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On-line clean-up by multidimensional liquid chromatography–electrospray ionization tandem mass spectrometry for high throughput quantification of primary and secondary phthalate metabolites in human urine

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Abstract

We developed a new and fast multidimensional on-line HPLC-method for the quantitative determination of the secondary, chain oxidized monoester metabolites of diethylhexylphthalate (DEHP), 5-hydroxy-mono-(2-ethylhexyl)-phthalate (5OH-MEHP) and 5-oxo-mono-(2-ethylhexyl)-phthalate (5oxo-MEHP) in urine samples from the general population. Also included in the method were the simple monoester metabolites of DEHP, dioctylphthalate (DOP), dibutylphthalate (DBP), butylbenzylphthalate (BBzP) and diethylphthalate (DEP). Except for enzymatic hydrolysis for deconjugation of the metabolites no further sample pre-treatment step is necessary. The phthalate metabolites are stripped from urinary matrix by on-line extraction on a restricted access material (LiChrospher® ADS-8) precolumn, transferred in backflush-mode and chromatographically resolved by reversed-phase HPLC. Eluting metabolites are detected by ESI-tandem mass spectrometry in negative ionization mode and quantified by isotope dilution. Within a total run time of 25 min we can selectively and sensitively quantify seven urinary metabolites of six commonly occurring phthalate diesters including the controversial di(2-ethylhexyl)phthalate (DEHP). The detection limits for all analytes are in the low ppb range (0.5–2.0 μg/l urine). First results on a small non-exposed group ($n=8$) ranged for 5OH-MEHP from 0.59 to 124 μg/l, for 5oxo-MEHP from <LOQ to 73.0 μg/l, and for MEHP from <LOQ to 41.1 μg/l. The other short chain monoester metabolites were detectable in every sample with mean concentrations for MnBuP of 36.5 μg/l, for MBzP of 7.19 μg/l and MEP of 1.0 mg/l. With this rapid and economic method we can determine the internal exposure of the general population to DEHP and other phthalates as well as the body burden of occupationally and medically exposed subjects. The results can help to rank the risks of phthalates in the areas of carcinogenesis, peroxisome proliferation and endocrine disruption. Since secondary, functionalized metabolites of DEHP are included in the method an enduring problem of the past is excluded: sample contamination in the pre-analytical and analytical phase by both di- and monoesters.

Keywords: Biological monitoring; Plasticizers; Phthalates

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1. Introduction

Plastics have become an almost irreplaceable component of our modern world. And so have the plasticizers giving the synthetic material its desired flexibility. Phthalates, the dialkyl or alkyl aryl esters of phthalic acid have with 93% the lion's share in the plasticizers' segment. Each year 900 000 t are produced in Western Europe [1]. In Germany di(2-ethylhexyl)phthalate (DEHP) with ~250 000 t accounts for 60% of phthalate production. It is estimated that an additional 100 000 t of DEHP alone is emitted into the environment through DEHP containing waste every year in Germany [2–4]. Other important phthalates application- and production-wise are diethylphthalate (DEP), dibutylphthalate (DBP), butylbenzylphthalate (BBzP), dinonylphpthalate (DNP) and dioctylphthalate (DOP). In addition to being used as plasticizers, phthalates are also used as industrial solvents and lubricants, additives in the textile industry and pesticide formulations, and as components in consumer products such as deodorants and perfumes [5–8].

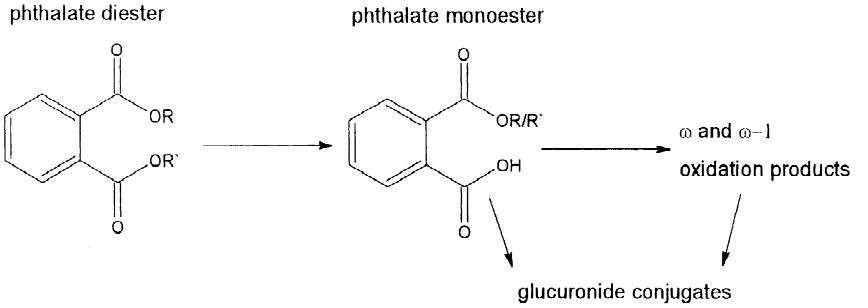
Humans are exposed to phthalates in numerous ways, e.g. by migration of phthalates into foodstuffs, by dermal absorption of cosmetics or by inhaling phthalate containing air [9–11]. In addition, specific groups of the population such as workers in the PVC-industry or medical patients undergoing dialysis, blood transfusions or having implants are potentially more heavily exposed [12–17].

Several phthalates and some of their monoester metabolites have shown teratogenic, reproductive and developmental effects, toxicity to the testes and liver carcinoma in rodents [18–20]. Effects on humans in the areas of carcinogenesis, peroxisome proliferation and endocrine disruption are controversial [5,21–28].

External exposure scenarios have always been difficult to evaluate. This is partly due to the fact that phthalates have become ubiquitous in the environment and so phthalate diester measurements have been hampered. It is also due to the fact that it has always been difficult to deduce from measurements of environmental contamination to actual individual intake. An unambiguous assessment of the exposure of the population to phthalates can only be achieved by measuring specific metabolites of the phthalates, preferably in urine, in a biological monitoring study [29–34]. In humans, phthalates are rapidly cleaved to their respective monoesters and a portion is further metabolized to oxidation products. These products are then mainly excreted through the urine [35–39].

Analytical methods determining diesters in blood or even monoesters in urine have been flawed in the past due to the contamination factor. Sjöberg [38] and Dirven et al. [39] performed biological monitoring studies after DEHP exposure and determined four DEHP metabolites. Detection limits however were not suitable for the determination of baseline excretion in the general population. Neither were the metabolite standards made available nor their synthesis published. Since then no ongoing studies have been performed on phthalate exposure by unequivocal determination of secondary phthalate metabolites. No reference values exist today for the excretion of secondary metabolites of DEHP or other phthalates for the general population. Blount et al. [40] determined only the monoester metabolites of the most applied phthalates. They circumvented a major contamination problem of monoester analysis by introducing a β-glucuronidase enzyme with no non-specific lipase activity. Conjugated phthalate monoesters in urine could be deconjugated even in the presence of phthalate diesters (being ubiquitously present) without generating monoesters out of the diesters. However, to definitely rule out contamination in the pre-analytical phase during sample collection and transportation by phthalate monoesters generated through simple chemical or microbiological ester cleavage of the diesters and not human metabolism, we decided to focus our method on the secondary metabolites of the most applied phthalate diester, di(2-ethylhexyl)phthalate (DEHP). Those secondary metabolites are 5-hydroxy-mono-(2-ethylhexyl)-phthalate (5OH-MEHP) and 5-oxo-mono-(2-ethylhexyl)-phthalate (5oxo-MEHP). They are generated in human metabolism by ω-1 oxidation of the monoester alkyl chain. Only by implementing those secondary metabolites of DEHP in the analytical method is the risk of external contamination ruled out. Synthesis of those secondary metabolites was published by Gilsing et al. [41] and the standards are also available D4-isotopically labelled for internal standard use. Fig. 1 illustrates the general human metabolism of phthalates and the resulting metabolites analysed in our method.

