Addendum to: Di(2-ethylhexyl) phthalate (DEHP) metabolites in urine Mono-n-butyl phthalate (MnBP) and mono-iso-butyl phthalate (MiBP) in urine

Matrix:	Urine
Substance:	Di-n-butyl phthalate, diisobutyl phthalate
Analytical principle:	High-performance liquid chromatography with tandem mass spectrometric detection (LC–MS/MS)
Completed in:	November 2007

Overview of the parameters that can be determined with this method and the corresponding hazardous substances:

Hazardous substance	CAS	Parameter	CAS
Di-n-butyl phthalate	84-74-2	Mono-n-butyl phthalate	131-70-4
Diisobutyl phthalate	84-69-5	Mono-iso-butyl phthalate	30833-53-5

Summary

The procedure for the determination of Di-n-butyl phthalate (DnBP) and diisobutyl phthalate (DiBP) metabolites corresponds to the HPLC–MS/MS method for the determination of the main metabolites of Di(2-ethylhexyl) phthalate (DEHP) in the urine of occupationally or environmentally exposed persons. The present method is, therefore, an addendum to the procedure for the determination of the DEHP metabolites [Koch et al. 2007].

For calibration, aqueous standard solutions spiked with the target analytes [mono-n-butyl phthalate (MnBP) and mono-iso-butyl phthalate (MiBP)] and the internal standards (d_4-mono-n-butyl phthalate and d_4-mono-iso-butyl phthalate) are processed and analyzed in the same way as the urine samples.

Reliability data of the method

Mono-n-butyl phthalate

Within day precision:	Standard deviation (rel.)	s_w = 5.1 or 5.1%
	Prognostic range	u = 11.8 or 11.8%
	at a native concentration of 10.3 or 136 µg MnBP per litre urine and where n = 9 determinations	
Day to day precision:	Standard deviation (rel.)	s_w = 15.1 or 6.7%
	Prognostic range	u = 35.7 or 15.8%
	at a native concentration of 10.3 or 136 µg MnBP per litre urine and where n = 8 determinations	
Accuracy:	Recovery rate (rel.)	r = 99.6%
	at a nominal concentration of 50 µg MnBP per litre urine and where n = 8 determinations	
Detection limit:	1 µg MnBP per litre urine	
Quantitation limit:	3 µg MnBP per litre urine	

Mono-iso-butyl phthalate

Within day precision:	Standard deviation (rel.)	s_w = 6.5 or 8.1%
	Prognostic range	u = 15.0 or 18.7%
	at a native concentration of 4.7 or 21.5 µg MiBP per litre urine and where n = 9 determinations	
Day to day precision:	Standard deviation (rel.)	s_w = 14.8 or 11.3%
	Prognostic range	u = 35.0 or 26.7%
	at a native concentration of 4.7 or 21.5 µg MiBP per litre urine and where n = 8 determinations	
Accuracy:	Recovery rate (rel.)	r = 102.9%
	at a nominal concentration of 50 µg MiBP per litre urine and where n = 8 determinations	
Detection limit:	1 µg MiBP per litre urine	
Quantitation limit:	3 µg MiBP per litre urine	

General Information on mono-n-butyl phthalate and mono-iso-butyl phthalate

MnBP and MiBP are metabolites of DnBP and DiBP, which are plasticizers of industrial importance.

Di-n-butyl phthalate

The total production volume of DnBP was estimated to be 26,000 tons in the EU, in 1998. However, a falling trend can be observed over the preceding years: 49,000 tons/year (1994) – 37,000 tons/year (1997) – 26,000 tons/year (1998) [ECB 2003]. More recent data are available only for the sum of C4 phthalates (i.e. above

all DnBP and DiBP). The total consumption of these phthalates in the EU has continuously fallen from 35,000 tons/year (2005) to 25,000 tons/year (2008). For 2013 a consumption of 18,000 tons is predicted. At the same time the share of DnBP/DiBP in the overall phthalate market declined by half from 6% in 1998 to 3% in 2008 [Bizzari et al. 2009].

