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Biological monitoring of the five major metabolites of di-(2-ethylhexyl)phthalate (DEHP) in human urine using column-switching liquid chromatography–tandem mass spectrometry

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Abstract

We present a fast and reliable on-line clean-up HPLC-method for the simultaneous determination of the five major urinary metabolites of di-(2-ethylhexyl)phthalate (DEHP) namely mono-(2-ethyl-5-carboxypentyl)phthalate (5carboxy-MEPP), mono-[2-(carboxymethyl)hexyl]phthalate (2carboxy-MMHP), mono-(2-ethyl-5-hydroxyhexyl)phthalate (5OH-MEHP), mono-(2-ethyl-5-oxohexyl)phthalate (5oxo-MEHP) and mono-(2-ethylhexyl)phthalate (MEHP). These metabolites represent about 70% of an oral DEHP dose. We for the first time succeeded to reliably quantify 5carboxy-MEPP and to identify 2carboxy-MMHP as major metabolites in native urines of the general population. The analytical procedure consists of an enzymatic hydrolysis, on-line extraction of the analytes from urinary matrix by a restricted access material column (RAM), back-flush transfer onto the analytical column (betasil phenylhexyl), detection by ESI–tandem mass spectrometry and quantification by isotope dilution (limit of detection (LOD) 0.25 μg/l). Median concentrations of a small collective taken from the general population ($n = 19$) were 85.5 μg/l (5carboxy-MEPP), 47.5 μg/l (5OH-MEHP), 39.7 μg/l (5oxo-MEHP), 9.8 μg/l (MEHP) and about 37 μg/l (2carboxy-MMHP). The presented method can provide insights into the actual internal burden of the general population and certain risk groups. It will help to further explore the human metabolism of DEHP—an occupational and environmental toxicant of great concern.

Keywords: DEHP; Phthalates; Urine; Plasticizer; Metabolites; Biological monitoring

1. Introduction

Today's life is inconceivable without plastics. Many of its versatile properties are due to the integration of plasticizers giving the synthetic material its desired flexibility. Phthalates, the di-alkyl or alkyl-aryl esters of phthalic acid, account for 93% in the plasticizers segment. Di-(2-ethylhexyl)phthalate (DEHP) is one of the most widespread phthalate plasticizer. It is extensively used in flexible polyvinylchloride (PVC) products such as wire and cable insulations, wallpapers, vinyl upholstery, car seats, footwear, raincoats, packagings, children's toys and all sorts of medical devices (tubings, blood storage bags, etc.) [1–3]. At least 244,000 t of DEHP have been produced in Europe in 2003 [4]. Sooner or later, the plasticizer will be released into the environment because it is not covalently bound to the plastic materials.

DEHP is a known reproductive and developmental toxicant in rodents [3,5]. Observed effects are reduced testosterone production [6,7], reduced anogenital distance, reduced testis weight and reduced reproductive performance [8–11]. Developing males were found to be more sensitive to testicular toxicity than sexually mature animals [8,10–13]. Effects

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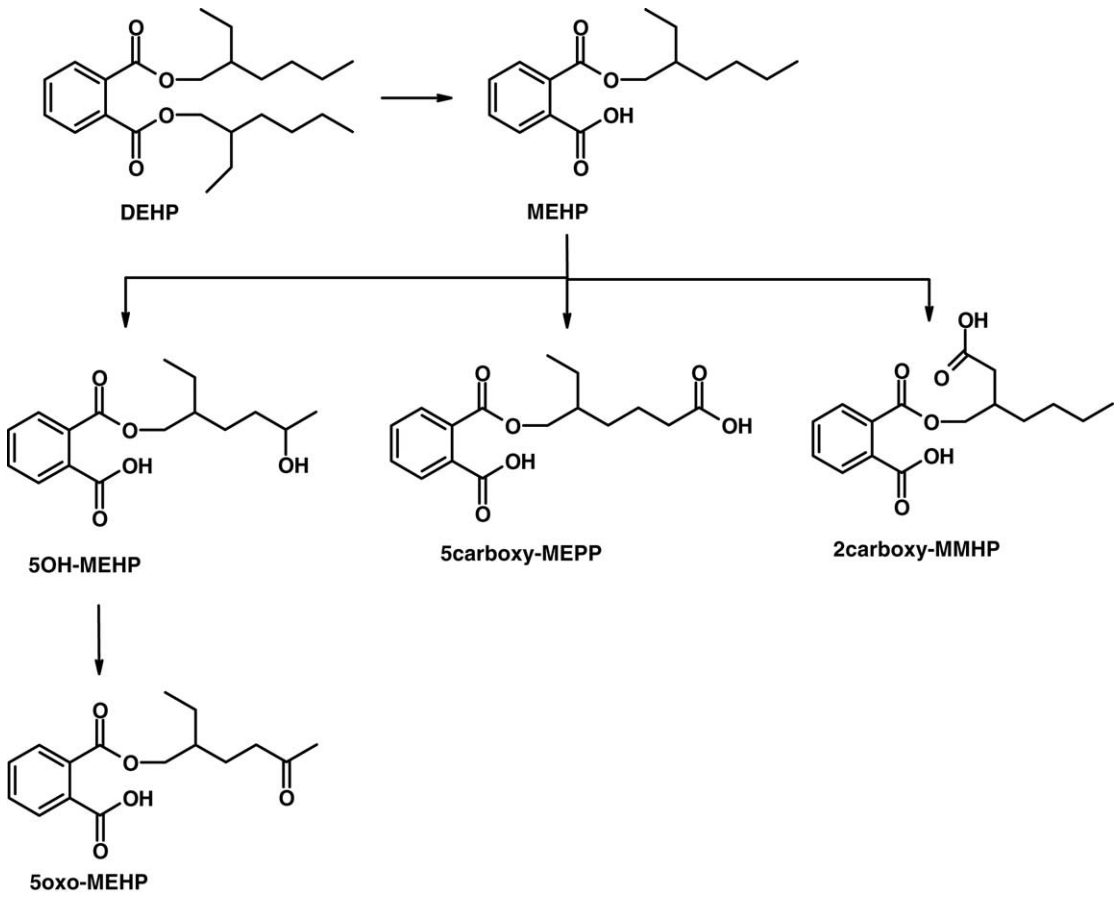

Fig. 1. Simplified metabolic pathways of DEHP covering about 70% of an incorporated DEHP dose.

on humans concerning endocrine disruption are still under controversial discussion [6,14–19].

Diet seem to be the predominant exposure source of the general population. This causes a ubiquitous background burden of the general population with DEHP [20–23]. Young children, workers in the PVC-industry or medical patients undergoing dialysis, blood transfusion, intensive care, intravenous therapy, enteral and parenteral nutrition support are expected to be more heavily exposed [21,24–27].