With our method and subsequent results we hope



phthalate diester		phthalate monoester		ω-1 oxidation products		
DEP	R, R`:	$-CH_2CH_3$	**MEP**			
DnBP	R, R`:	$-CH_2(CH_2)_2CH_3$	**MnBP**			
BBzP	R:	$-CH_2(CH_2)_2CH_3$	**MnBP**			
	R`:	$-CH_2C_6H_5$	**MBzP**			
DEHP	R, R`:	$-CH_2CH(C_2H_5)CH_2(CH_2)_2CH_3$	**MEHP**			
				R:	$-CH_2CH(C_2H_5)CH_2CH_2CHOCH_3$	**5oxo-MEHP**
				R:	$-CH_2CH(C_2H_5)CH2CH2CH(OH)CH_3$	**5OH-MEHP**
DOP	R:	$-CH_2(CH_2)_6CH_3$	**MOP**			

Fig. 1. Metabolic pathway of phthalates. In the first step the alkyl- or alkylaryldiesters are hydrolyzed to form monoesters. In a second phase-I-reaction monoesters react in part further to form ω- and ω-1 oxidation products. In a phase-II-reaction the phase-I-reaction products can be conjugated to glucuronic acid. The actual metabolites analysed are the phase-I-reaction products described by the single R bound to phthalic acid.

to provide insights into the actual exposure of the general population and certain risk groups to phthalates.

2. Experimental

2.1. Chemicals

5-Hydroxy-mono-(2-ethylhexyl)-phthalate (5OH-MEHP), 5-oxo-mono-(2-ethylhexyl)-phthalate (5oxo-MEHP) and their D4-ringlabelled analogues were synthesized in cooperation with the ‘‘Institut für Dünnschichttechnologie und Mikrosensorik e.V. (IDM)’’ according to Gilsing et al. [41]. All four compounds had 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.

Monoethylphthalate (MEP), mono-*n*-butylphthalate (MnBuP), mono-isobutylphthalate (MiBuP), monobenzylphthalate (MBzP), mono-(2-ethylhexyl)phthalate (MEHP), and mono-*n*-octylphthalate (MOP) were synthesized in our laboratory from phthalic anhydride and the respective alcohols by esterification according to Dirven et al. [39]. The products were characterized by mass spectrometry, ^{13}C- and ^{1}H-NMR. D4-monoethylphthalate, D4-mono-*n*-butylphthalate, D4-monobenzylphthalate and D4-mono-(2-ethylhexyl)-phthalate were synthesized in our laboratory from 3D-, 4D-, 5D-, 6D-ringlabelled phthalic anhydride and the respective alcohols again according to Dirven et al. [39]. The products were characterized by mass spectrometry

and ^{1}H-NMR. All 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, ethanol (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.

2.2. Standard preparation

The stock solution for the native standards was prepared by dissolving 10 mg MEP, MnBuP, MBzP, MEHP, 5OH-MEHP, 5oxo-MEHP and MOP in 25 ml acetonitrile (400 mg/l). This stock solution was stored at −18 °C in a Teflon-capped glass vial until further use. A 0.5-ml aliquot of this stock solution was placed in a 100-ml glass volumetric flask and diluted to the mark with water (2 mg/l). This solution served as the highest standard and the working solution for the preparation of the other standard concentrations (2 mg/l–0.4 μg/l). The stock solution for the internal standards was prepared by dissolving 25 mg D4-MEP and 5 mg D4-MnBuP, D4-MBzP, D4-MEHP, D4-5OH-MEHP and D4-5oxo-MEHP in 25 ml acetonitrile. This stock solution was stored at −18 °C in a Teflon-capped glass vial until further use. A 1.0-ml aliquot of this stock solution was placed in a 100-ml glass volumetric flask and diluted to the mark with water (10 mg/l D4-MEP and 2 mg/l for the other D4-standards).

2.3. Sample preparation

Frozen urine samples were allowed to equilibrate to room temperature. The samples were vortex mixed and 1-ml aliquots were then transferred to 1.8-ml glass screw-cap vials. Then 200 μl ammonium acetate (1 *M*, pH 6.5), 50 μl of the D4-internal standard solution and 5 μl β-glucuronidase were added to the samples. The samples were incubated for 1.0 h at 37 °C in a drying oven. After hydrolysis each sample was vortex mixed, ultrasonicated and frozen at −18 °C, thawed again, centrifuged at 3000 *g* for 10 min and the supernatant transferred into another 1.8-ml glass screw-cap vial. A 600-μl aliquot was then injected into the LC–MS–MS system for quantitative analysis.

Urinary creatinine concentrations were determined according to Larsen [42].

2.4. Calibration procedure and quality control

A stock solution was prepared containing 400 mg of each monoester per liter acetonitrile. From this standard stock solution 11 calibration standards were prepared by diluting the solution with water. The solutions were spiked with concentrations in the range from 0.4 to 2000 μg/l. Unspiked water was used as a blank. The standards were processed as described in Section 2.3. Linear calibration curves were obtained by plotting the quotients of the peak areas of the monoesters and the respective D4-internal standards as a function of the concentrations used. These graphs were used to ascertain the unknown concentrations of the monoesters in urine samples. D4-MEHP was used as an internal standard for both MEHP and MOP.

As there was no quality control material commercially available it had to be prepared in the laboratory. For the low-concentration quality control material we spiked water with 10 μg of each monoester per liter. For the high-concentration quality control material we spiked pooled urine from laboratory personnel with 100 μg monoester per liter after freezing and filtering the urine. Due to different background levels of each monoester in the pooled urine this resulted in concentrations ranging from ~100 to 900 μg/l. The pools were divided into aliquots and stored at −18 °C. For quality assurance one low- and one high-concentration control sample was included in each analytical series.

