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# Analytical method for the sensitive determination of major di-(2-propylheptyl)-phthalate metabolites in human urine

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ABSTRACT

Di-(2-propylheptyl)-phthalate (DPHP) is a specific phthalic acid ester of isomeric C10 alcohols. It is classified as high molecular weight phthalate and marketed as plasticizer for polyvinyl chloride (PVC). The increase of its production volume and its wide field of application suggest a possible background exposure of the human population as found for other phthalates, making suitable analytical methods necessary. The aim of the presented analytical report is the sensitive and selective determination of the three major DPHP metabolites mono-2-(propyl-6-hydroxy-heptyl)-phthalate (OH-MPHP), mono-2-(propyl-6-oxoheptyl)-phthalate (oxo-MPHP) and mono-2-(propyl-6-carboxy-hexyl)-phthalate (cx-MPHxP) in human urine. Most of the published analytical methods for phthalate metabolites use high pressure liquid chromatography tandem mass spectrometry (HPLC–MS/MS). The methods presented here allow a comparison of chromatographic separation between HPLC–MS/MS and gas chromatography high resolution mass spectrometry (GC–HRMS), which is useful to distinguish between DPHP and DIDP. The enhanced detection limits range between 0.05–0.1 μg/L for GC–HRMS and 0.1–0.2 μg/L for HPLC–MS/MS.



## 1. Introduction

Several biomonitoring studies indicate that the general population is exposed ubiquitously with low molecular weight phthalates (e.g. di-(2-ethylhexyl)-phthalate) [1–6]. Di-(2-propylheptyl)-phthalate (CAS No. 53306-54-0) is a substitution product of di-(2-ethylhexyl)-phthalate (DEHP), which is listed in Annex XIV of REACH as SVHC (substance of very high concern) [7].

DPHP is produced and commercialized by BASF [8] as plasticizer for technical applications in polyvinyl chloride (PVC) and other vinyl chloride (VC) polymers. There are two different C 10 phthalates on the market: DPHP and DIDP (CAS No. 68515-49: 1,2-benzenedicarboxylic acid, di-C9-11-branched alkyl esters, C10-rich and CAS No. 26761-40-0: di-isodecyl-phthalate). Due to their low volatility they are used in high temperature resistant products such as cables, carpet backing, roofing membranes and car interiors [9,10]. These plasticizers are not chemically bound in PVC products and hence an exposure of humans cannot be excluded [11]. In Europe DPHP is not approved for contact to foodstuffs, toys and medical products [10]. Moreover, the substance is not listed in Commission Regulation (EU) No. 10/2011 as it has not been evaluated by European Food Safety Agency (EFSA) [12].

In 2008 the production volume of DPHP was 105000 t in the USA and 55000 t in Europe [13], with upward tendency. Also at this time the shift from the low molecular weight phthalates (e.g. di-(2-ethylhexyl)-phthalate or dibutyl phthalate) to the high molecular weight phthalates (DINP, DIDP and DPHP) represented 65% of the overall consumption in Western Europe [14].

DPHP, which is marketed under the trade name "Palatinol® 10-P", consists of about 99.5% phthalic ester. The esterified alcohol mainly consists of 90% 2-propyl-heptanol and 10% 2-propyl-4-methylhexanol or 2-propyl-5-methylhexanol. From this follows the isomer distribution ~81% DPHP, 18% phthalic acid, 2-propyl-heptyl-ester, 2-propyl-(4/5-methylhexyl)-ester and 1% bis-(2-propyl-4/5-methylhexyl)-ester. The DPHP metabolism takes place in analogy to other previously studied phthalates [15–18], primarily via mono ester, followed by secondary ω- and ω-1 oxidation, yielding main metabolites OH-MPHP, oxo-MPHP and cx-MPHxP (Fig. 1), which were excreted into the urine in amounts of up to 34% of the applied DPHP dose [19]. The mono ester (MPHP) concentration is insignificant with a clearance rate of less than 1% and is therefore disregarded in the analysis. This enables an analysis focused on the three oxidized DPHP metabolites as it was published for other phthalates [4,20].

The published analytical methods for phthalate analytics utilized HPLC–MS/MS detection with column switching techniques [21] and quantification of oxidative DIDP metabolites in urine is based on peak summation of DPHP and DIDP because of the complex mixture of isomers [4]. Silva et al. [17] reported urinary