Today, it is widely accepted that an unambiguous assessment of the human exposure to DEHP can only be achieved by biological monitoring studies measuring specific secondary oxidized metabolites of the phthalates preferably in urine [2,22,23,28,29]. In humans, DEHP is rapidly cleaved to ethylhexanol and its respective monoester MEHP. MEHP is for the most part further metabolized to oxidized products by ω- and ω–1-oxidation leading to mono-(2-ethyl-5-carboxypenty)phthalate (5carboxy-MEPP), mono-(2-ethyl-5-hydroxyhexyl)phthalate (5OH-MEHP) and mono-(2-ethyl-5-oxohexyl)phthalate (5oxo-MEHP). ω-Oxidation of the ethyl side chain leads to mono-[2-(carboxymethyl)hexyl]phthalate (2carboxy-MMHP). These products are mainly excreted via urine [26,30–32].

Previous analytical procedures for internal DEHP exposure have only covered MEHP and the two ω–1-oxidation products 5OH-MEHP and 5oxo-MEHP [18,20,21,28,33,34], These urinary metabolites represent about 44% of an oral dose of DEHP [33]. Now, in order to cover the majority of urinary DEHP metabolites, to implement also ω-oxidation products and to further contribute to the exploration of the human metabolism of DEHP, we enhanced this method by introducing for the first time the ω-oxidation products of DEHP: 5carboxy-MEPP and 2carboxy-MMHP. Metabolism studies indicate, that these urinary metabolites represent about 70% of an oral DEHP dose [35–37]. Fig. 1 illustrates a simplified diagram of the metabolism of DEHP with the metabolites analyzed in our method.

The presented method will provide insights into the actual exposure situation of the general population and certain risk groups and it will help to further explore the human metabolism of DEHP—an occupational and environmental toxicant of great concern.

2. Experimental

2.1. HPLC–MS/MS method

2.1.1. Chemicals

Mono-(2-ethyl-5-carboxypentyl)phthalate (5carboxy-MEPP), mono-(2-ethyl-5-hydroxyhexyl)phthalate (5OH-

Table 1
MRM-parameters of the HPLC–MS/MS method for the new metabolite 5carboxy-MEPP and its internal standard D4-5carboxy-MEPP

Analyte	Parent ion (Q1)	Daughter ion (Q3)	Retention time (min)	DP	FP	EP	CE
5carboxy-MEPP	307	159*	14.55	−6	−340	5.5	−20
		121		−16	−260	3.5	−28
		113		−6	−350	4.0	−40
D4-5carboxy-MEPP	311	159	14.50	−6	−350	7.5	−18
5OH-MEHP	293	77	14.77	−16	−330	8.5	−40
		121*		−16	−330	8.5	−24
D4-5OH-MEHP	297	125	14.75	−21	−340	11.5	−26
5oxo-MEHP	291	77*	15.16	−11	−340	9	−40
		121		−16	−310	9	−20
D4-5oxo-MEHP	295	125	15.11	−31	−350	11.5	−26
MEHP	277	127	17.91	−21	−350	9.5	−26
		134*		−21	−350	9.5	−26
D4-MEHP	281	138	17.85	−21	−340	10	−20

Specific parent and daughter ion combinations for the remainder of the metabolites are according to [28]. The combinations used for quantification purposes are marked with an asterisk (*). DP: declustering potential [V]; FP: focussing potential [V]; EP: entrance potential [V]; CE: collision energy [V].

MEHP), mono-(2-ethyl-5-oxohexyl)phthalate (5oxo-MEHP) and their D4-ringlabelled analogues were synthesized in cooperation with the "Institut für Dünnschichttechnologie und Mikrosensorik e.V. (IDM)" [38–40]. All four compounds had a chemical purity >95%. The isotopic purity of each labelled internal standard was tested by LC–MS/MS and contained no measurable unlabelled or partially labelled (D3 or D2) compound. Mono-(2-ethylhexyl)phthalate (MEHP) and D4-mono-(2-ethylhexyl)phthalate (D4-MEHP) were synthesized in our laboratory from phthalic anhydride and from 3D, 4D, 5D, 6D-ringlabelled phthalic anhydride and the respective alcohols by esterification according to Dirven [41], respectively. The products were characterized by mass spectrometry, ^{13}C- and ^{1}H-NMR. The monoesters had a chemical purity >95%. The isotopic purity of each labelled internal standard was tested by LC–MS/MS and contained no measurable unlabelled or partially labelled (D3 or D2) compound. Acetonitrile, water, methanol (all of HPLC-grade), acetic acid (glacial, extra pure) and ammonium acetate p.a. were purchased from Merck, Darmstadt, Germany. β-Glucuronidase from *Escherichia coli* K12 was purchased from Roche Biomedical, Mannheim, Germany.

2.1.2. Apparatus

Liquid chromatography was carried out on a Hewlett-Packard HP 1100 Series HPLC apparatus (auto sampler, quaternary pump, vacuum degasser) and an additional isocratic Merck-Hitachi L6000A pump from Merck (Darmstadt, Germany).

The MS/MS detection was performed on a Sciex API 2000 LC–MS/MS system (PE Biosystems, Langen, Germany) equipped with a software controlled 10-port valve (only 6 ports were used). The source specific parameters were optimized manually for the LC conditions used during analysis depending on flow rate and eluent composition. The ion source-dependent (turbo ion spray) conditions were the same for all of the analytes with an ion spray (IS) voltage of −4500 V in the negative ionization mode. Nitrogen as nebulizer and turbo heater gas (temperature TEM: 475 °C) was set to a pressure of 35 and 70 psi (ion source gas GS1/GS2), respectively. The curtain gas (CUR) was set to 45 psi. The collision gas (N_2) for the MS/MS mode at quadrupole Q2 was set to a flow of five instrument units (CAD). Compound specific mass spectrometer parameters were optimized for each compound by the Quantitative Optimization Wizard of the Sciex Analyst software. Continuous flow injections of standard solutions for all analytes were performed to establish the MS/MS operating conditions with the syringe pump system of the API 2000. For each analyte, at least two specific parent and daughter ion combinations were monitored with one combination being used for quantification ("quantifier") and the other(s) for verification ("qualifier"). MS/MS operating conditions in the MRM-mode for all parameters were as follows: resolution Q1: unit; resolution Q3: low; settling time: 5 ms; MR pause: 5 ms; scan time: 100 ms (period duration: 22 min, cycles: 967, cycle time: 1.365 s). Analyte specific parameters are shown in Table 1.