Within-day repeatability was determined by analysing the low- and high concentration quality control urine eight times in a row. Between-day repeatability was determined by analysing the quality control samples on 8 different days. Furthermore imprecision was determined using eight spiked individual urine samples with a creatinine content from 0.24 to 2.12 g/l. The monoester content of the non-spiked specimens varied strongly from subject to subject. Spiked specimens (spiked concentration 400 μg/l for MEP and 50 μg/l for the other monoesters)

and the same specimens without the addition of monoesters were analysed.

2.5. LC–MS–MS analysis

2.5.1. Liquid chromatography

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 latter was used to load the sample (600-μl aliquot) on a RAM (restricted access material) phase, a LiChrospher® RP-8 ADS (25 μm) 25×4 mm RAM from Merck (Darmstadt, Germany) using a 1% aqueous solution of acetic acid and methanol (90:10, v/v) as the mobile phase and a flow-rate of 0.8 ml/min. After this clean-up and enrichment step the analytes were transferred to a reversed-phase HPLC column (Luna Phenyl-Hexyl 150×4.6 mm, 3 μm particle size from Phenomenex, Aschaffenburg, Germany) in backflush mode through a time controlled ten-port valve on the API 2000 Sciex MS–MS (PE Biosystems, Langen, Germany) and an LC pump gradient described in Table 1. Fig. 2 shows the backflush arrangement. All steps were controlled by Analyst 1.1 Software from Perkin-Elmer except the isocratic pump. A guard column (phenylpropyl, 4×3 mm, Phenomenex) was placed in front of the analytical column to extend its lifespan. During routine measurements it was replaced after ~100 injections.

2.5.2. Mass spectrometry

The MS–MS detection was performed on a Sciex API 2000 LC–MS–MS system. The ion source-dependent (turbo ion spray) conditions were the same for all of the analytes with an electrospray needle voltage of −4000 V in the negative ion mode. Nitrogen as nebulizer and turbo heater gas (500 °C) was set at a pressure of 35 and 68 p.s.i., respectively. The curtain gas was set to 45 p.s.i.. The collision gas (N_2) for the MS–MS mode at quadrupole Q2 was set to a flow of 4 (instrument units). 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-daughter ion combinations were monitored with one combination being used for quantification and the other(s) for verification. MS–MS operating conditions in the MRM-mode for all parameters were as follows: resolution Q1, unit; resolution Q3, low;

Table 1
Analysis program of the gradient pump

Program step	Time (min)	Eluent A (vol. %)	Eluent B (vol. %)	Eluent C (vol. %)	Flow-rate (ml/min)	Position of value	Analysis step
1	0	45	45	10	0.6	A	RAM-charging
2	6					B	Transfer
3	8					A	Separation
4	10						
5	10.1	12.5	77.5				
6	13	10	85	5			
7	13.5	0	100	0			
8	15.6					B	Washing
9	15.9				0.8		
10	16		0	100			
11	21					A	Washing
12	21.1		100	0			
13	24						
14	24.4	45	45	10	0.6		Reconditioning
15	25						

The second pump (isocratic precolumn pump) continuously pumps the mobile phase (water, 10% vol. MeOH, 1% acetic acid) for the RAM charging/fractionation step at a flow-rate of 0.8 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.

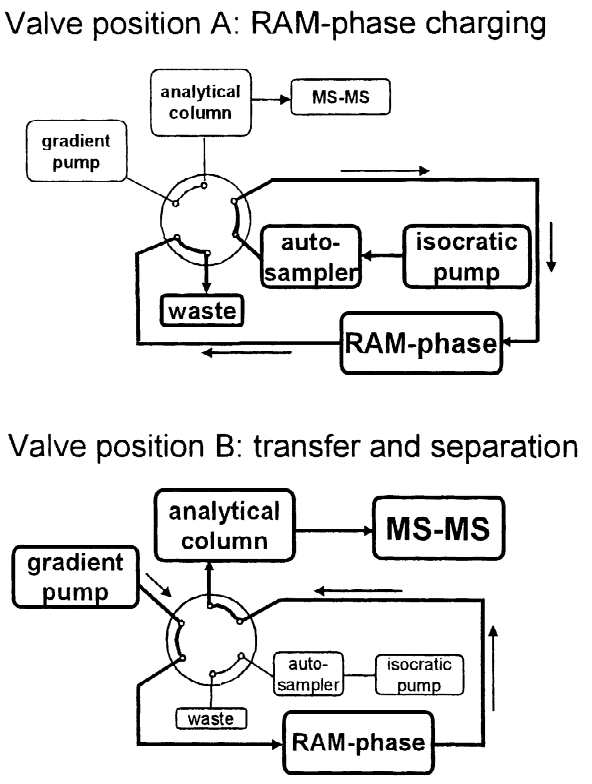


Fig. 2. Two-column HPLC system with backflush arrangement for the analyte transfer step in valve position B. The system is automatically controlled via a six-port switching valve.

settling time, 5 ms; MR pause, 5 ms; and scan time, 75 ms. Analyte specific parameters are shown in Table 2.

2.6. Study subjects

For a pilot study we investigated eight spot urine samples from members of our laboratory staff. They were all male, aged between 26 and 41 and occupationally not exposed to phthalates. Two of them were smokers.

3. Results and discussion

3.1. General considerations

Method development was led by three requirements: minimization of contamination factors, coverage of the most important phthalates and effectiveness.

In order to build a fast and easy method we applied novel HPLC on-line enrichment and column-switching techniques and combined them with state-of-the-art MS–MS detection. The applied RAM-phase enabled us to extract the analytes online out of the matrix and transfer them with a switching valve directly onto the analytical column. This effective clean-up and the use of the highly sensitive and selective ESI-MS–MS-detection ensured a smooth and false-positive-free performance. Because of the limited number of off-line sample preparation steps we not only minimized the input of manpower, but also the risks of contamination with various phthalates in the analytical phase. Yet for the first time we present a method measuring not only the monoester metabolites of the most applied phthalates, but also secondary metabolites of DEHP, one of the most controversially discussed phthalates, in one analytical run.