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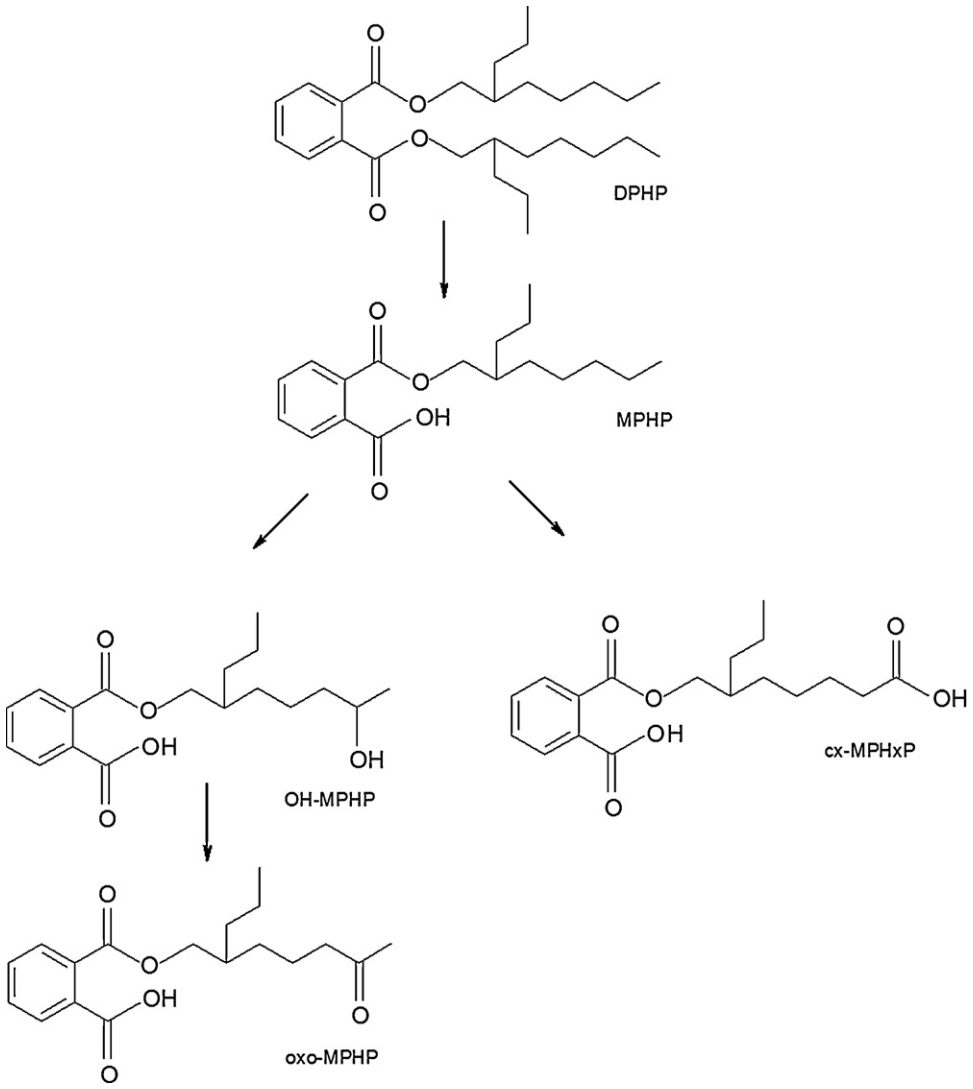


**Fig. 1.** Proposed metabolic pathway of DPHP.

concentrations of DIDP/DPHP metabolites in a group of 129 US adults (median: cx-MIDP 4.4 μg/L, OH-MIDP 4.9 μg/L and oxo-MIDP 1.2 μg/L). Koch et al. [4] showed data of 45 urines out of German population (median: cx-MIDP 0.7 μg/L, OH-MIDP 1.0 μg/L and oxo-MIDP 0.3 μg/L). Data distinguishing between DPHP and DIDP metabolites in a single sample has not been reported, because the analytical separation of these compounds is rather difficult.

The aim of the analytical method described here is a specific determination of DPHP metabolites in human urine at the background level. Possible analytical separation problems are discussed by comparison of different analytical procedures.

## 2. Experimental

### 2.1. Chemicals

Acetonitrile (supra solv), glacidic acid (p.a.), sodium hydrogencarbonate (p.a.) and hydrochloric acid 37% (p.a.) were purchased from Merck, Darmstadt, Germany.

N,N-Diisopropylcarbodiimide, 1,1,1,3,3,3-hexafluoro isopropanol, iso-octane (for residue analysis), tert-butyl methylether (for residue analysis) and ammonium acetate (p.a.) were purchased from Fluka, Taufkirchen, Germany.

The used water was obtained from a millipore water cleaning system, whereas the β-glucuronidase from *Escherichia coli* K12 was purchased from Roche, Mannheim, Germany.

The following standards were synthesized at the Institut für Dünnschichttechnologie e.V. (IDM), Teltow, Germany: mono-2-(propyl-6-hydroxy-heptyl)-phthalate, mono-2-(propyl-6-oxo-heptyl)-phthalate, mono-2-(propyl-6-carboxy-hexyl)-phthalate, mono-2-(propyl-6-hydroxy-heptyl)-phthalate-d4 ring deuterated, mono-2-(propyl-6-oxo-heptyl)-phthalate-d4 ring deuterated and mono-2-(propyl-6-carboxy-hexyl)-phthalate-d4 ring deuterated. The purity of all compounds was determined by $^{1}$H NMR and was found as ≥95%.

### 2.2. Standard preparation

For the preparation of the stock solutions needed for the standards approximately 10 mg of OH-MPHP, oxo-MPHP and cx-MPHxP were weighed separately into 10 mL glass volumetric flasks and diluted to volume with acetonitrile (1000 mg/L). From these three stock solutions, a multi-component starting solution was prepared by diluting 100 μL of each in a 10 mL glass volumetric flask filled to the mark with acetonitrile. This starting solution (10 mg/L) was gradually diluted for the preparation of the working standards to

**Table 1**
HPLC gradient program with eluent A (methanol), eluent B (water) and eluent C (1% formic acid in water).

| Time (min) | Eluent A (%) | Eluent B (%) | Eluent C (%) | Flow rate (mL/min) |
|---|---|---|---|---|
| 0 | 10 | 80 | 10 | 0.2 |
| 15 | 90 | 0 | 10 | 0.2 |
| 17 | 90 | 0 | 10 | 0.2 |
| 18 | 10 | 80 | 10 | 0.2 |
| 25 | 10 | 80 | 10 | 0.2 |

achieve standard concentrations of 1 mg/L, 0.1 mg/L, 0.01 mg/L and 0.001 mg/L.

The preparation of the internal standard stock solutions was done by dilution of approximately 10 mg of OH-MPHP-d4, oxo-MPHP-d4 and cx-MPHxP-d4 separate in 10 mL volumetric flasks with acetonitrile (1000 mg/L). Starting solution A was prepared by diluting 100 μL of each of the three stock solutions into a 10 mL volumetric flask (10 mg/L) to the mark with acetonitrile. For the preparation of solution B 1 mL of solution A was diluted in a 10 mL volumetric flask to its nominal volume with acetonitrile (1 mg/L).

Calibration standards were prepared with spiked water samples for HPLC–MS/MS and unexposed urine for GC–HRMS.

### 2.3. Sample collection and preparation

Human urine samples were collected in 100 mL polyethylene bottles and stored at −20 °C prior analysis.