2.1.3. HPLC columns

We used two different columns. First column was a restricted access material (RAM) phase LiChrospher RP-8 ADS, 25 μm, 25 mm × 4 mm from Merck (Darmstadt, Germany). Second column was the analytical reversed-phase HPLC column betasil phenyl-hexyl, 3 μm, 150 mm × 4.6 mm from Thermo Electron Corporation (Darmstadt, Germany). A guard column (phenylpropyl, 4 mm × 3 mm, Phenomenex, Aschaffenburg, Germany) was placed in front of the analytical column to extend its lifespan. During routine measurements, the guard column was replaced after around 100 injections.

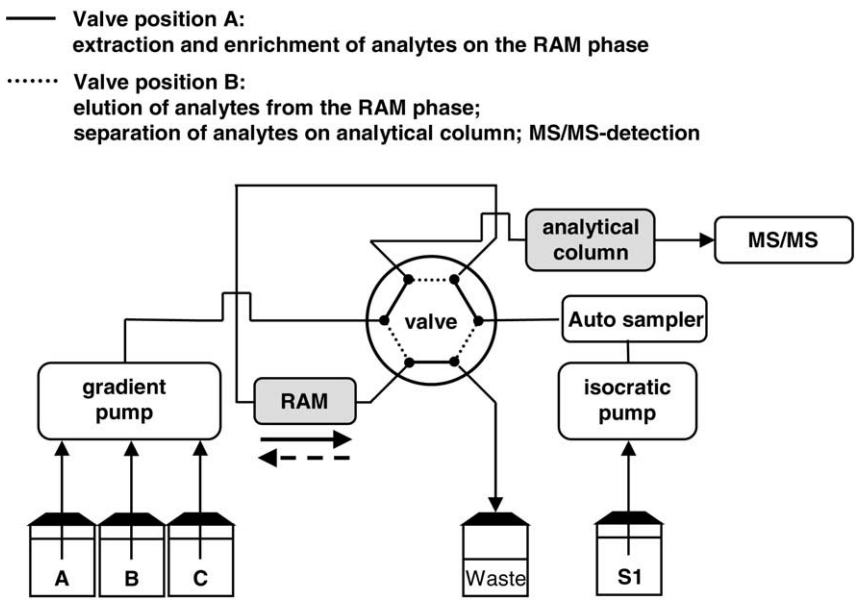

Fig. 2. Software controlled column switching HPLC system with back-flush arrangement.

2.1.4. HPLC solvents

HPLC solvent S1 consisted of a 1% aqueous solution of acetic acid and methanol (95:5 (v/v)). The isocratic pump was solely operated with this mobile phase. The gradient pump was operated with solvents A, B and C. Solvent A consisted of 1% aqueous solution of acetic acid and acetonitrile (90:10 (v/v)). Solvent B was a mixture of 1% aqueous solution of acetic acid and acetonitrile (10:90 (v/v)). Solvent C was 100% of methanol. The gradient used is described in Table 2.

2.1.5. Analytical procedure

Fig. 2 shows a schematic diagram of the whole assembly. The timetables of the analysis program of the gradient HPLC pump and the automated switching procedure are given in Tables 2 and 3. All steps were controlled by Analyst 1.1 Software from Perkin-Elmer except the isocratic pump which was operated in isocratic mode with a constant flow rate.

The primary isocratic pump was used to load the sample (500 μl aliquot) on the RAM phase using solvent S1 with a flow rate of 1.0 ml/min. After this clean-up and enrichment step, the analytes were transferred to the reversed-phase analytical column in back-flush mode by means of the time controlled switching valve on the API 2000 Sciex MS/MS. The LC pump gradient used mobile phases A and B with a constant flow rate of 1.0 ml/min. Both columns were rinsed as soon as the analytes were detected using 75% of solvent C and 25% of B for 3 min. Both columns were then re-equilibrated with the respective mobile phases in preparation for the next sample injection.

Table 2
Analysis program of the gradient pump (flow rate: 1.0 ml/min)

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0.0–11.5	50	50	0
11.6–14.0	25	75	0
14.1–17.0	18	82	0
17.1–18.0	0	100	0
18.1–21.0	0	25	75
21.1–22.0	50	50	0

The isocratic pump continuously pumps the mobile phase S1 (water, 5% vol. MeOH, 1% acetic acid) for charging the RAM phase (flow rate: 1.0 ml/min). Solvent A: 10% acetonitrile in a 1% aqueous solution of acetic acid. Solvent B: 90% acetonitrile in a 1% aqueous solution of acetic acid. Solvent C: 100% methanol.

2.1.6. Sample collection and preparation

Urine samples were collected in 250 ml polyethylene bottles and frozen immediately until analysis. In preparation for the analysis, urine samples were thawed and equilibrated to room temperature. The samples were vortex mixed and aliquots of 1 ml were then transferred to 1.8 ml glass screw-cap vials. Two hundred microliters of ammonium acetate buffer solution (1 M, pH 6.5), 50 μl of the D4-internal standard solution (1.5 mg/l) and 10 μl β-glucuronidase were added to the samples. The samples were incubated for 1.5 h at 37 °C in a drying oven. After hydrolysis, each sample was thoroughly mixed and centrifuged at 1500 × *g* for 10 min. The supernatant was transferred into another 1.8 ml glass screw-cap vial and 500 μl were then injected into the

Table 3
Timetable for the automatic column switching valve

Time (min)	Valve position	Event
0–9.9	A	RAM charging
10.0–12.0	B	Analyte transfer
12.1–18.0	A	Analyte separation
18.1–21.0	B	washing
21.1–22.0	A	Re-equilibrating

LC–MS/MS system for quantitative analysis. Urinary creatinine concentrations were determined according to Larsen [42].

2.1.7. Standard preparation and calibration procedure

The initial stock solution for the standards was prepared by dissolving 10 mg of 5carboxy-MEPP, 5OH-MEHP, 5oxo-MEHP and MEHP in 25 ml of acetonitrile (400 mg/l). This stock solution was stored at −18 °C in a Teflon-capped glass vial until further use.

During method development two calibration curves were simultaneously prepared in water and in urine to verify a possible influence of the urinary matrix on slope and linearity.

In order to prepare six calibration standards during routine operation of the method, the initial stock solution was gradually diluted with water to achieve standard concentrations in the range from 0.5 to 500 μg/l.

An aliquot of 1 ml of these standard solutions was used and processed in the same way as described in Section 2.1.6. Unspiked water, which was processed in the same way, was used as a blank. Linear calibration curves were obtained by plotting the quotients of the peak areas of the metabolites and the respective D4-internal standards as a function of the concentrations. The coefficients of correlation for all calibration curves were higher than 0.99. These graphs were used to ascertain the unknown concentration of the metabolites in urine samples. Samples with concentrations above the highest calibration standard have to be diluted to fit the calibration range.