The major part of DnBP is used in the production of plastics and synthetic resins (mainly PVC and polyvinyl acetate). In addition, DnBP is used in printing inks, adhesives, sealants, thin-film coatings, car underbody coatings, and in nitrocellulose paints. DnBP is further applied as solvent for insecticides, peroxides, and other organic compounds. Moreover, it is used as anti-foaming and wetting agent in the textile industry. DnBP is often combined with other plasticizers [ECB 2003, Bizzari et al. 2009, EFSA 2005, Kavlock et al. 2002].

The widespread application of DnBP in consumer products is demonstrated by its use in cosmetic products: as perfume solvent and fixative, as suspension agent for solids in aerosols, as lubricant for aerosol valves, as antifoamer, as skin emollient, and as plasticizer in nail polish and hairspray. In a study by the US Food and Drug Administration (FDA) [Hubinger and Havery 2006] dibutyl phthalates (presumably DnBP) were found in 4 of 6 nail polish products with maximum concentrations of up to 59 g/kg (5.9%), in 3 of 8 hairsprays (up to 54 mg/kg), in 1 of 9 deodorants (104 mg/kg) and in 2 of 5 hair mousses (up to 43 mg/kg). In Europe, today the use of DnBP is prohibited in cosmetic products due to its classification as a reproductive toxicant (see below). Despite this ban, DnBP may still be detected in perfumes and cosmetic products in Europe up to date [SCCP 2007].

Furthermore, DnBP has been approved for its use as an adjuvant in medicinal products and also in food supplements. DnBP is commonly used in the enteric-coated encapsulation of essential oils, herbal extracts, enzymes, vitamins and iron species. After the capsules have passed the stomach DnBP controls the release of the contained active substance into the small intestine or into the colon. According to the German Medicines Compendium book of 2012 [Rote Liste der Arzneimittel und Arzneimittelzusatzstoffe), 11 preparations still contain DnBP or DiBP (generic term dibutyl phthalate (DBP)] as adjuvant [ROTE LISTE® 2012]. According to the Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, BfArM) [BfArM 2007], altogether 132 of such drugs were approved for use in Germany in 2007. The BfArM stated that the DBP content in these drugs was between 0.009 and 10.9 mg DBP per unit. Currently, however, the BfArM assumes that in 2012 most companies will develop new formulations that do no longer contain DBP as an adjuvant [BfArM 2011].

The toxicity of DnBP has been presented in detail in the MAK documentation from 2013 [Hartwig 2013]. DnBP has been classified in Carcinogen category 3B. Thus, a MAK value of 0.05 mL/m^3 or 0.58 mg/m^3 was established for DnBP [DFG 2014]. In June 2005, based on recent toxicological studies, the European Food Safety Authority (EFSA) lowered the TDI (tolerable daily intake) for DnBP from 100 µg/kg body weight day to 10 µg/kg body weight day with a margin of safety (MOS) of 200 [EFSA 2005].

As early as in 1999, the use of DnBP and other phthalates in certain toys and child care products was prohibited by the European Commission (1999/815/EG). In 2004, the ban was extended on all toys and child care articles (2004/781/EG). Further decisions were made in 2004 to ban its application in cosmetic products and to restrict its use in other consumer products such as paints and adhesives (Directive 2004/93/EG). However, the use of DBP in pharmaceutical drugs is still allowed.

After oral administration to laboratory animals, dibutyl phthalate is rapidly absorbed, metabolized and eliminated. In rats and mice, more than 90% of the oral dose is excreted within 24 to 48 hours as metabolites via urine. Excretion with the feces is low (1.0 – 8.2%). In humans, oral absorption is high as well. According to Koch et al. [2012] about 84% of an orally administered DBP dose is excreted within 24 hours in the urine as monoester metabolite MnBP (in free and glucuronidated form). Further oxidized metabolites increase the percentage of the renally excreted dose to more than 92% [Koch et al. 2012]. An in vitro study shows that dermal absorption of DnBP through the human skin (2.40 µg/cm$^{2)}$ h) is more slowly than through the rat skin (93.35 µg/cm$^{2)}$ h). No data are available for the absorption of the substance after inhalative exposure [ECB 2003].