Initially, the samples were thawed and equilibrated to room temperature. One milliliter of the homogenized urine was transferred into a 12 mL screw cap glass test tube. After addition of 10 μL internal standard solution B (10 μg/L), 2 mL 1 M ammonium acetate buffer (pH 6.5) and 10 μL β-glucuronidase, the sample was incubated at 37 °C over night.

The cold sample (room temperature) was acidified at pH 2 with 200 μL hydrochloric acid (37%). Four milliliters of tert-butyl-methyl-ether were added to the sample and the test tube sealed with a screw cap. Subsequently, the sample was mixed vigorously for 10 min on a shaker, followed by centrifugation for 10 min at 10 °C and 2200 × *g*. The supernatant was transferred to a new glass test tube with a Pasteur pipette and the lower urine phase discarded. A nitrogen evaporator was used for sample drying in a 35 °C tempered water bath with a nitrogen stream of 5 psi for 8 min.

For LC–MS/MS determination the sample residue was dissolved in 200 μL methanol and transferred into a micro vial which was sealed with a crimp cap.

In case of GC–MS detection the sample residue was dissolved in 150 μL acetonitrile. For derivatization 20 μL 1,1,1,3,3,3-hexafluoroisopropanol and 10 μL N,N-diisopropylcarbodiimide were added and the solution was slightly mixed for 15 min at room temperature. Subsequently 1 mL 1 M sodium hydrogen carbonate solution and 500 μL iso-octane were added. The test tube was sealed with a screw cap and the sample was mixed vigorously for 10 min followed by centrifugation at 10 °C and 2200 × *g*. Finally 200 μL of the supernatant was transferred into a micro vial.

### 2.4. Calibration procedure and quantification

Calibration was carried out by spiking 1 mL water (HPLC–MS/MS) or 1 mL urine samples (GC–HRMS) at twelve calibration points with concentrations ranging from 0.05 μg/L to 100 μg/L. All calibration samples were analyzed as described in Section 2.3. Linear calibration curves were obtained by plotting the quotient of the peak area of the target analytes and the corresponding deuterated internal standards against the standard concentrations.

### 2.5. Quality control and validation

As there is no control material available, it was prepared in the laboratory with spiked urine samples at different concentration ranges (1 μg/L, 10 μg/L and 100 μg/L of each metabolite). 1 mL aliquots of these control samples were stored in a refrigerator at −20 °C. Two samples of each concentration were analyzed during the analysis sequences on five different days to determine between day precision data. The within-day precision was obtained by analyzing pooled urine samples in three concentrations as described above. These samples were analyzed ten times in a row and all samples were quantified against the calculated calibration curve. Moreover, the influence of the urine matrix was tested by using 10 individual urines with creatinine levels between 0.52 and 3.74 g/L spiked with 10 μg/L of each metabolite.

### 2.6. Gas chromatography high resolution mass spectrometry

Analysis was done with HP 5890 II gas chromatograph (Agilent, Waldbronn, Germany) equipped with a split/split less injector (total flow 40 mL/min, septum purge 3 mL/min, split less time 1 min), a CTC A 200 S auto sampler (CTC Analytics, Zwingen, Switzerland) and a AutoSpec Ultima high resolution mass spectrometer (Micromass/Waters, Manchester, UK). Ionization was done in negative chemical ionization mode (transferline temperature 250 °C, ion source temperature 230 °C, accelerating voltage 8000 V, multiplier voltage 350 V, electron energy 100 eV, filament 0.5 mA and resolution 10000) with perfluorokerosene (PFK) as calibration gas and ammonia as reactant gas. Helium (99.999%) was used as carrier gas and the sample separation was performed on a 30 m × 0.25 mm × 0.25 μm Rxi 17 (alternative 30 m × 0.25 mm × 0.25 μm Rtx 65) fused silica column (Restek, Bad Homburg, Germany).

**Table 2**
Substance specific parameters for the DPHP metabolites measured by HPLC–MS/MS determination.

| Analyte | Retention time (min) | Parent ion (*m*/*z*) | Daughter ion (*m*/*z*) | Dwell time (s) |
|---|---|---|---|---|
| cx-MPHxP | 19.83 | 335.16 | 187.04 | 0.1 |
| cx-MPHxP-d4 | 19.81 | 339.12 | 187.04 | 0.1 |
| OH-MPHP | 20.15 | 321.13 | 121.02 | 0.1 |
| OH-MPHP-d4 | 20.13 | 325.16 | 125.04 | 0.1 |
| oxo-MPHP | 19.65 | 319.12 | 121.02 | 0.1 |
| oxo-MPHP-d4 | 19.60 | 323.15 | 124.98 | 0.1 |