The stock solution for the internal standards was prepared by dissolving 10 mg D4-5carboxy-MEPP, D4-5OH-MEHP, D4-5oxo-MEHP and D4-MEHP in 25 ml of acetonitrile (400 mg/l). This stock solution was stored at −18 °C in a Teflon-capped glass vial until further use. An aliquot of 375 μl was placed in a 100 ml glass volumetric flask and diluted to the mark with water (1.5 mg/l). This solution served as the working solution.

2.1.8. Quality assurance

As there was no quality control material commercially available it had to be prepared in the laboratory. For low concentration quality control material (Q_{low}), we spiked pooled urine from laboratory staff with 10 μg of each metabolite per litre after freezing and filtering the urine. For high concentration quality control material (Q_{high}), we spiked the pooled urine with 500 μg/l of each metabolite. The pools were divided into aliquots and stored at −18 °C. For precision and accuracy experiments, these low and high concentrated control samples were included in each of the following analytical series:

- Within-series imprecision was determined by preparing and analyzing Q_{low} and Q_{high} eight times in a row.
- Between-day imprecision was determined by preparing and analyzing the quality control samples Q_{low} and Q_{high} on nine different days.

Furthermore, inaccuracy which might be due to the influence of the urinary matrix was determined using eight different individual urine samples with a creatinine content ranging from 0.07 to 3.24 g/l. These urine samples were spiked with 50 μg/l of each metabolite and analyzed. The same specimens without the addition of the metabolites were analyzed and the recovery of the spiked amount of each metabolite was determined.

2.2. Identification of 2carboxy-MMHP by LC–MS/MS and GC–MS/MS

During method development, a peak of an unknown compound was observed in every native urine sample eluting 0.7 min after 5carboxy-MEPP. This peak was assumed to represent a further metabolite of DEHP—possibly an isomer of 5carboxy-MEPP. In order to prove that this unknown compound is unequivocally a metabolite of DEHP, we analysed a urine sample of a person who had incorporated D4-ring labelled DEHP in the context of a metabolism study [33]. This urine sample also showed the so far unidentified peak at the same retention time with mass fragments containing this time the D4-labelling. In order to explore the identity of this metabolite, we proceeded as follows: The respective peak in the LC–MS/MS chromatogram was isolated by collecting the eluting compound within a retention time slot of 14.9–15.5 min. This solution was brought to dryness by a gentle stream of nitrogen and re-dissolved with 0.5 ml of acetonitrile. This solution was injected into the ion source of the LC–MS detector by a syringe pump to generate mass spectra of the isolated native peak.

Furthermore, an aliquot of the collected HPLC fraction was mixed with 50 μl of diazomethane-toluene derivatization reagent to methylate a putative carboxy group. An aliquot of 1 μl of this derivatized fraction was then injected into the GC–MS/MS ion trap system. The diazomethane-toluene derivatization reagent was prepared by adding 5 ml of a 20% potassium hydroxide solution to 20 ml of toluene at 10 °C. One gram of nitroso-*N*-methylurea was slowly added since it was completely solved. Fifty microliters of the toluene phase was then used for derivatization purposes.

GC ion trap analyses were carried out on a Varian Chrompack® system consisting of a CP-3800 gas chromatograph, a Saturn 2000 GC–MS/MS ion trap detector, a CP-8200 autosampler and a Saturn system control software version 5.41. The GC column was a Hewlett-Packard HP5MS (60 m × 0.25 mm, 0.25 μm). The injector was set to a temperature of 275 °C and 1.2 ml/min was set as constant column flow rate. The column oven temperature gradient was as follows: 60 °C was initially held for 1 min, then temperature increases with 25 °C/min up to 300 °C within 9.6 min and held for 5.4 min. Acetonitrile was used as reagent gas. MS scan runs were performed in CI ionization mode, with a scan time of 0.5 s within a mass range between segment low mass of 70 *m*/*z* and segment high mass of 380 *m*/*z*. Multiplier offset was 150 V. For MS runs in multi-reaction-monitoring

(MRM) mode the parent ion masses were 157 and 167 *m/z*. Resonant wave form was used with excitation storage levels set to 48.0 *m/z* and excitation amplitude set to 0.80 and 0.85 V, respectively. Segment low mass was set to 60 *m/z* and high mass was 199 *m/z* in this mode.

2.3. Study subjects

For a pilot study, we investigated 19 spot urine samples randomly taken from a collective representing the general population in Southern Germany (including young children and adults). All of them were occupationally not exposed to phthalates. Creatinine levels were in the range from 0.57 to 2.27 g/l.

3. Results and discussion

3.1. On-line clean-up HPLC–MS/MS method

After hydrolysis and centrifugation, the aliquots of the samples were injected into the HPLC–MS/MS apparatus. Sample clean-up and separation of the analytes were performed on-line and automatically (Fig. 2). Using this procedure, we succeeded in analyzing the five major DEHP metabolites in one analytical run. Fig. 3 shows a typical chromatogram of a processed native urine sample. The analytical background noise level is very low. This is due to the application of the RAM phase leading to a very good on-line sample clean-up. The column-switching procedure includes a change in flow direction (back-flush) on the RAM phase during the transfer step which causes the analytes to refocus on the analytical column leading to very sharp analyte peaks. This procedure has been proven to be a very efficient one in the analytical treatment of body fluids [28,43–45]. The chromatographic separation had to be especially optimized to distinguish the so far unidentified structural isomer of 5carboxy-MEPP eluting 0.7 min later (Fig. 3a) since they resulted in the same daughter ion spectra. This also applies to the MEHP isomer called mono-octylphthalate (MOP) which can occur in urine samples of dioctylphthalate exposed humans. A good baseline separation could be achieved in both cases although MOP was not detected in native urine samples of the general population collective investigated in the present study.

The chosen LC conditions on the phenyl-hexyl column also showed peaks of putative isomers of 5OH- and 5oxo-MEHP (Fig. 3b and c). Those isomers (e.g. 4OH-MEHP, 6OH-MEHP, 4oxo-MEHP) are known from metabolism studies to be minor metabolites of DEHP [33–37].

Q1 ESI negative ion mass spectra with fragment structures for the new compound 5carboxy-MEPP and its deuterated internal standard D4-5carboxy-MEPP are shown in Fig. 4a and b, respectively. Loss of the 2-ethyl-5-carboxypentyl side chain leads to the main mass fragment 159 *m/z* in both cases (307 → 159 *m/z* and 311 → 159 *m/z*). Subsequent loss of carbon dioxide means a decarboxylation of the side chain (159 → 113 *m/z*). The fragments 77 (81) and 121 (125) *m/z* represent (D4-)benzene and (D4-)benzoic acid, respectively. Mass spectra and fragmentation patterns for the other compounds 5OH-MEHP, 5oxo-MEHP and MEHP have been previously shown [28].