A large part of the DnBP is hydrolyzed to MnBP and its corresponding alcohol already prior to absorption in the small intestine. Hydrolysis, however, can also take place in the liver and kidney or in the blood. The metabolites excreted via urine are MnBP, MnBP glucuronide and various ω- and ω-1-oxidation products of MnBP (possibly also partially glucuronidated).

The metabolism of DnBP (and DiBP) is represented in Figure 1.

Despite its potential susceptibility to exogenous contamination, the monoester MnBP is nevertheless the most suitable parameter for the determination of DnBP exposure to date. This is due to its relatively high concentration in urine.

Diisobutyl phthalate

The available data on DiBP is far less comprehensive than on DnBP. Additionally, many studies do not distinguish clearly between DnBP and DiBP and often only use the generic terms dibutyl phthalate (DBP) or monobutyl phthalate (MBP).

The production volume of DiBP in Germany was clearly below 10,000 tons in 1994. The domestic consumption was estimated to be under 5,000 tons/year [BUA 1997]. More recent data are not available or are only reported in association with DnBP (see above). After banning DnBP in some products, a substitution of DnBP by DiBP took place, which was reflected by a slight increase of the internal exposure to MiBP. This could be found in urine samples from the German Environmental Specimen Bank [Wittassek et al. 2007] and other samples taken from the general population [Koch and Calafat 2009].

Like DnBP, DiBP is used as a specialty plasticizer mainly in combination with other higher-molecular plasticizers. The properties of DiBP are very similar to those of DnBP. DiBP can therefore substitute DnBP in most, but not all applica-

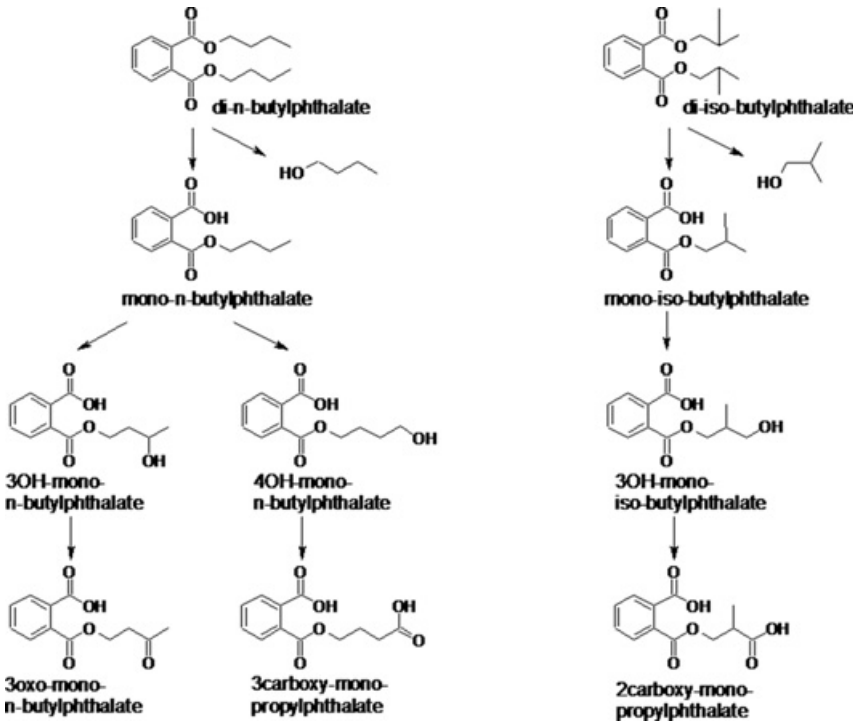

Figure 1 Metabolism of DnBP and DiBP (according to [ECB 2003, Koch et al. 2012]).

tions. Its possible applications range from plasticizing to the production of paints and adhesives. According to the monograph by Sears and Darby [1982], DiBP as plasticizer is suitable for the following plastics: polyvinyl chloride, polyvinyl acetate, polyvinyl butyrate, cellulose nitrate, cellulose acetate, ethyl cellulose, polyurethane, acrylates, chlorinated rubber and nitrile rubber.