3.2. Enzymatic hydrolysis

Phthalate metabolites are excreted in urine partially as glucuronides. Deconjugation of the glucuronic acid has posed a great problem in the past since simple acid hydrolysis could also cause cleavage of the phthalate ester bond. The same has been the case with previously applied glucuronidase-enzymes which also had considerable non-specific lipase activity. As a result there has always been great potential for contamination in the analytical process since phthalate monoesters could be degraded out of the ubiquitously present diesters in laboratory environment. Blount et al. [40] were the first to introduce a method applying an enzyme with no measurable lipase activity, *E. coli* β-glucuronidase (K12 from Roche Biomedical). We applied that enzyme and checked it for unspecific lipase activity. Incubated solutions of the phthalate diesters (10 mg/l in water) showed no measurable monoester formation under our experimental conditions. Enzymatic deconjugation of the glucuronic acid was found to be complete after 30 min for all analytes. Urine specimens however were incubated with the enzyme for 1 h to ensure complete deconjugation. Yet one has to keep in mind that the introduction of

Table 2
MRM-parameters for each specific parent and daughter ion combination

Analyte	Parent ion (Q1)	Daughter ion (Q3)	Retention time (min)	DP	FP	EP	CE
MEP	193	77[a]	11.05	−16	−320	9.5	−22
	193	77		−16	−310	4	−14
D4-MEP	197	81	11.00	−21	−350	10	−16
5-OH-MEHP	293	77	12.30	−16	−330	8.5	−40
	293	121[a]		−16	−330	8.5	−24
D4-5OH-MEHP	297	125	12.25	−21	−340	11.5	−26
5oxo-MEHP	291	77[a]	13.05	−11	−340	9	−40
	291	121		−16	−340	9	−20
D4-5oxo-MEHP	295	125	13.00	−31	−350	11.5	−26
MnBP	221	71	13.35	−16	−320	6	−20
	221	121[a]		−21	−320	6.5	−20
	221	134		−21	−320	6.5	−20
MiBP	(see MnBP)		13.10				
D4-MnBP	225	81	13.30	−21	−300	9	−24
MBzP	225	77[a]	13.80	−21	−350	4	−30
	225	107		−21	−350	4	−18
D4-MBzP	259	107	13.77	−26	−330	10	−18
MEHP	277	127	15.18	−21	−350	9.5	−26
	277	134[a]		−21	−350	9.5	−26
D4-MEHP	281	138	15.15	−21	−340	10	−20
MOP	277	127[a]	15.37	−21	−350	9.5	−26
	277	134		−21	−350	9.5	−26

CE, collision energy (V); DP, declustering potential (V); EP, entrance potential (V); FP, focusing potential (V).
[a] Parent–daughter ion combination used for quantification.

E. coli β-glucuronidase K12 only minimizes the within laboratory contamination risks, but not any possible contaminations in the pre-analytical phase. There is still the risk of measuring false elevated monoester content in some cases. This problem can only be circumvented by including secondary metabolites in the method. Secondary metabolites for DEHP are e.g. ω, ω-1 and β oxidation products of the monoesters alkyl chain.

3.3. Liquid chromatography

In preparation for liquid chromatography the hydrolyzed samples were frozen, thawed and centrifuged in order to precipitate cryophilic proteins and particulate matter. This procedure proved not to influence the precision and accuracy of the method as was shown by tests with spiked individual specimens. Precipitation was considerable for some specimens. Since no guard column could be placed in front of the RAM-phase due to adsorption effects and the backflush arrangement this step was essential for extension of the RAM-phase lifetime.

We applied an LC–LC column switching method which enabled us to perform an online sample extraction/fractionation step with subsequent chromatographic separation of the analytes on an analytical column. The elution power of the gradient solvent was sufficiently strong to desorb the analytes from the pre-column, but not strong enough to start significant chromatographic migration for most of the analytes on the analytical column. A change in flow direction (backflush) in the transfer step caused

the analytes to refocus on the analytical column and increased sensitivity.

LiChrospher® ADS8 (alkyl-diol-silica) material is a special reversed-phase sorbent for LC-integrated sample preparation. Extraction is based on two chromatographic processes: reversed-phase and size exclusion chromatography. LiChrospher® ADS thus belongs to the class of restricted access materials (RAM). Macromolecules with a molecular weight greater than 15 kDa are not able to penetrate the pores and are so eluted with the void volume of the column. Analytes migrating into the pores are retained by C8–RP-interactions. All analytes were quantitatively retained on the ADS material. As an alternative to LiChrospher® ADS8, LiChrospher® ADS18 material also proved to be suitable.

Analytes were transferred onto the analytical column by a ten-port switching valve integrated into the Sciex API 2000 mass spectrometer. A six-port switching valve would have been sufficient to perform the task as Fig. 2 shows.

Due to the on-line sample extraction and switching procedure several chromatographic requirements had to be taken into account. The isocratic solvent was optimized to a 1% aqueous solution of acetic acid and methanol (90:10, v/v) ensuring maximum clean-up on the RAM-phase and no analyte losses. The starting conditions for the gradient solvent with 45% water, 45% acetonitrile and 10% methanol were found to be optimal for transferring the analytes from the RAM-phase and focusing on the analytical phase. LC–LC parameters are abstracted in Table 1. Washing of the RAM- and the analytical phase was performed by the gradient pump providing the needed ratio of organic solvent. A second gradient pump can reduce the time for the washing step significantly since the RAM-phase can be washed directly after the transfer.

The chromatographic separation had to be optimized to distinguish the structural isomers MnBP and MiBP and the structural isomers MEHP and MOP since they resulted in the same daughter ion spectra. For MnBP and MiBP a peak but not a full baseline separation could be achieved. Yet the isomers could also be distinguished because of their different daughter ion intensities. Best peak shapes and separation properties were obtained on a phenyl-hexyl column. An Xterra RP18 column 2.5 μm (4.6×50 mm) from Waters also showed excellent performance in half the time per analytical run, but with the compromise of almost no separation of the two MBP isomers.

Yet we decided to stay with the phenyl-hexyl column since in almost all specimens considerable amounts of both MnBP and MiBP in varying ratios could be detected.