For quantification purposes, the peak area ratio of analyte to D4-internal standard was used. All calibration curves were linear within the given concentration range (0.5–500 μg/l) and led to linear correlation coefficients greater than 0.99. This wide working range was necessary to cover the variable levels of DEHP metabolites in human urine. Urinary matrix did not influence slope and linearity of the calibration curve. Calibration with the standards spiked in water gave the same slopes as a calibration with spiked urinary standards. This means that due to a very efficient on-line clean-up, effects of the matrix were eliminated. Therefore, calibration graphs were obtained by analyzing spiked water standards.

Reagent blanks were found to contain no traces of any of the analyzed secondary phthalate metabolites. No unlabelled isotope fragments were detectable in the labelled standards even at high concentrations (10 mg/l) and after going through the analytical process. No D–H isotope exchange interfering with the analytical precision was observed. In order to deconjugate the glucuronic acid derivatives of the phthalate metabolites without contamination of the simple monoester (MEHP) in the analytical process, we applied the method introduced by Blount et al. [46] using *E. coli* β-glucuronidase K12 enzyme. Hydrolysis was found to be complete after one and a half hours.

In order to assess the within-series and between-day imprecision, we analyzed two different pooled control urine samples containing a high and a low concentration of each compound (Q_{low} and Q_{high}). Different background levels of each metabolite in this urine were determined to be 14.2 μg/l (5carboxy-MEPP), 11.7 μg/l (5OH-MEHP), 6.7 μg/l (5oxo-MEHP) and 6.4 μg/l (MEHP). All data are presented in Table 4.

Within-series imprecision and accuracy: Q_{low} revealed relative standard deviations (R.S.D.s) for the various compounds in the range 4.0–5.6% and for Q_{high} R.S.D. were 2.5–4.9% demonstrating perfect reproducibility of this method over the whole concentration range down to trace levels. Mean relative recoveries were 97.3–106.1% for Q_{low} and 87.2–104.6% for Q_{high}, respectively.

Between-day imprecision and accuracy: R.S.D. exhibited 3.5–6.5% for Q_{low} and 2.8–9.7% for Q_{high}. Mean relative recoveries were 92.3–105.8% for Q_{low} and 92.4–103.0% for Q_{high}.

Interindividual matrix dependent reliability: Eight different individual urine specimens were spiked with 50 μg/l of each of the phthalate metabolites. In that way, we can verify a possible influence of different compositions of different urine samples on the analytical result. The R.S.D.s for the compounds at the spiked concentrations were 3.0–10.1%. Relative recovery rates of the spiked amount were in the range from 91.2 to 113.3% for all compounds and all specimens.

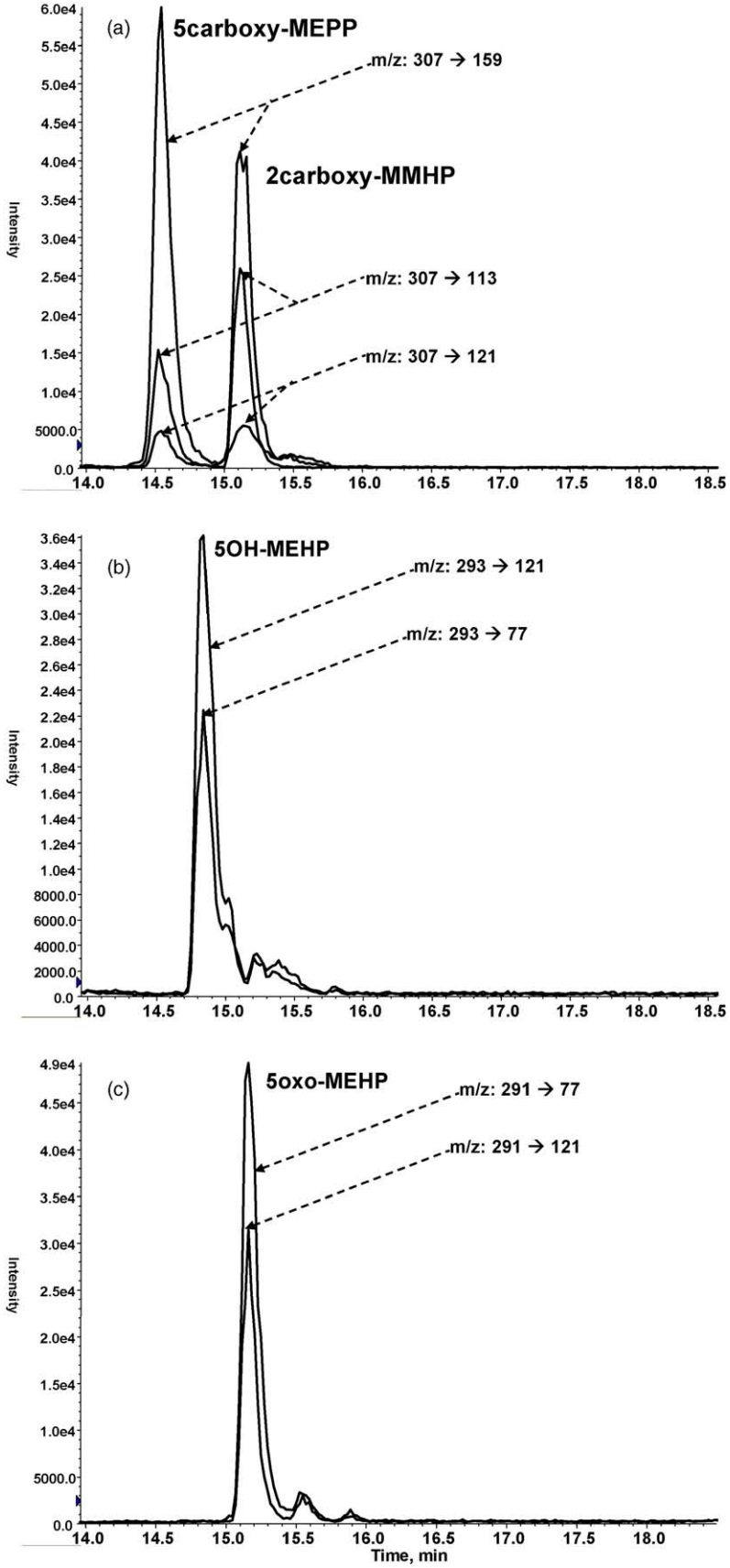

Fig. 3. Chromatogram of a processed urine sample (creatinine: 1.05 g/l). Concentrations were as follows: (a) 87.0 μg/l (5carboxy-MEPP) and 61.8 μg/l (2carboxy-MMHP); (b) 47.5 μg/l (5OH-MEHP); (c) 45.6 μg/l (5oxo-MEHP).