In Germany in 1996, about 40% of the DiBP was used as adhesives ingredient, 21.5% as PVC plasticizer, 6% as plasticizer for rubber, 2% as plasticizer for dispersions (Latex), 10% as stabilizer for organic peroxides, and 0.5% in car underbody coatings [BUA 1997]. Owing to its toxicity, DiBP must no longer be used in cosmetic and personal care products in the EU. However, DiBP is not one among the six phthalates whose use in children's toys is prohibited.

A MAK documentation on the toxicity of DiBP is not available to date. So far an assessment on DiBP from other scientific boards is not available either. The toxicity classification of the two phthalates according to EU criteria and the restrictions of use are shown in Table 1.

A current study on the metabolism of DiBP in humans is published by Koch et al. [2012]. After oral administration of DiBP, 70.3% of the dose was recovered in the urine as its monoester metabolite MiBP within 24 hours. About 20% of the dose

Table 1 Classification of DnBP and DiBP according to Annex I of Council Directive 67/548/EWG and, with regard to their reproductive and developmental toxicity and restrictions, according to Directive 2005/84/EG.

Phthalate	Year	Reproduction	Development	Restrictions acc. to 2005/84/EG
DiBP	2009[a]	Kat. 3 (R 62)	Kat. 2 (R 61)	–
DnBP	2001[b]	Kat. 3 (R 62)	Kat. 2 (R 61)	+

R 61: may cause harm to the unborn child.
R 62: possible risk of impaired fertility.
[a] Directive 2009/2/EG (31st adaptation directive)
[b] Directive 2001/59/EG (28th adaptation directive)

was excreted as oxidized metabolite (2-hydroxy-MiBP). Altogether, more than 90% of the dose was metabolized and excreted in the urine within 24 hours. After dermal application of ^{14}C-labeled DiBP on rats, 50 – 60% of the dose is excreted with

Table 2 Overview of some biomonitoring studies and the determined urinary concentrations of MnBP and MiBP in the general population.

Study	Country	Number (Age [y])	MnBP [µg/L]	MiBP [µg/L]
			Median (95th percentile)	
Environmental Speci-men Bank 1988 – 2003 [Wittassek et al. 2007]	Germany	634 (20 – 29)	112 (604)	34.5 (176)
Environmental Survey for Children 2002/2003 [Koch et al. 2007]	Germany	254 (3 – 14)	166 (624)	n.a.
General population 2003 [Koch et al. 2003]	Germany	85 (7 – 63)	181 (825)	n.a.
Environmental Survey for Children 2003 – 2006 [Becker et al. 2011]	Germany	599 (3 – 14)	93.4 (166)	88.1 (308)
Environmental Speci-men Bank 2002 – 2008 [Göen et al. 2011]	Germany	240 (19 – 29)	32.8 (132)	28.3 (107)
NHANES 2001 – 2002 [CDC 2012]	USA	2782 (from 6)	20.4 (108)	2.7 (17.9)
NHANES 2003 – 2004 [CDC 2012]	USA	2605 (from 6)	23.2 (122)	4.2 (21.3)
NHANES 2005 – 2006 [CDC 2012]	USA	2548 (from 6)	20.6 (107)	5.8 (36.1)
NHANES 2007 – 2008 [CDC 2012]	USA	2604 (from 6)	20.0 (110)	8.0 (39.1)
General population [Itoh et al. 2005]	Japan	36 (4 – 70)	43	n.a.

* NHANES: National Health and Nutrition Examination Survey, n.a.: not analysed.

the urine [Elsisi et al. 1989]. The metabolism of DiBP is basically analogous to that of DnBP (see Figure 1), the share of oxidized metabolites, however, being clearly higher for DiBP (more than 20%) than for DnBP (below 9%). First results of biomonitoring studies show that, in addition to MiBP, these oxidative metabolites can be detected in the urine of the general population [Koch et al. 2003, Koch et al. 2012, Kasper-Sonnenberg et al. 2012].

