a second solid phase extraction. The main characteristics of the methods are the high reproducibility, reliability, robustness, high sensitivity and high selectivity. Minimal system maintenance is necessary due to a high degree of sample clean up. The developed method is sensitive enough to determine OPFR metabolites in native human urine samples and to ascertain background exposure levels of OPFR metabolites in the German general population. A first glance on the metabolism of OPFR in humans is possible. Dialkyl- and diaryl-metabolites of TCEP and TPP are formed and excreted via urine. It is assumed that the present method can be extended to determine other metabolites of OPFR if they are structural similar to the examined metabolites and if appropriate internal standards are available.

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