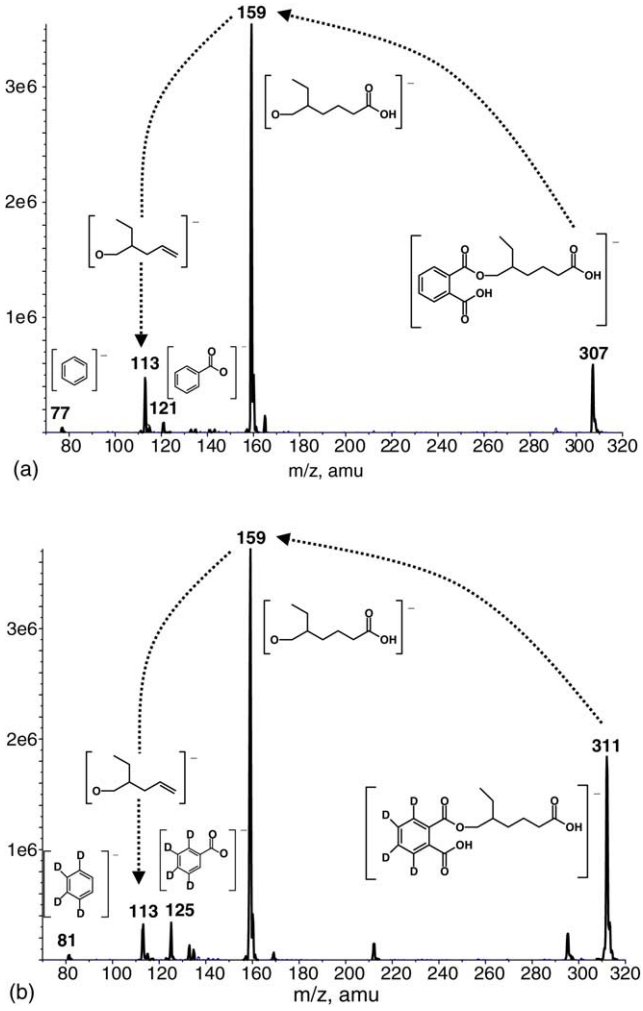

Fig. 4. ESI-negative Q1 mass spectra of 5carboxy-MEPP (a) and D4-5carboxy-MEPP (b) with the predicted structures of the fragments.

Autonomy from the urinary matrix was hereby proven. Detailed data are shown in Table 5.

The limits of detection, defined as a signal to noise ratio of three for the registered fragment ions were estimated to be 0.25 μg/l for 5carboxy-MEPP, 5OH-MEHP and 5oxo-MEHP. At this concentration, both registered mass fragments of each metabolite could be detected. The limits of quantification, defined as a signal-to-noise ratio of six, were estimated to be 0.5 μg/l. The limit of detection and the limit of quantification for MEHP was estimated to be 0.5 and 1.0 μg/l, respectively. Hence, this method is sensitive enough to quantify the background exposure of the general population to DEHP.

In summary, we present a method covering the most important urinary metabolites of the most applied phthalate DEHP in one analytical run with a total run time of only 22 min. To our knowledge, it is the first time that the secondary DEHP metabolites 5carboxy-MEPP and 2carboxy-MMHP were determined in human urine samples. With the presented method, we combined novel HPLC on-line enrichment and column-switching techniques with state-of-the-art MS/MS detection. The automatic column-switching procedure minimizes the input of manpower and it allows a high sample throughput (approximately 50 samples per 24 h). Precision and accuracy of the method are very good over the whole concentration range. The on-line clean-up procedure is very efficient so that the analytical result and the reliability are not influenced by varying compositions of urinary species. The developed HPLC–MS/MS method has therefore proven to be robust and reliable and no deterioration of quality control urine was observed. Results can be applied in the field of environmental medicine for assessing the body burden of the general population but also of occupational or medically exposed subjects as well as young children. Using this method, we were able for the first time to detect 5carboxy-MEPP and 2carboxy-MMHP (see below) in combination with 5OH-MEHP and 5oxo-MEHP in every urine sample of the general population together with MEHP.

3.2. Identification of 2carboxy-MMHP

The putative 2carboxy-MMHP peak which eluted 0.7 min after 5carboxy-MEPP showed the same fragmentation pattern as 5carboxy-MEPP (Fig. 4). We therefore assumed it to arise from a structural isomer of 5carboxy-MEPP. This assumption was proven by the fact that the chromatogram of the urine sample of a person who had incorporated D4-ring labelled DEHP exhibited D4-5carboxy-MEPP (MS parent–daughter ion combination 311/159 *m/z*) as well as the second peak 0.7 min after it with the same parent daughter ion combination (including the D4-labelling). Thus, it was proven that the unknown compound is clearly a metabolite of the administered D4-DEHP.

In order to further identify this unknown D4-DEHP metabolite, the respective peak was isolated by collecting it during the HPLC run when it was eluting from the column. The peak intensity of the unknown compound was compared with a 5carboxy-MEPP peak of known concentration. The concentration of the unknown compound in the final solution was estimated to be about 1 mg/l. A Q1-scan was generated with this HPLC fraction by injecting it directly into the ion source of the LC–MS/MS system by means of a syringe pump. The corresponding mass spectrum showed the same main mass fragments like D4-5carboxy-MEPP (Figs. 4b and 5a). It showed the parent ion mass 311 *m/z* and several daughter ion masses of which 159 *m/z* was the most abundant one. The masses 125 and 81 *m/z* are D4-benzoic acid and D4-benzene. The fragmentation pattern (311/159/113)—as it was already seen in the case of D4-5carboxy-MEPP—represented primarily the loss of the side chain (311 → 159 *m/z*) and subsequently the loss of carbon dioxide of this side chain (159 → 113 *m/z*). The product ion scan of the collected HPLC fraction (parent mass 311 *m/z*) also showed this typical fragmentation pattern. The product ion scan of 159 *m/z* led to only one mass fragment 113 *m/z* shown in Fig. 5b (decarboxylated side chain). The parent

Table 4
Results concerning imprecision and recovery for within-series and between-day quality assurance experiments