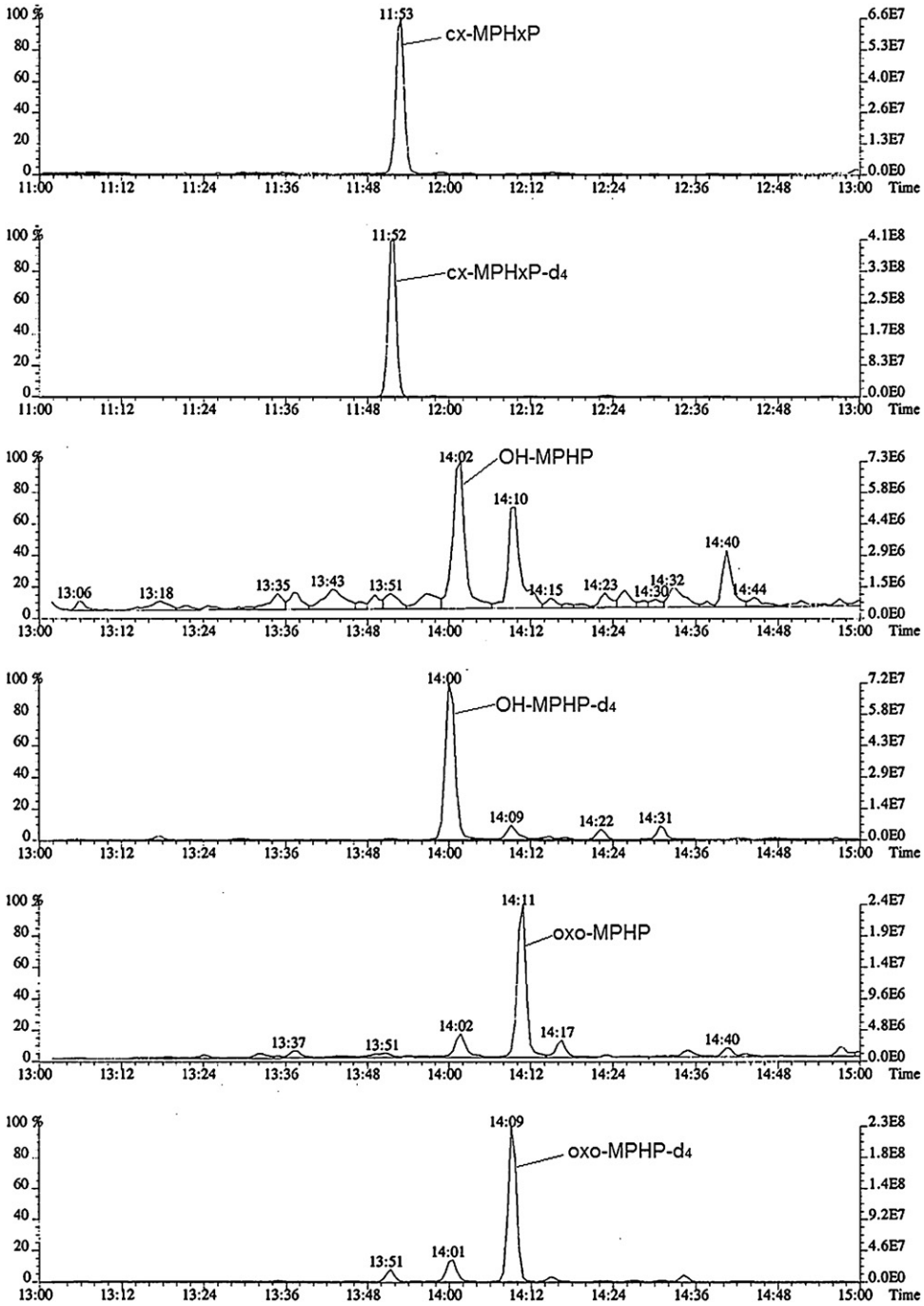


**Fig. 2.** GC–HRMS chromatogram of a pool urine spiked with 1 μg/L of each metabolite after derivatization with HFIP.

One microliter was injected split less (1 min) in a 280 °C heated split/split less injector equipped with a 4 mm double gooseneck liner (Restek, Bad Homburg, Germany), a gold plated injector inlet seal (Restek, Bad Homburg, Germany) and helium pressure was set to linear gas velocity of 30 cm/s. The oven temperature program was ramped from 90 °C (hold time 1 min) to 250 °C (12 °C/min) and finally ramped with 30 °C/min to 280 °C (hold time 5 min). This method was used for routine analysis of human urines at the background level.

For a specific determination of DPHP metabolites in a complex mixture of metabolites of DINP and DIDP isomers, a special chromatographic separation on a 60 m × 0.25 mm × 0.25 μm Rxi 17 fused silica column was applied. The oven temperature program started at 90 °C for 1 min then ramped with 15 °C/min to 150 °C (hold time 3 min) then ramped with 5 °C/min to 220 °C (hold time 5 min) and finally ramped with 5 °C/min up to 250 °C (hold time 5 min). A special pressure program was used for helium as carrier gas. The program started with 180 kPa for 1 min and was then ramped with 2 kPa/min up to 230 kPa till the end of the chromatographic program.

Instrument processing and data handling was done by use of the instrument's software Opus V3.6X.

### *2.7. High pressure liquid chromatography tandem mass spectrometry*

LC–MS/MS analysis was done on a Waters HPLC system Alliance 2695 coupled to a Waters Quattro Ultima tandem MS. The HPLC was

equipped with a quaternary pump, an auto sampler and a degasser. Separation of the target metabolites was performed on an Agilent Zorbax Eclipse Plus C18 2.1 mm × 150 mm × 3.5 μm by injection of 10 μL at 30 °C and using of a ternary gradient system consisting of eluent A (methanol), eluent B (water) and eluent C (1% formic acid in water) at a flow rate of 0.2 mL/min (Table 1).

Mass fragmentation (Table 2) and mass detection were done in electrospray ionization negative mode (ESI) with argon as collision gas by using the multiple reaction monitoring mode (MRM). The source conditions were: capillary 3.5 kV, cone 120 V, Hex (1) 0.8 V, aperture 0 V, Hex (2) 1 V, source temperature 150 °C, desolvation temperature 300 °C, cone gas flow 75 L/h and desolvation gas flow 650 L/h while nitrogen was used as desolvation gas. The typical condition for the analyser were LM (1) resolution 15, HM (1) resolution 15, ion energy (1) 2, entrance −2, collision 16, exit 2, LM (2) resolution 15, HM (2) resolution 15, ion energy (2) 1 and multiplier 650. Instrument processing and data handling were performed by using the instrument's software Mass Lynx 4.1.

### 2.8. Biological monitoring

To demonstrate the applicability of the method for quantifying values in the environmental range, spot urine samples (May 2011, creatinine 0.18–3.74 μg/L) of 40 persons of our department not knowingly exposed to DPHP were investigated. Furthermore 12 urine samples of persons who were exposed against DINP and DIDP were analyzed to get information about the selectivity of the analytical methods [22].