Biomonitoring studies exploring the metabolites of the dibutyl phthalates in the general population yielded the urinary metabolite concentrations given in Table 2.

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Addendum to: Di(2-ethylhexyl) phthalate (DEHP) metabolites in urine Mono-n-butyl phthalate (MnBP) and mono-iso-butyl phthalate (MiBP) in urine

Matrix:	Urine
Substance:	Di-n-butyl phthalate, diisobutyl phthalate
Analytical principle:	High-performance liquid chromatography with tandem mass spectrometric detection (LC–MS/MS)
Completed in:	November 2007

Contents

1 General principles

The urine samples are buffered to pH 6.5 and subjected to enzymatic hydrolysis, together with the deuterium-labeled internal standard. An arylsulfatase-free ß-glucuronidase is used for hydrolysis. After separation of possible precipitates by centrifugation, the hydrolysate is injected into the LC–MS/MS system. The analytes are concentrated and higher molecular matrix compounds and more polar substances are separated using a RAM phase column. By employing a backflush, the analytes are rinsed off onto an analytical phenyl-hexyl column where they are chromatographically separated. Tandem mass spectrometry allows for highly selective detection of the analytes. For calibration, aqueous standard solutions with known concentrations of MnBP and MiBP are used and processed in the same way as the urine samples. For quality control, pooled urines containing high and low native MnBP and MiBP levels were used.

2 Equipment, chemicals and solutions

2.1 Equipment

See the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2007].

2.2 Chemicals

- Mono-n-butyl phthalate (MnBP, synthesis according to Dirven et al. [1993], >95% or e.g. Cambridge Isotope Laboratories (Andover, USA), No. ULM-6148)
- Mono-iso-butyl phthalate (MiBP, synthesis according to Dirven et al. [1993], >95% or e.g. Cambridge Isotope Laboratories (Andover, USA), No. ULM-7919)
- d_4-Mono-n-butyl phthalate (d_4-MnBP, ring-labeled, synthesis according to Dirven et al. [1993], >95%) or $^{13}C_4$-MnBP e.g. Cambridge Isotope Laboratories (Andover, USA), No. CLM-4590

- d_4-Mono-iso-butyl phthalate (d_4-MiBP, ring-labeled, synthesis according to Dirven et al. [1993], >95%)
- ß-Glucuronidase E. coli K12, 50% (v/v) in glycerin, ~100 U/mL (e.g. Roche, No. 03707598001)

For further information see the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2008].

2.3 Solutions

See the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2007].

Eluents for HPLC
See the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2007].

2.4 Internal Standards

- Stock solution of the internal standards (0.2 g/L):
  About 5 mg d_4-MnBP and 5 mg d_4-MiBP are weighed exactly into a 25 mL volumetric flask. The flask is then made up to the mark with acetonitrile.
- Working solution of the internal standards (2 mg/L):
  1 mL of the stock solution of the internal standards is pipetted into a 100 mL volumetric flask. The flask is then made up to the mark with ultrapure water.

The solutions are stored at –18°C in screw cap glass vials with Teflon lined caps.

2.5 Calibration Standards

- Stock solution of the analytes (0.4 g/L):
  10 mg MnBP and 10 mg MiBP are weighed exactly into a 25 mL volumetric flask. The flask is then made up to the mark with acetonitrile.
- Working solution I (WS I, 2 mg/L):
  0.5 mL of the stock solution of the analytes is transferred to a 100 mL volumetric flask and made up to the mark with ultrapure water.
- Working solution II (WS II, 200 µg/L)
  20 mL of working solution I are pipetted into a 200 mL volumetric flask. The flask is then made up to the mark with ultrapure water.
- Working solution III (WS III, 20 µg/L)
  20 mL of working solution II are pipetted into a 200 mL volumetric flask. The flask is then made up to the mark with ultrapure water.

The solutions are stored at –18°C in screw cap glass vials with Teflon-lined caps.