3.4. Mass spectrometry

Blount et al. [40] achieved excellent results in phthalate monoester detection applying atmospheric pressure chemical ionization (APCI). We also tested APCI and compared it to electrospray ionisation (ESI) on the API 2000. With ESI in the negative mode we got excellent limits of detection without extensive co-optimization of related APCI parameters. Compound specific mass spectrometer parameters were optimized for each compound automatically by the Quantitative Optimization Wizard of the Sciex AnalySt™ software. The source specific parameters were optimized manually for the LC conditions used during analysis depending on flow-rate and eluent composition. Q_1 ESI negative ion mass spectra with tentative fragment structures for MEHP, 5OH-MEHP, 5oxo-MEHP and D4-5OH-MEHP are shown in Fig. 3. The chosen parent ions for the MS–MS fragmentation of all analytes were $[M–H]^-$. At least two daughter ions were monitored for each analyte ensuring maximum selectivity. With a scan time for each fragmentation of 75 ms, 20 parent–daughter ion combinations were registered simultaneously in the MRM (multiple reaction monitoring) mode of the API 2000. All combinations were registered within the same time-window. MRM-parameters are illustrated in Table 2. The identity of each analyte peak was confirmed by at least two parent–daughter ion combinations and by matching its retention time with its D4-labelled internal standard. For MnBP and MiBP three parent–daughter ion combinations were registered, since MnBP and MiBP are structural isomers with rather similar retention times at the given conditions and unequivocal identification was performed by taking into account differences in the relative daughter ion abundance. Only MnBP was determined quantitatively since only D4-MnBP was present as internal

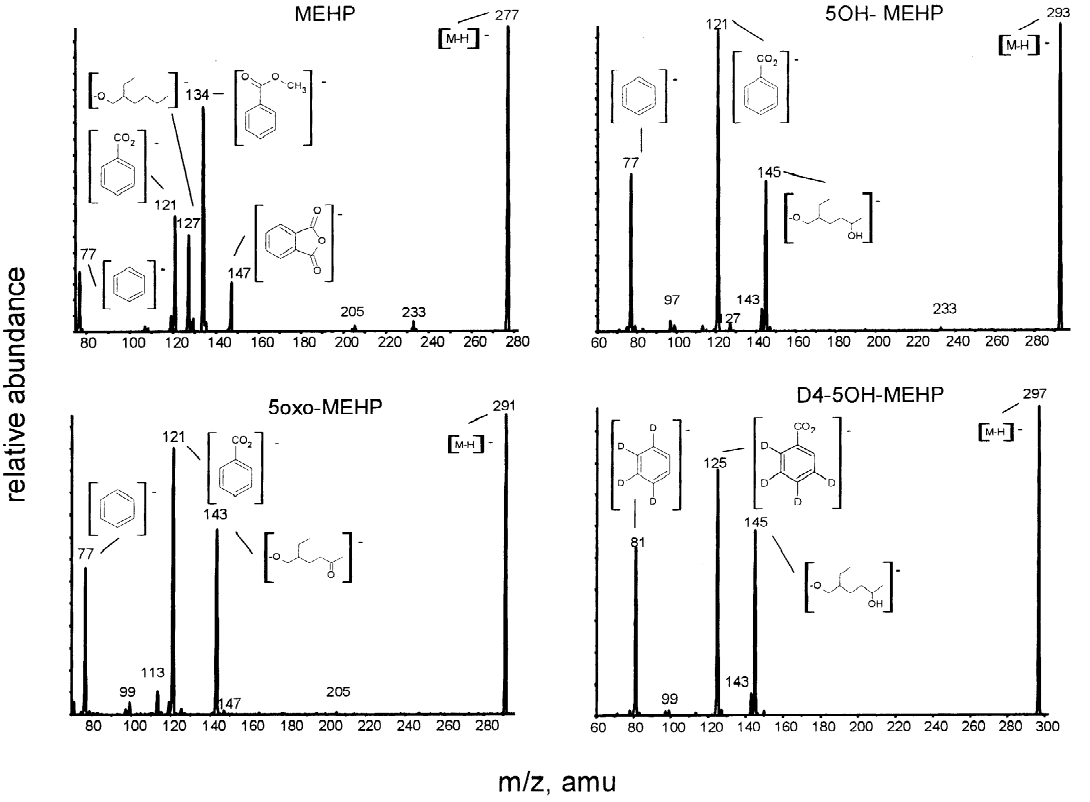


Fig. 3. ESI-negative Q1 mass spectra of MEHP, 5OH-MEHP, 5oxo-MEHP and D4-5OH-MEHP with the predicted structures of the fragments.

standard. We however found that MiBP was present in concentrations in the same order of magnitude as MnBP yet in varying ratios. In a future method MiBP will be implemented as an independent parameter. For MOP D4-MEHP was used as the internal standard. In contrast to MEHP, its isomer MOP was detected only in a small fraction of the analysed specimens.

For quantification the peak area ratio of analyte to D4-internal standard was used. 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.

For MEP and D4-MEP in some cases ionisation was strongly suppressed resulting in an up to ten-fold loss of sensitivity. This was probably due to co-eluting substances in the urinary matrix. Since quantification was based on isotope dilution this variation in absolute signal intensity did not significantly deteriorate quantitative accuracy unless ionization was dramatically suppressed. Due to that and because of the high variability in urinary MEP content (53.7–3050 μg/l) 400 μg/l of D4-MEP were spiked.

3.5. Calibration graphs

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. Autonomy from the urinary matrix was also demonstrated by determining the phthalates out of eight spiked individual urines with a wide range of creatinine content (0.24–2.12 g/l). Therefore calibration graphs were obtained by analyzing spiked water standards.

All calibration curves were linear within the given concentration range (0.4–2000 μg/l) and produced linear correlation coefficients greater than 0.99. This wide working range was necessary to cover the highly variable levels of the different phthalates in human urine.

Samples with concentrations above the highest calibration standard were diluted to fit the calibration range.

Reagent blanks were found to contain traces of MnBuP and MiBuP. For MnBuP blank concentrations ranged from 0.4 to 2.0 μg/l. The results were corrected by background subtraction. The actual source of contamination could not be located. No other monoester or secondary phthalate metabolites were found in water reagent blanks.

3.6. Reliability of the method

3.6.1. Precision and accuracy

As no certified reference material was commercially available, accuracy had to be checked by special recovery experiments. We apply those recovery experiments as strong acceptance criteria, which have proven to be very effective. Eight different urine specimens were spiked with the phthalates in question (300 μg/l for MEP and 50 μg/l for the other parameters). The urine specimens were selected to reflect a composition as different as possible. As an indicator we use the creatinine content which for the eight urine specimens ranged from 0.24 to 2.12 g/l. In that way we could also check for a possible influence of the complex biological matrix on the analytical result. For that experiment relative recoveries were in the range from 84.6 to 106.0% for all parameters and all specimens.

In order to assess the within-day repeatability, quality controls $Q1_{low}$ and $Q2_{high}$ were analysed eight times in a row. $Q1_{low}$ contained ~10-times the LOQ for each analyte and was prepared in water. $Q2_{high}$ contained ~100–500-times the LOQ for each analyte and was prepared from pooled urine (creatinine content 1.08 g/l). For $Q1_{low}$ relative standard deviations for the various parameters were 2.5–8.3%, while for $Q2_{high}$ the relative standard deviations were 2.4–5.1%, demonstrating perfect repeatability of this method over the whole concentration range down to trace levels.