## 3. Results and discussion

### 3.1. Mass spectrometry

In GC–HRMS major mass fragments of the three DPHP metabolites were built by elimination of the hexafluoroisopropyl-moiety (*m*/*z* 150.9982) introduced in the derivatization process. Quantification was done by single ion resolution on mass fragment *m*/*z* 485.1399 for cx-MPHxP (11:53 min), *m*/*z* 319.1545 for oxo-MPHP (14:11 min) and *m*/*z* 321.1762 or *m*/*z* 319.1545 (Qualifier) for OH-MPHP (14:02 min) respectively. Based on a similar fragmentation pattern of the deuterated internal standards fragment ion *m*/*z* 489.1650 was used for cx-MPHxP-d4 (11:52 min), *m*/*z* 325.1953 for OH-MPHP-d4 (14:00 min) and *m*/*z* 323.1797 for oxo-MPHP-d4 (14:09 min) respectively.

In HPLC–MS/MS the daughter ion of OH-MPHP and oxo-MPHP were obtained at *m*/*z* 121 based on the benzoic acid fragment (base peak), whereas lower intensities were found for the hydroxyl alkyl chain and oxo-alkyl chain at *m*/*z* 173 and *m*/*z* 171 respectively. In contrast to this cx-MPHxP showed a base peak built of the carboxy-alkyl chain at *m*/*z* 187, whereas the benzoic acid moiety at *m*/*z* 121 plays a minor role. As a consequence to this the fragmentation of the deuterated internal standards proceeds similarly to the original compounds (cx-MPHxP-d4: *m*/*z* 187, OH-MPHP: *m*/*z* 125 and oxo-MPHP: *m*/*z* 125).

### 3.2. Detection limit and quantification limit

All detection limits were calculated according the calibration curve method by use of the six lowest calibration points. The established LOD in HR-GCMS were 0.05 μg/L for cx-MPHxP, 0.1 μg/L for OH-MPHP and 0.08 μg/L for oxo-MPHP, whereas the corresponding LOQ were calculated as 0.15 μg/L for cx-MPHXP, 0.3 μg/L for OH-MPHP and 0.25 μg/L for oxo-MPHP, respectively.

For the determination with HPLC–MS/MS LOD of 0.1 μg/L for cx-MPHxP and 0.2 μg/L for OH-MPHP and oxo-MPHP were calculated.

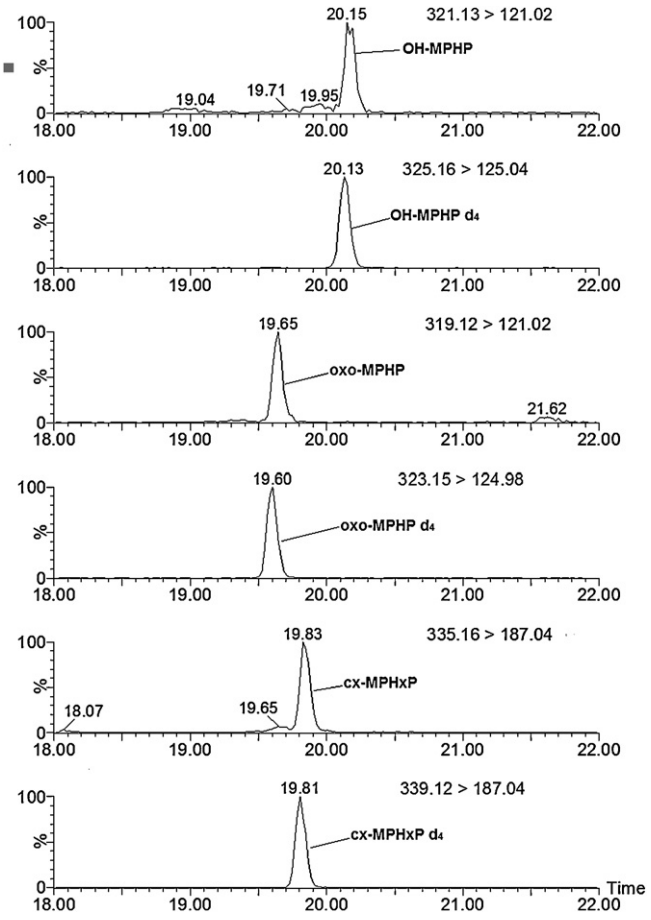


**Fig. 3.** HPLC–MS/MS chromatogram of a pool urine spiked with 1 μg/L DPHP-metabolites each.

The corresponding LOQ were 0.3 μg/L, 0.5 μg/L and 0.3 μg/L for cx-MPHxP, OH-MPHP and oxo-MPHP respectively.

### 3.3. Reliability of the method

Both analytical methods showed well resolved peaks without any interference (Figs. 2 and 3). All tested urine samples used for the method development showed no significant background exposure of DPHP and DIDP metabolites.

Based on the stereochemistry of OH-MPHP two chiral centers with R- or S-configuration are possible. By application of the presented method a chromatographic separation of these stereoisomers is not possible to achieve and the determined OH-MPHP represents the sum of them. As a side note in case the OH-group would be derivatized additionally by silylation, a separation of the stereoisomers could be obtained (data not shown).

The reaction with 1,1,1,3,3,3-hexafluoroisopropanol by implementation of the CH-$(CF_3)_2$ group is very specific for esterification of carboxyl groups and works only quantitatively in water free samples [23]. Therefore it is important to transfer the tert-butyl-methyl-ether phase without any water residues. Peak separation by application of medium polar fused silica columns with Rxi-17 or Rxi-65 phases yields the best peak resolution without implementation of a second derivatization step for the hydroxyl group of OH-MPHP. The separation power of Rxi 17 and Rxi 65 column phases is similar, but Rtx 17 has an advantage due to its higher stability. In contrast to this, an also tested fused silica column coated with unpolar DB 5 phase showed incomplete peak resolution between OH-MPHP and oxo-MPHP, which implies mass interference.