Calibration standards are prepared in ultrapure water, containing analyte levels between 1.0 and 1000 µg/L. The pipetting scheme is given in Table 3.

Table 3 Pipetting scheme for the preparation of calibration standards in water.

Volume of the working solutions	Ultrapure water [mL]	Final volume of the calibration standard [mL]	Analyte level in the calibration standard [µg/L]
50 mL WS I	50	100	1000
25 mL WS I	75	100	500
10 mL WS I	90	100	200
25 mL WS II	75	100	50
10 mL WS II	90	100	20
5 mL WS II	95	100	10
20 mL WS III	80	100	4
5 mL WS III	95	100	1

3 Specimen collection and sample preparation

3.1 Specimen collection

See the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2007].

3.2 Sample preparation

See the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2007].

It should be ensured that an arylsulfatase-free ß-glucuronidase is used for hydrolysis, to avoid any MnBP/MiBP release from the ubiquitous DnBP/DiBP. Moreover, the effect of pH on the hydrolysis step should be tested. The optimum pH for the hydrolysis using the Escherichia coli K12 ß-glucuronidase by the supplier mentioned was found to be pH 6.5. When other ß-glucuronidases are used, the pH optimum may be checked. Additionally, when changing the amount of urine or buffer used, the pH value should be checked, the amount of enzyme adjusted and the completeness of the hydrolysis ensured.

4 Operational parameters

4.1 High-performance liquid chromatography

See the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2007].

4.2 Tandem mass spectrometry

See the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2007].

Quadrupole settings:

Q1 Resolution:	Unit
Q3 Resolution:	Low
Settling time:	5 msec
MR pause:	5 msec
Collision gas Q2:	Nitrogen, 4 instrumental units
Scan time:	75 msec
Scan type:	MRM (Multi-Reaction-Mode)
Dwell time:	300 msec

Parameter-specific settings:

see Table 4

Table 4 MRM parameters for the analytes and internal standards (DP = declustering potential, FP = focussing potential, EP = entrance potential, CE = collision energy).

Analyte	Q1 [m/z]	Q3 [m/z]	DP [V]	FP [V]	EP [V]	CE [V]
MiBP	221	77.1*	–16	–320	–9.5	–26
		121.1	–21	–350	–6.5	–20
		134.1	–21	–330	–6.5	–20
MnBP	221	77.1*	–16	–320	–9.5	–26
		121.1	–21	–350	–6.5	–20
		134.1	–21	–330	–6.5	–20
d4-MiBP	225	81.1*	–6	–340	–9	–24
d4-MnBP	225	81.1*	–6	–340	–9	–24

The ion transitions used for quantitation are marked with an asterisk (*).

All ion sources and MRM parameters are instrument specific and need to be individually adjusted by the user via the corresponding calibration routines of the MS/MS system.

The fragment patterns of MnBP, d_4-MnBP, MiBP and d_4-MiBP in Q1 are shown in Figure 2 (in the Appendix).

5 Analytical Determination

100 µL each of the urine samples prepared according to Section 3, are injected into the HPLC system. The time courses of the ion transitions listed in Table 5 are recorded in the MRM mode of the tandem mass spectrometer (ESI negative mode).

The retention times given in Table 5 are intended to be a rough guide only. Users

Table 5 Retention times and detected ion transitions.

Analyte	Retention time [min]	Q1 [m/z]	Q3 [m/z]
MiBP	12.99	221	77.1*
			121.1
			134.1
MnBP	13.18	221	77.1*
			121.1
			134.1
d4-MiBP	12.92	225	81.1*
d4-MnBP	13.12	225	81.1*

The ion transitions used for quantitation are marked with an asterisk (*).

of the method must ensure proper separation performance of the analytical columns used and the resulting retention behavior of the analytes. If necessary, the analytical separation of the two isomeric monoesters on the respective analytical column is to be optimized by adjusting the gradient and the solvent mixture. Figure 3 (in the Appendix) shows the chromatogram of a processed native urine sample.

6 Calibration

See the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2007].