The relative standard deviation of the between-day repeatability was determined on 8 different days and was 2.7–15.1% for $Q1_{low}$ and 2.3–13.8% for $Q2_{high}$. All data are presented in Table 3.

Imprecision was also calculated based on the eight individual urine samples from the recovery experiments described above. The relative standard deviations for the parameters at the spiked concentrations were 4.1–15.1% and thus within the analytical error resulting from the precision of the method.

3.6.2. Detection limit and quantification limit

The limits of detection (LOD), defined as a signal-to-noise ratio of three for the registered fragment ions, were estimated to be 0.25–1.0 μg/l. At this

Table 3
Quality control data and quantification limits of this method for the determination of primary and secondary phthalate metabolites in human urine

Analyte	Precision						Accuracy		LOQ (6×SD)
	$Q1_{low}$			$Q2_{high}$			Relative recovery (n=8)		
	Conc. (μg/l)	RSD (%), intra-day	n=8, inter-day	Conc. (μg/l)	RSD (%), intra-day	n=8, inter-day	Mean	Range	
MEP	9.0	3.7	4.7	890	5.0	10.0	91.7	76.6–103	2.0
5OH-MEHP	10.3	2.5	2.7	125	3.6	2.3	90.9	84.8–95.0	0.5
5oxo-MEHP	10.1	5.1	5.1	102	2.6	2.7	84.6	76.9–101	0.5
MnBuP	10.4	5.1	15.1	142	5.1	6.7	97.2	76.9–117	2.0
MBzP	9.0	3.9	3.6	98	2.5	4.7	99.6	87.2–122	0.5
MEHP	10.8	3.7	5.3	120	4.7	5.7	106	97.3–110	0.5
MOP	7.1	8.3	8.5	84	2.4	13.8	103	97.3–111	1.0

RSD, relative standard deviation; SD, standard deviation.

concentration both or all three registered mass fragments of each metabolite could be detected. The limits of quantification (LOQ), defined as a 6× standard deviation of a blank sample were estimated to be 0.5–2.0 μg/l (Table 3).

3.6.3. Sources of error

The developed LC–LC-MS–MS method has proven to be robust and reliable. The lifetime of the ADS8 column has exceeded 250 injections and no deterioration of quality control data has been observed. The guard column was replaced after 100 real life sample injections.

The highest priority was given to contamination control. Polypropylene 100-ml specimen cups were batch-screened for phthalate mono- and diesters and found to contain acceptable traces of phthalate diesters. Only approved material was used for sample collection. All other utilised chemicals and materials were screened for the presence of monoesters. Only the approved chemicals and materials were used for the sample work-up. Total exclusion of phthalate diesters could never be achieved. Reagent blanks were run within each batch. Except for MnBuP and MiBuP no background contamination was observed. For MnBuP background contamination was between 0.4 and 2.0 μg/l in the reagent blanks and was subtracted from all data. The actual source for this contamination could not be located. By this strict laboratory management we could minimize the risk of contamination for the monoester determinations in the analytical phase. Yet contamination could still occur in the pre-analytical phase during sample collection and transportation by phthalate monoesters generated through simple chemical or microbiological ester cleavage of the diesters, e.g. on the skin and hands of the subjects. To definitely rule out this exogenous contamination we incorporated the secondary metabolites of DEHP into the new method.

3.7. Results of biological monitoring

The results of the biomonitoring of the eight persons not occupationally exposed to phthalates are summarised in Table 4. All urine samples contained the phthalate metabolites in question in different concentrations, except MOP which was not found in any urine sample. The concentrations of the metabolites were found to differ 100-fold from subject to subject, while for MEP the range was almost 1000-fold. Fig. 4 shows a chromatogram of a processed spot urine sample. The results suggest the exposure

Table 4
Results of biological monitoring (n=8): phthalate metabolites in human urine

Analyte	Mean (μg/l)	Range (μg/l)
5OH-MEHP	23.0	0.59–124
5oxo-MEHP	13.8	>LOD–73.5
MEHP	8.87	>LOD–41.1
MEP	1000	53.7–3050
MBzP	7.19	>LOD–35.4
MnBuP	36.5	8.48–101
MOP	<LOD	<LOD

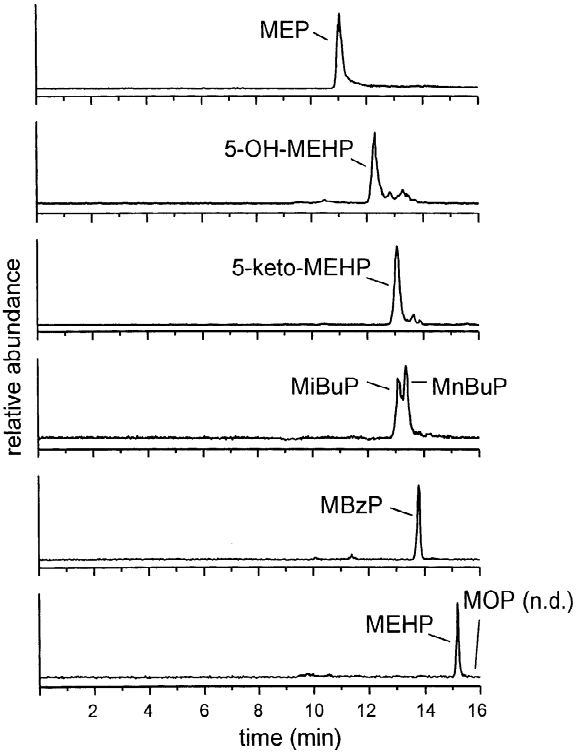


Fig. 4. Chromatogram of a processed urine sample. Only the quantifier parent–daughter ion combinations for the analytes are shown. Concentrations were as follows: 5OH-MEHP, 41.4 μg/l; 5oxo-MEHP, 48.6 μg/l; MEHP, 15.0 μg/l; MnBuP, 47.9 μg/l; MBzP, 18.9 μg/l; MEP, 404 μg/l; MOP, not detected. Creatinine content was 0.72 g/l.

of the general population to phthalates is not negligible and in some cases extremely high. This exposure is probably caused by dietary intake, the use of cosmetics etc. We checked the correlation coefficients for the excretion of the DEHP-metabolites and found an almost perfect correlation between 5OH-MEHP and 5oxo-MEHP with a correlation coefficient of 0.9994. Correlations between 5OH-MEHP, 5oxo-MEHP and the primary metabolite MEHP were evident but had weaker correlation coefficients (Fig. 5). One possible reason for the perfect correlation of 5OH- and 5oxo-MEHP is the close proximity in the metabolic pathway. This may also explain the weaker correlations between the primary metabolite MEHP and the secondary ones. We also can not totally exclude partial contamination of the samples with phthalate di- or monoesters during collection. This may cause a higher variation in monoester content and artificially elevated MEHP concentrations in real life samples, as the offset of the correlation curve for MEHP and 5OH-MEHP in the direction of MEHP might indicate. The secondary metabolites are not susceptible in this way. The correlation curve for 5oxo- with 5OH-MEHP almost exactly passes through the point of origin.