**Table 3**
LC–MS/MS, quality control data for precision within-day and between-day ($n = 10$).

| Analyte | Within-day precision | | | | Between-day precision | | | |
|---|---|---|---|---|---|---|---|---|
| | Conc. (μg/L) | Recov. (%) | R.S.D. (%) | Progn. range (%) | Conc. (μg/L) | Recov. (%) | R.S.D. (%) | Progn. range (%) |
| OH-MPHP | 1 | 106 | 5.9 | 13.0 | 1 | 105 | 8.0 | 17.8 |
| | 10 | 104 | 4.3 | 9.6 | 10 | 104 | 5.4 | 12.0 |
| | 100 | 103 | 5.5 | 12.3 | 100 | 102 | 3.8 | 8.5 |
| oxo-MPHP | 1 | 100 | 7.9 | 17.5 | 1 | 112 | 3.4 | 7.8 |
| | 10 | 100 | 2.1 | 4.8 | 10 | 99 | 4.5 | 9.9 |
| | 100 | 99 | 4.2 | 9.3 | 100 | 99 | 4.9 | 10.8 |
| cx-MPHxP | 1 | 100 | 5.1 | 11.3 | 1 | 104 | 6.2 | 13.9 |
| | 10 | 96 | 3.7 | 8.1 | 10 | 97 | 7.0 | 15.7 |
| | 100 | 97 | 3.2 | 7.1 | 100 | 102 | 6.0 | 13.3 |

**Table 4**
GC–HRMS, quality control data for precision within-day and between-day ($n = 10$).

| Analyte | Within-day precision | | | | Between-day precision | | | |
|---|---|---|---|---|---|---|---|---|
| | Conc. (μg/L) | Recov. (%) | R.S.D. (%) | Progn. range (%) | Conc. (μg/L) | Recov. (%) | R.S.D. (%) | Progn. range (%) |
| OH-MPHP | 1 | 88 | 3.8 | 8.5 | 1 | 98 | 7.4 | 16.4 |
| | 10 | 90 | 2.4 | 5.3 | 10 | 101 | 4.8 | 10.7 |
| | 100 | 108 | 2.9 | 6.4 | 100 | 106 | 1.8 | 4.1 |
| oxo-MPHP | 1 | 100 | 1.7 | 3.9 | 1 | 104 | 2.6 | 5.7 |
| | 10 | 100 | 1.2 | 2.7 | 10 | 104 | 1.3 | 2.8 |
| | 100 | 106 | 2.0 | 4.4 | 100 | 102 | 1.4 | 3.0 |
| cx-MPHxP | 1 | 102 | 1.0 | 2.3 | 1 | 100 | 2.2 | 4.9 |
| | 10 | 102 | 0.8 | 1.8 | 10 | 103 | 4.5 | 10.0 |
| | 100 | 105 | 2.5 | 5.5 | 100 | 100 | 5.2 | 11.7 |

**Table 5**
Background concentration of DPHP metabolites in 40 individual human spot urines.

| | GC–HRMS | | | HPLC-MS/MS | | |
|---|---|---|---|---|---|---|
| | cx-MPHxP | OH-MPHP | oxo-MPHP | cx-MPHxP | OH-MPHP | oxo-MPHP |
| Median (μg/L) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Mean (μg/L | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Range (μg/L) | <LOQ | <LOQ–0.51 | <LOQ–0.93 | <LOQ | <LOQ–1.25 | <LOQ–1.22 |
| Detection rate (%) | 0 | 8 | 38 | 0 | 25 | 10 |

For both instruments the coefficients of determination of the calibration curves were higher than $r^2 = 0.995$. Each instrument used showed specific linearity ranges: HRGC–MS 0.05–20 μg/L for cx-MPHxP and 0.05–50 μg/L for OH-MPHP and oxo-MPHP. HPLC-MS/MS also yielded a linear range between 0.1 and 200 μg/L for all analytes, but due to its high dynamic range a calibration range up to 2000 μg/L can be obtained if a quadratic curve fit is used.

All calibration curves were tested in urine and water. No significant influences of matrix could be observed. Hence, we prefer calibration in matrix because this is analogical with the samples (e.g. suppress of quenching or adsorption effects).

As described in Section 2.5, several quality control samples were analyzed to determine the reliability of the analytical methods. The relative standard deviation of LC–MS/MS and GC–HRMS were found in a range between 0.8 and 7.9% for the within day precision and 1.3–8.0% for the between day precision respectively (Tables 3 and 4). Furthermore, recovery experiments in different urines (GC–HRMS: RSD 3.8–4.0%, recoveries 100–103%; HPLC–MS/MS: RSD 3.7–5.6, recoveries 89–110%) demonstrate that no influence of different creatinine concentration could be observed.

An optionally accomplished internal laboratory validation procedure of the GC-method confirmed the successful application of the presented method. In this experiment a GC–MS/MS was applied and the received within day precision and LOQ were in the same range as reported for GC–HRMS (data not shown).

Nevertheless, the analytical determination of 12 urine samples received out of a human biomonitoring study [22] indicates that a specific determination of DPHP metabolites in presence of DINP and DIDP metabolites is not specific if LC–MS/MS is used (Fig. 4). This is due to the poor peak resolution of the complex mixture of these phthalates and yields giving the sum of the integrated peaks as it were published [4].