	5carboxy-MEPP		5OH-MEHP		5oxo-MEHP		MEHP	
	Q_{low}	Q_{high}	Q_{low}	Q_{high}	Q_{low}	Q_{high}	Q_{low}	Q_{high}
Basal + spiked conc.	14. 2 + 10.1	14.2 + 505.7	11.7 + 10.5	11.7 + 524.2	6.7 + 9.6	6.7 + 477.4	6.4 + 10.0	6.4 + 500.5
= target conc.	24.3	519.9	22.2	535.9	16.3	484.1	16.4	506.9
Within-series (n = 8)								
Measured conc.								
Mean	25.2	453.6	21.6	488.0	16.1	437.0	17.4	530.3
Range	23.3–27.6	433.0–467.0	20.1–23.0	474.0–520.0	15.3–17.0	407.0–467.0	16.2–18.7	505.0–568.0
Imprecision								
R.S.D. (%)	5.6	2.5	4.0	3.4	4.5	4.9	5.3	3.6
Relative recovery								
Mean (%)	103.7	87.2	97.3	91.1	98.8	90.3	106.1	104.6
Between-day (n = 9)								
Measured conc.								
Mean	25.7	520.1	20.5	495.0	15.8	445.4	15.3	522.0
Range	23.8–27.1	458.9–643.0	18.4–22.1	466.0–538.0	14.2–17.1	380.9–473.0	14.6–16.3	500.0–547.0
Imprecision								
R.S.D. (%)	4.6	9.7	6.5	5.6	6.5	6.3	3.5	2.8
Relative recovery								
Mean (%)	105.8	100.0	92.3	92.4	96.9	92.0	93.3	103.0

All concentrations in μg/l. Q_{low} and Q_{high}: pooled quality control urine spiked with 10 μg (Q_{low}) and 500 μg (Q_{high}) of each compound per litre, R.S.D.: relative standard deviation, conc.: concentration.

daughter ion combination which is specific for a decarboxylation of the side chain of DEHP (159 → 113 m/z) was herby clearly identified. That means that the unknown compound must be a carboxylated DEHP metabolite structurally isomeric to 5carboxy-MEPP but not identical with it (different retention time). Hence, it seems reasonable to us that the "unknown" peak belongs to the ethyl side chain carboxylated MEHP derivative called 2carboxy-MMHP. This metabolite is the only thinkable carboxy-isomer of 5carboxy-MEPP. With respect to the chemical structure, we expect 2carboxy-MMHP to be more hydrophobic than 5carboxy-MEPP leading to a retention time later than 5carboxy-MEPP under the given LC conditions.

In addition, to verify the identity of 2carboxy-MMHP with an independent method, we carried out a GC–MS/MS ion trap analysis with a derivatizised aliquot of the isolated HPLC fraction (see Section 2.2). The derivatization with diazomethane leads to methylated carboxy groups. It was proven that neither an oxo group (of 5oxo-MEHP) nor a hydroxy group (of 5OH-MEHP) can be methylated by this procedure (data not shown). Hence, only the carboxy groups of the DEHP metabolites were methylated. Fig. 6 shows the results of the GC–MS/MS run of a methylated D4-5carboxy-MEPP standard (dotted chromatogram) in comparison to the methylated HPLC fraction. The putative 2carboxy-MMHP eluted earlier than 5carboxy-MEPP in the GC chromatogram. This is due to the fact that the methylated 2-ethyl-5-carboxypentyl side chain can be expected to be more hydrophobic than the methylated 2-(carboxymethyl)hexyl side chain. The mass spectra of both peaks are also presented in Fig. 6 showing exactly the same mass fragments with 157 m/z as the most abundant one (representing the methylated side chain).

Table 5
Results concerning imprecision and recovery for the interindividual quality assurance experiment

	5carboxy-MEPP	5OH-MEHP	5oxo-MEHP	MEHP
Interindividual (n = 8)				
Basal conc. range	0.7–145.0	0.4–151.0	0.3–103.0	<LOD–36.5
+ spiked conc.	50.5	52.4	47.7	50.0
Measured conc.				
Total range	50.8–191.0	53.8–205.0	45.0–145.0	60.1–90.3
Spiked conc.	46.0–55.2	44.4–61.0	39.4–48.3	53.8–59.8
Imprecision				
R.S.D. (%)	5.3	10.1	6.6	3.0
Relative recovery				
Mean (%)	99.2	98.0	91.2	113.3

All concentrations in μg/l. LOD: Limit of detection; R.S.D.: relative standard deviation; conc.: concentration.

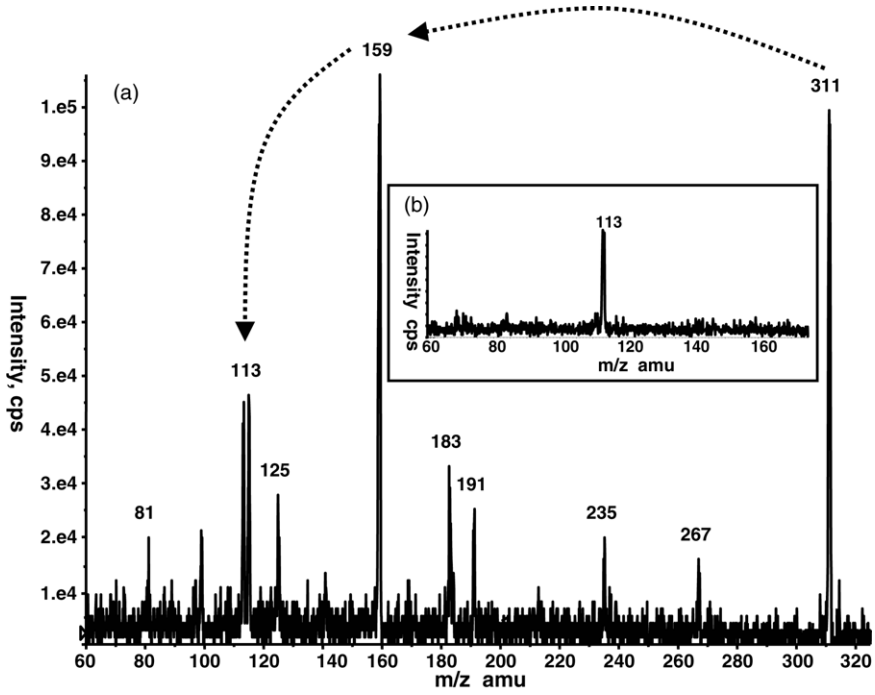

Fig. 5. ESI-negative mass spectra of the collected HPLC fraction containing approximately 1 mg/l of the unknown D4-DEHP metabolite: (a) Q1 scan of the HPLC fraction; (b) product ion scan of the mass fragment 159 *m/z*.

The mass fragments 167 and 185 *m/z* represent the D4-label-carrying benzene ring moiety of the metabolites. A product ion scan of the alkyl chain fragment (157 *m/z*) led to the same mass fragments for the D4-5carboxy-MEPP standard (Fig. 7a) and for the HPLC fraction (shown in Fig. 7b). The methylated side chains of the two isomers can be expected to fragment to the same masses, however, due to their isomeric structure with different likelihoods. This can be seen in the different ratios of the fragment masses in Fig. 7.