Samples showing concentrations above the calibration range (>1000 µg/L) are diluted with ultrapure water, re-processed and analyzed again. A reagent blank is run

with each set of samples, using ultrapure water instead of urine. The calibration graphs are linear between the detection limit and analyte levels of 1000 µg/L water.

7 Calculation of the analytical results

See the original method published in "The MAK Collection for Occupational Health and Safety Part IV: Biomonitoring Methods" [Koch et al. 2007].

For both analytes a reagent blank around the quantitation limit may occur. The blank values are accounted for by subtraction.

8 Standardization and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the Bundesärztekammer (German Medical Association) and in a general chapter of the MAK-Collection for Occupational Health and Safety Part IV: Biomonitoring Methods [Bundesärztekammer 2008, Bader et al. 2010]. For quality control, two quality control samples with a low and a high analyte level are analyzed within each analytical run. As quality control material is not commercially available, it must be prepared in the laboratory. For this purpose, native urine samples with different analyte levels can be taken. If such native material is not available, pooled urine can be spiked with defined amounts of the analyte standards. Aliquots of these solutions are stored at −20°C and included as quality control samples in each analytical run. The nominal value and the tolerance ranges of this quality control material are determined in a pre-analytical period (one analysis of the control material on each of 10 different days) [Bader et al. 2010].

9 Evaluation of the method

9.1 Precision

To determine the within day precision, two native urine samples (creatinine levels of 0.7 and 1.4 g/L) with different analyte concentrations were prepared and analyzed nine times in a row to obtain the within day precision documented in Table 6.

Table 6 Within day precision for the determination of MnBP and MiBP in urine (n = 9).

Analyte	Mean value [µg/L]	Standard deviation (rel.) [%]	Prognostic range [%]
MnBP	10.3	5.1	11.8
	136.2	5.1	11.8
MiBP	4.7	6.5	15.0
	21.5	8.1	18.7

For the determination of the day to day precision, the same sample material was used as for determining the within day precision. The quality control samples were prepared and analyzed on eight different days. The resulting precision data are shown in Table 7.

Table 7 Day to day precision for the determination of MnBP and MiBP in urine (n = 8).

Analyte	Mean value [µg/L]	Standard deviation (rel.) [%]	Prognostic range [%]
MnBP	10.3	15.1	35.7
	136.2	6.7	15.8
MiBP	4.7	14.8	35.0
	21.5	11.3	26.7

9.2 Accuracy

Accuracy of the method was determined by carrying out recovery experiments. For this purpose, eight individual urine samples (creatinine levels from 0.4 to 2.6 g/L) were spiked with 50 µg/L MnBP and MiBP each. The spiked and unspiked urine samples were then processed and analyzed. The background levels of MnBP ranged between 8.5 and 102 µg/L, those of MiBP between 4.7 and 54.7 µg/L. The relative recovery rates obtained are given in Table 8.

Table 8 Mean relative recovery rates for MnBP and MiBP in eight individual urine samples.

Analyte	Spiked analyte level [µg/L]	Mean rel. Recovery [%]	Range [%]
MnBP	50	99.6	87.6 – 122.3
MiBP	50	102.9	97.3 – 111.4

9.3 Detection and quantitation limits

Under the mentioned conditions of sample preparation and high-performance liquid chromatography, the detection limit of MnBP and MiBP was 1.0 µg/L urine. The detection limit was estimated on the basis of a signal to noise ratio of 3 : 1. The quantitation limit was 3.0 µg/L urine estimated on the basis of a signal to noise ratio of 9 : 1.