In comparison to LC–MS/MS the analytical determination with GC–HRMS allows to distinguish between DIDP and DPHP

**Table 6**
Results of DIDP/DPHP metabolite analysis in 12 individual human urines after plastisol exposure.

| | GC–HRMS | | | HPLC–MS/MS | | |
|---|---|---|---|---|---|---|
| | cx-MPHxP | OH-MPHP | oxo-MPHP | Sum of cx | Sum of OH | Sum of oxo |
| Median (μg/L) | <LOQ | <LOQ | 0.26 | 15.7 | 44.9 | 6.1 |
| Mean (μg/L) | <LOQ | <LOQ | 0.31 | 28.3 | 127.7 | 16.9 |
| Range (μg/L) | <LOQ | <LOQ | <LOQ–0.72 | 2.1–99.7 | 7.7–337 | 1.1–49.2 |
| Detection rate (%) | 0 | 0 | 58 | 100 | 100 | 100 |

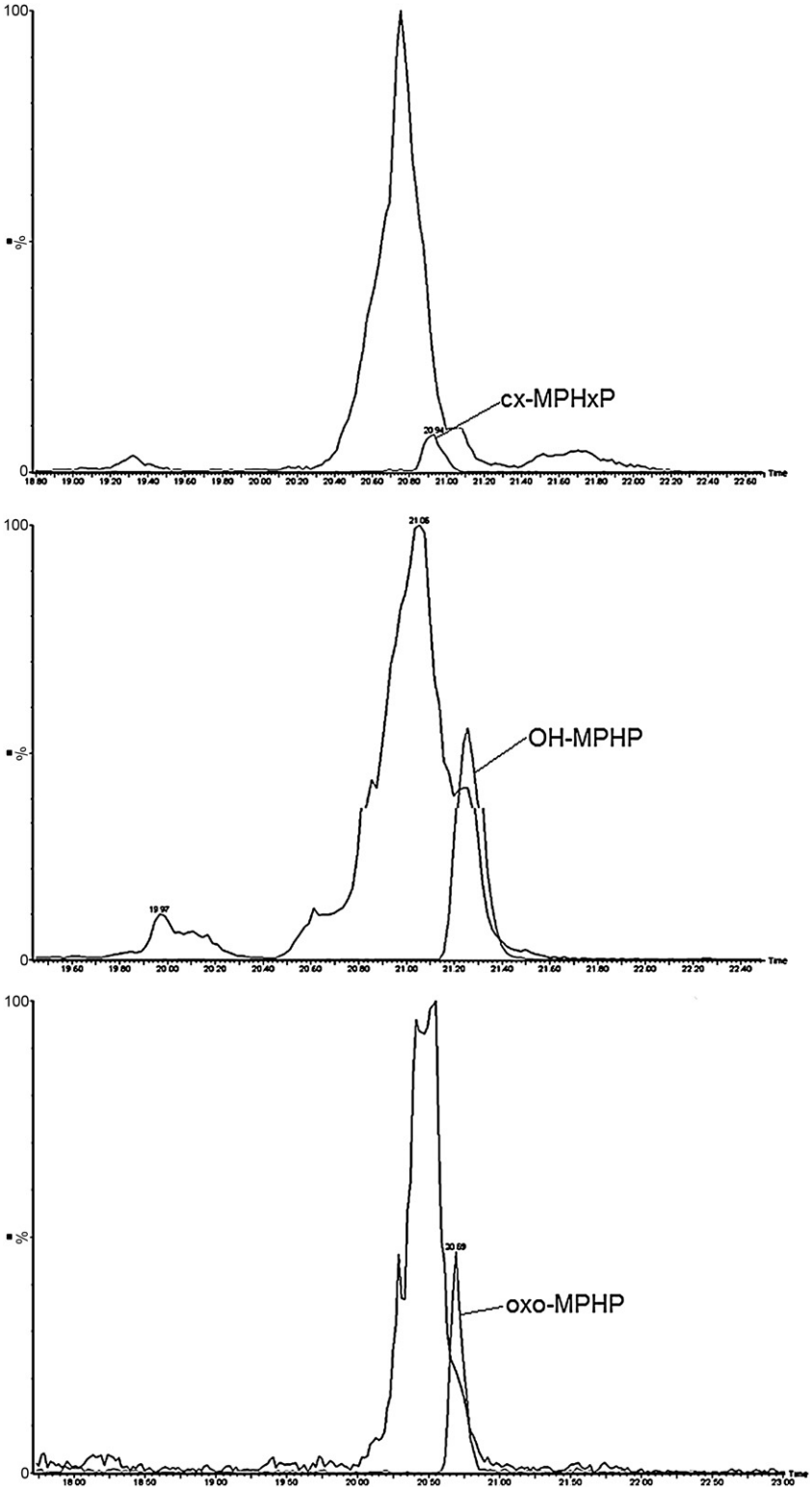


**Fig. 4.** HPLC–MS/MS chromatogram of a human urine with DINP/DIDP metabolites overlaid with a 1 μg/L standard of DPHP metabolites.

metabolites due to higher separation power. The complex isomer mixture of DIDP is distributed and resolved over a period of more than a minute for each metabolite, whereas DPHP metabolites shows only single narrow peaks. This enables a separation of DPHP metabolites in presence of DINP/DIDP, which is useful for a selective determination.

Based on the peak separation of DIDP in presence of high DINP and DIDP background levels a special gas chromatography program using a 60 m Rxi 17 column could be applied optionally (Section 2.6). The described analytical condition represents the best obtained peak resolution. On the one hand, OH-MPHP and oxo-MPHP must be separated, because they generate same mass