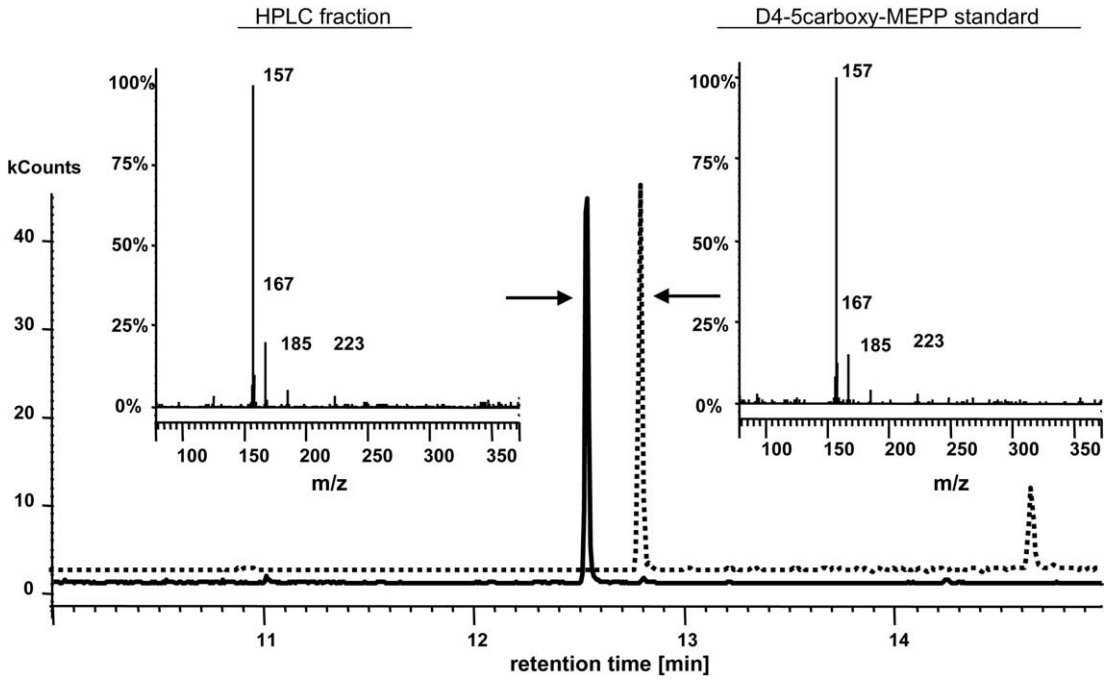

Fig. 6. GC–MS/MS chromatograms (monitoring 157 *m/z*) and mass spectra of the methylated HPLC fraction containing the unknown D4-DEHP metabolite (retention time: 12.55 min) and of a methylated D4-5carboxy-MEPP standard solution (retention time: 12.8 min, dotted line).

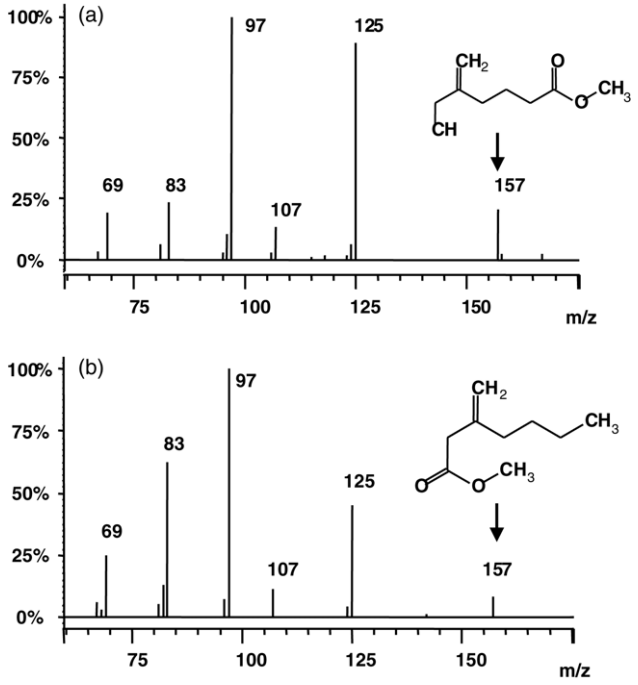

Fig. 7. GC–MS/MS product ion scan mass spectra of the alkyl side chain fragment 157 *m/z*. (a) Methylated D4-5carboxy standard solution; (b) methylated HPLC fraction.

In summary, although we had no reference standard of 2carboxy-MMHP available to unambiguously confirm the identity of the unknown peak, we conclude that the second peak occurring 0.7 min after 5carboxy-MEPP in the HPLC run is 2carboxy-MMHP. This is the only possible carboxylated isomer of 5carboxy-MEPP and it is already a proven DEHP metabolite [35,37]. We therefore included it half-quantitatively into the method until the reference standard will be available. Quantification of 2carboxy-MMHP was accomplished by referring its peak area to the calibration obtained for 5carboxy-MEPP.

3.3. Results of biological monitoring

Results of biological monitoring of 19 persons occupationally not exposed to phthalates are summarized in Table 6. All urine samples contained the phthalate metabolites above the detection limits. This pilot study indicates that 5carboxy-MEPP is the major urinary metabolite of DEHP, being found in about two-times the concentration of 5OH- and 5oxo-MEHP. The estimated concentrations of 2carboxy-MMHP are reaching and in some cases surpassing the concentrations of 5OH- and 5oxo-MEHP. Both facts are remarkable for two metabolites which have only been insufficiently investigated in humans so far. The relevance and diagnostic sensitivity of these two new ω-oxidized metabolites has to be investigated in the future as well as their exact quantitative relationship towards the other metabolites. Our results for 5OH-MEHP, 5oxo-MEHP and MEHP are in accordance with recently published data [18,20,22,24,25,34]. Our results for the new compounds 5carboxy-MEPP and 2carboxy-MMHP are unprecedented at the present time.

Table 6
Results of biological monitoring (n = 19): DEHP metabolites in human urine

DEHP metabolite	Median (μg/l)	Mean (μg/l)	Range (μg/l)
5carboxy-MEPP	85.5	84.3	12.8–164.0
2carboxy-MMHP	36.6	41.2	6.3–87.7
5OH-MEHP	47.5	52.1	7.9–96.1
5oxo-MEHP	39.7	41.3	8.1–72.5
MEHP	9.8	14.0	3.7–49.9

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