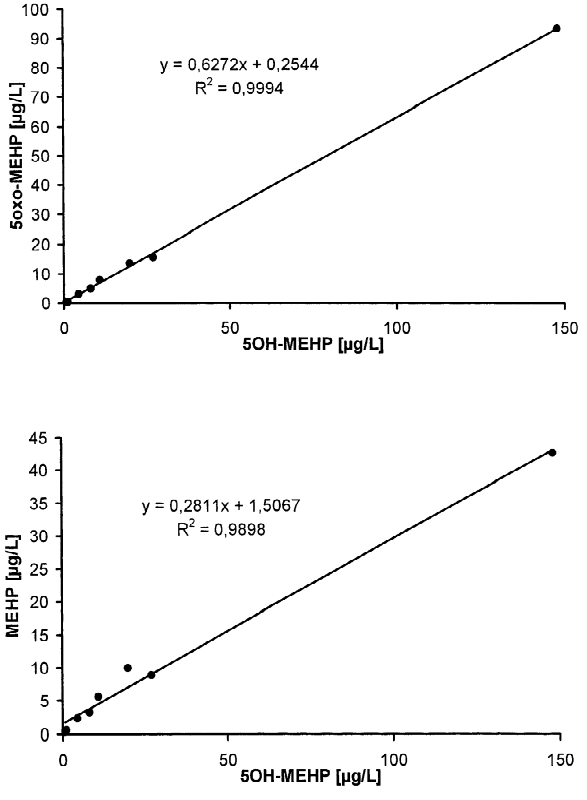


Fig. 5. Urinary excretion of the secondary DEHP metabolites compared with each other and comparison of the excretion of the primary metabolite MEHP with the secondary metabolite 5OH-MEHP. Linear correlation coefficients and linear functions are shown for each graph.

A recent study also showed there to be a baseline concentration for the excretion of phthalates. An American study [43] which investigated the general population using a more complex analytical procedure with similar detection limits also found phthalates within the same concentration range in urine samples. They however only measured phthalate monoesters and no secondary metabolites. We were able to confirm the monoester results by also determining the secondary metabolites of DEHP and definitely rule out such contamination. Metabolism studies of Peck and Albro [12], Schmid and Schlatter [37] and Dirven et al. [39], found excretion ratios of MEHP to 5oxo-MEHP to 5OH-MEHP of 1 to 1.1 to 3.4, 1 to 2.3 to 3.1 and 1.0 to 0.7 to 1.5, respectively. The excretion ratio we found for MEHP to 5oxo-MEHP to 5OH-MEHP was 1 to 1.6 to 3.0 fitting well into that scheme. Schmid and Schlatter state that 10–25% of orally ingested DEHP is excreted in urine as metabolites. Calculating with a 25% excretion according to Schmid and Schlatter, 8.1% of the ingested DEHP is excreted as 5OH-MEHP, 6.0% as 5oxo-MEHP and 2.6% as MEHP. Under the assumption that the average person excretes 1.25 l urine per day this would indicate a daily intake for DEHP of 288–425 μg/day in the mean depending on 5oxo-, 5OH- or MEHP as the basis for calculation. The highest intake value for our group would indicate an intake of 1.5–1.9 mg DEHP per day. Calculating with only a 10% urinary excretion of metabolites would more than double those values. For Blount et al. [43], a maximum intake was calculated to be 46 μg/kg body-weight per day which would indicate an intake of 3.5 mg per day for a person of 75 kg body weight.

4. Conclusions

We have developed a reliable, easy and fast analytical procedure for the determination of various phthalates in human urine samples. To our knowl-

edge this is the first time that secondary phthalate metabolites have been used to unequivocally determine the exposure of the general population. Within-day and between-day repeatability is very good, even at low concentrations but also over the whole concentration range. The on-line clean-up procedure is very efficient, so that no interfering effect of the matrix on the analytical results was observed. As a consequence the analytical background interference is very low yielding 0.25–1.0 μg/l as limits of detection and 0.5–2.0 μg/l as limits of quantification. Only for MEP was ionisation suppression observed in some cases resulting in an up to ten-fold loss of sensitivity. The detection of the secondary metabolites of DEHP minimizes the risk of false positive results due to contamination. This is also proven by the perfect correlation of the two secondary DEHP metabolites.

The method is perfectly suited for determining levels of phthalates over a wide concentration range. Results can be applied in the field of environmental medicine for assessing the body burden of the general population but also of occupationally or medically exposed subjects. Using this method, we were able for the first time to detect 5OH-MEHP and 5oxo-MEHP in every urine sample of the general population together with MEHP and the other monoester metabolites of the most important phthalates. Our findings for the urinary distribution of the DEHP metabolites MEHP, 5oxo-MEHP and 5OH-MEHP are well in accordance with the distribution found in metabolism studies and our results for the monoester excretion of various phthalates are also well in accordance with a recent study of Blount et al. [43]. These first results indicate that there might be considerable background exposure to phthalates in the general population. This calls for further research in this field of environmental medicine.