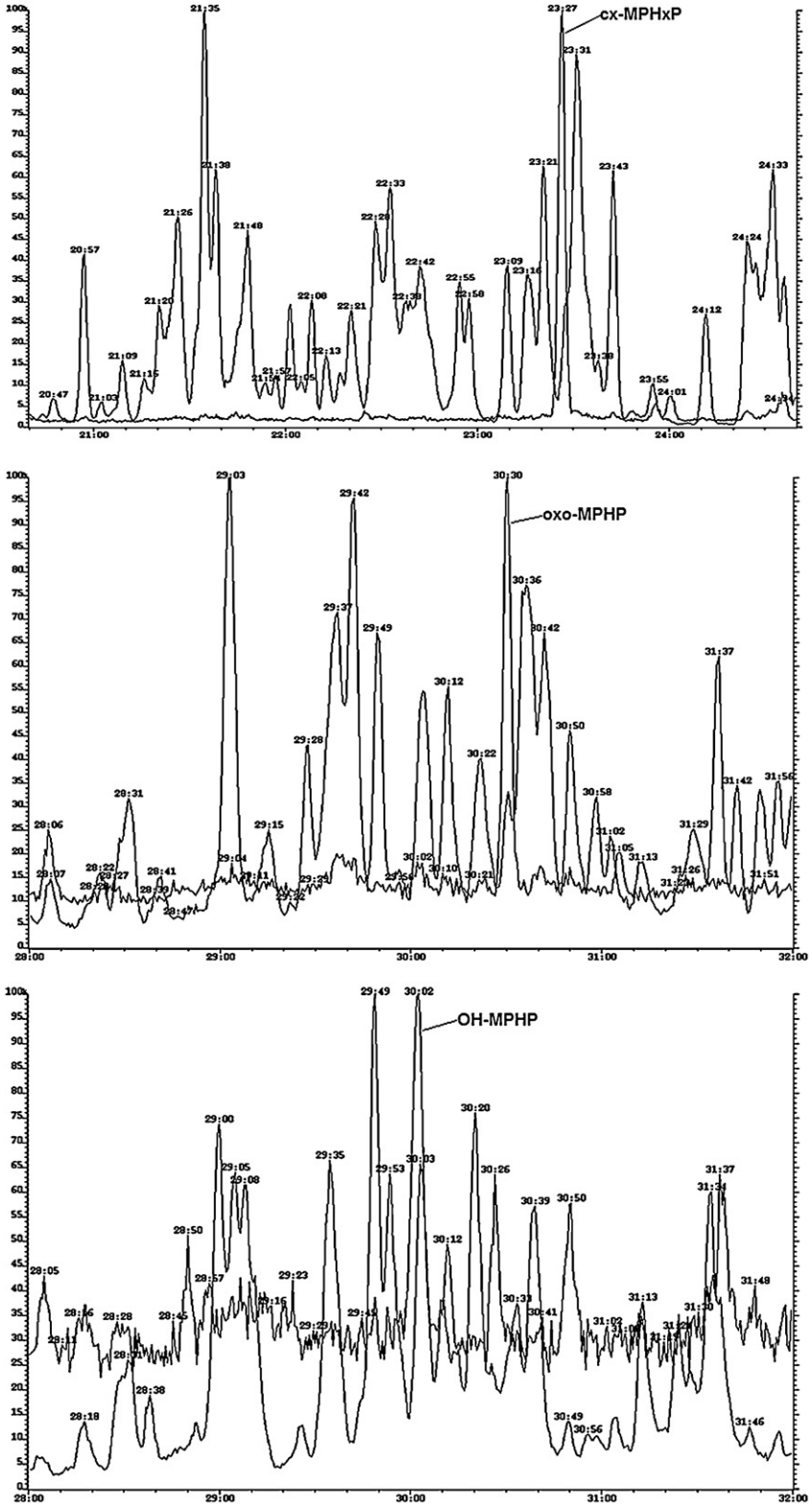


**Fig. 5.** GC–HRMS chromatogram of a human urine with DINP/DIDP metabolites overlaid with a 1 μg/L standard of DPHP metabolites.

fragments, and on the other hand both components must be separated from all of the various isomers. This results in well separated cx-MPHxP and oxo-MPHP peaks, whereas only a slight separation could be achieved for OH-MPHP (Fig. 5). With the help of this peak separation it could be shown that DPHP played only a minor rule in these phthalate mixtures. Due to missing commercially available standards for each possible isomer it has to be noticed that it could not be clarified with absolute certainly, if the detected small amounts of isomers belongs to DPHP or DINP/DIDP. Based on the isomeric composition published in the EU risk assessment

report on DIDP and on the production process it is unlikely that the isodecanol in DIDP contains 2-propylheptanol or 2-propyl-4/5-methylhexanol.

### 3.4. Results of biological monitoring

Data from 40 urine samples showed that there was only a small background pattern of the DPHP metabolites. A maximum level of 0.93 μg/L was found for oxo-MPHP and 0.51 μg/L for OH-MPHP respectively, whereas the amount of cx-DPHP was in all cases below the LOQ (Table 5). Moreover, the amount of the detected "DPHP metabolites" in 12 urines of persons who were exposed against DINP/DIDP where in the background ranges too (Table 6).

## 4. Conclusions

The three major metabolites of DPHP OH-MPHP, oxo-MPHP and cx-MPHxP were selected as biomarker of body burden on the basis of human in vitro-studies [19]. Aim of the described report was the development of an analytical method that enables a selective and sensitive determination of the metabolites in urine at the environmental range.

In comparison to other published analytical methods, which used HPLC–MS/MS with column switching technique, alternative method variations are presented. The derivatization with 1,1,1,3,3,3-hexafluoroisopropanol after extraction of the liberated DPHP-metabolites enables the selective and sensitive detection by implementation of GC–MS in the negative chemical ionization mode. It could be demonstrated that the resolution power of capillary columns with a medium polar film in combination with sensitive detection technique enables a separation of DPHP metabolites in presence of metabolites of DINP/DIDP isomers.

Liquid/liquid extraction with t-BME is an alternative against online column separation for HPLC–MS/MS. As a result of the lower peak resolution power of HPLC–MS/MS it could be shown that these method techniques can be used for prescreening of urine samples. In case of special DPHP analysis or validation, a subsequently determination with GC–MS should be used.

For all metabolites detection limits at the background level could be reached (GC–HRMS: LOD's from 0.05 to 0.1 μg/L, HPLC–MS/MS: 0.1 to 0.2 μg/L).

It could be demonstrated by various validation experiments, that the presented method is robust, specific and sensitive for the analytical detection of DPHP metabolites at the background level. For the first time a method is presented that allows distinguishing between DPHP and DIDP metabolites. This enables a selective determination of DPHP metabolites in environmental studies to get an overview about possible human exposures to DPHP.

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