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References

[1] European Commission (Ed.), Green Paper on Environmental Issues of PVC. COM (2000), European Commission, Brussels, 2000.
[2] W.R. Schäfer, H.P. Zahradnik, N. Frijus-Plessen, K. Schneider, Umweltmed. Forsch. Prax. 1 (1996) 35.
[3] A. Leisewitz, in: Abwassertechn. Vereinigung (Ed.), Endokrine Stoffe, ATV-Schriftenreihe, 15, Hennef, 1999, p. 22.
[4] A. Leisewitz, W. Schwarz, in: Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit (Ed.), Forschungsbericht 106 01 076, Stoffströme wichtiger endokrin wirksamer Industriechemikalien (Bisphenol A; Dibutylphthalat/Benzyl-butylphthalat; Nonylphenol/Alkylphenolethoxylate), Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit, Bonn, 1997.
[5] International Agency for Research on Cancer, in: IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Some Industrial Chemicals and Dyestuffs, Vol. 29, IARC, Lyon, 1982, p. 416.
[6] C. Böhme, Bundesgesundheitsblatt 41 (1998) 340.
[7] H. Fromme, in: A. Beyer, A. Eis (Eds.), Praktische Umweltmedizin: 09.01 Chemische Faktoren Teil 4: Organische Stoffe-Phthalate, Loseblatt-Ausgabe, Springer, Berlin, 1999.
[8] G. Rippen (Ed.), Handbuch Umweltchemikalien, Di(2-ethylhexyl)phthalat, 3rd Edition, Ecomed, Landsberg, 2000, 49th supplement issue.
[9] J.H. Petersen, T. Breindahl, Food Addit. Contam. 17 (2000) 133.
[10] Agency for Toxic Substances and Disease Registry, in: Toxicological Profile for Di(2-ethylhexyl)phthalate, Final Report, ATSDR, Public Health Service, U.S. Department of Health and Human Services, Atlanta, 1993, p. 171, ATSDR/TP-92/05.
[11] W. Butte, W. Hofmann, O. Hostrup, A. Schmidt, G. Walker, Gefahrst. Reinh. Luft 61 (2001) 19.
[12] C.C. Peck, P.W. Albro, Environ. Health Perspect. 45 (1982) 11.
[13] P.O.J. Sjöberg, U.G. Bondesson, E.G. Sedin, J.P. Gustafson, Transfusion 25 (1985) 424.
[14] M.A. Faouzi, T. Dine, B. Gressier, K. Kambia, M. Luyckx, D. Paginez, C. Brunet, M. Cazin, A. Belabed, J.C. Cazin, Int. J. Pharmacol. 180 (1999) 113.
[15] J.A. Tickner, T. Schettler, T. Guidotti, M. McCally, M. Rossi, Am. J. Ind. Med. 39 (2001) 100.
[16] K. Kambia, T. Dine, B. Gressier, A.-F. Germe, M. Luyckx, C. Brunet, L. Michaud, F. Gottrand, J. Chromatogr. B 755 (2001) 297.
[17] K. Kambia, T. Dine, R. Azar, B. Gressier, M. Luyckx, C. Brunet, Int. J. Pharm. 229 (2001) 139.
[18] H.A.A.M. Dirven, P.H.H. van den Broek, M.C.E. Peeters, J.G.P. Peters, W.C. Mennes, B.J. Blaauboer, J. Noordhoek, F.J. Jongeneelen, Biochem. Pharmacol. 45 (1993) 2425.
[19] W.W. Huber, B. Grasl-Kraupp, R. Schulte-Hermann, Crit. Rev. Toxicol. 26 (1996) 365.
[20] J. Hellwig, H. Freudenberger, R. Jackh, Food Chem. Toxicol. 35 (1997) 501.
[21] J. Youssef, M. Badr, Crit. Rev. Toxicol. 28 (1998) 1.

[22] R.M. David, M.R. Moore, M.A. Cifone, D.C. Finney, D. Guest, Toxicoi. Sci. 50 (1999) 195.
[23] N.H. Kleinsasser, B.C. Wallner, E.R. Kastenbauer, H. Weissacher, U.A. Harräus, Teratog. Carcinog. Mutagen. 21 (2001) 189.
[24] P.B. Hoyer, Biochem. Pharmacol. 62 (2001) 1557.
[25] Beratergremium für umweltrelevante Altlasten (Ed.), Di-(2-ethylhexyl)phthalat, VCH, Weinheim, 1986, BUA-Stoffbericht 4.
[26] Beratergremium für umweltrelevante Altlasten (Ed.), Ergänzungsberichte 1, VCH, Weinheim, 1993, BUA-Stoffbericht 114.
[27] Beratergremium für umweltrelevante Altlasten (Ed.), Dibutylphthalat, VCH, Weinheim, 1987, BUA-Stoffbericht 22.
[28] Deutsche Forschungsgemeinschaft (Ed.), Toxikologisch-arbeitsmedizinische Begründung von MAK-Werten, Wiley–VCH, Weinheim, 2001.
[29] E.J. Sampson, L.L. Needham, J.L. Pirkle, W.H. Hannon, D.T. Miller, D.G. Patterson, J.T. Bernert, D.L. Ashley, R.H. Hill, E.W. Gunter et al., Clin. Chem. 40 (1994) 1376.
[30] J.L. Pirkle, E.J. Sampson, L.L. Needham, D.G. Patterson, D.L. Ashley, Environ. Health Perspect. 103 (Suppl. 3) (1995) 45.
[31] J. Angerer, K.H. Schaller, in: R.A. Meyers (Ed.), Encyclopedia of Environmental Analysis and Remediation (EEAR), Wiley, New York, 1998, p. 702, eight volume set.
[32] J. Angerer, Scand. J. Work Environ. Health 11 (Suppl. 1) (1985) 45.
[33] DFG, in: J. Angerer (Ed.), Biological Monitoring, Heutige und künftige Möglichkeiten in der Arbeits- und Umweltmedizin, Wiley–VCH, Weinheim, 2001.
[34] J. Angerer, J. Gündel, Ann. lst. Super. Sanita. 32 (1996) 199.
[35] P.W. Albro, R. Thomas, L. Fishbein, J. Chromatogr. 76 (1972) 321.
[36] P.W. Albro, S.R. Lavenhar, Drug Metab. Rev. 21 (1989) 13.
[37] P. Schmid, C. Schlatter, Xenobiotica 15 (1985) 251.
[38] P. Sjöberg, J. Chromatogr. 344 (1985) 167.
[39] H.A.A.M. Dirven, P.H.H. van den Broek, F.J. Jongeneelen, Int. Arch. Occup. Environ. Health 64 (1993) 555.
[40] B.C. Blount, K.E. Milgram, M.J. Silva, N.A. Malek, J.A. Reidy, L.L. Needham, J.W. Brock, Anal. Chem. 72 (2000) 4127.
[41] H.-D. Gilsing, J. Angerer, D. Prescher, Monatsh. Chem. 133 (2002) 1147.
[42] K. Larsen, Clin. Chim. Acta 41 (1972) 209.
[43] B.C. Blount, M.J. Silva, S.P. Caudill, L.L. Needham, J.L. Pirkle, E.L. Sampson, G.W. Lucier, R.J. Jackson, J.W. Brock, Environ. Health Perspect. 108 (2000) 979.