9.4 Sources of error

DnBP and DiBP as well as their monoesters are ubiquitous chemicals in the environment, but may occur in the laboratory as well. To avoid high analytical blank values or, in the case of actually occurring high blank values, laboratory equipment and chemicals used need to be tested for DnBP, DiBP and their monoesters. As DnBP and DiBP can occur as additives in various personal care and cosmetic products or in perfumes and deodorants, this possible way of contamination needs to be taken into account if high blank values are observed. The examiners of the method reported reagent blank values (after processing water or buffer in place of urine) of up to 2 µg monoester per liter. In addition, during examination of the method (from 2006 to 2008), the blank values for MnBP were found to decrease throughout that period (to about 0.5 µg/L at present), whereas the blank values for MiBP constantly increased (to about 1.5 µg/L at present). This may be related to the currently ongoing substitution of DnBP by DiBP. Due to the fact that the urinary background levels in the general population are, as a rule, clearly more than ten times higher, blank values seems to be a minor source of error. Exogenous contamination of the sample material might theoretically occur during sampling via phthalate diesters and phthalate monoesters adherent to the skin. This effect, however, cannot be controlled by quality assurance measures within the laboratory. Therefore, an additional determination of secondary, oxidative metabolites of DiBP and DnBP may be necessary to unambiguously confirm high MnBP and MiBP findings, prospectively (in analogy to the DEHP procedure).

In spite of direct urine sample injection on the RAM phase, the method proved to be extremely robust. In individual cases, protein precipitation may result in an increase of the RAM phase back-pressure. This can be avoided by prolonged isocratic washing with the eluent after sample injection onto the RAM phase (up to 10 min at a flow of 1.5 mL/min). Soiled RAM phases can be rinsed off by purging with protease.

When using ß-glucuronidases of Escherichia coli K12 from other manufacturers the volume applied need to be adjusted to the enzyme concentration.

10 Discussion of the method

The method enables the reliable and accurate determination of MnBP and MiBP in the concentration ranges relevant for environmental and occupational medicine. The analysis of MnBP and MiBP can easily be combined with the determination of the DEHP metabolites in urine. The reliability criteria are regarded as good not least because of the use of isotope-labeled internal standards. Metabolites of other relevant phthalates may be easily included in the method.

The analytical column and the operational parameters should ensure a satisfying separation of the two monobutyl phthalate isomers. In addition to the Luna phenyl-hexyl column (150 × 4 mm; 3 µm; Phenomenex) described, a baseline separation of the two isomers could be achieved during method examination using an Atlantis™ dC18 column (150 × 2.1 mm; 3 µm; Waters) in combination with a slight variation of the elution gradient and flow.

Variations of the present method are also possible with regard to the RAM phase used. While, with the relatively polar metabolites (such as the simple monoesters MnBP and MiBP), the RAM phase (ADS 18 or ADS 8) exhibits excellent retention power and matrix separation, RAM phases are limited when the method is extended to cover more polar oxidized metabolites (such as 3-carboxy-mono-n-propyl phthalate, the carboxylated metabolite of DnBP) or polar monoester metabolites of low-molecular phthalates (diethyl phthalate, dimethyl phthalate).

In this case, polymer phases (such as Oasis HLB™, Waters or CapCell PAK™, Shiseido) are advantageous for polar metabolites; however their matrix separation performance is worse.

Instruments used:

LC–MS/MS system consisting of LC HP 1100 with an autosampler, quaternary pump and eluent degassing device (Hewlett-Packard, USA), equipped with an injection system up to a sample volume of 900 µL and an isocratic pump L6000A (Merck-Hitachi), a tandem mass spectrometric detector API 2000 (Applied Biosystems) with 10-way valve and Turbo IonSpray Interface. The Analyst Software from Applied Biosystems was used for evaluation.

11 References

Bader M, Barr D, Göen Th, Schaller KH, Scherer G, Angerer J, Working group Analyses in Biological Materials (2010): Reliability criteria for analytical methods. In: Angerer J and Hartwig A (eds): The MAK-Collection for Occupational Health and Safety Part IV: Biomonitoring Methods, Vol. 12, Wiley-VCH, Weinheim
DOI: 10.1002/3527600418.bireliabe0012

Becker H, Herzberg F, Schulte A, Kolossa-Gehring M (2011): The carcinogenic potential of nanomaterials, their release from products and options for regulating them. *Int. J. Hyg. Environ. Health* 214, 231–238

BfArM (Bundesinstitut für Arzneimittel und Medizinprodukte) (2007): personal communication of 25. July 2007, Reference No.: 75.01-3822-V8254-170564/07

BfArM (Bundesinstitut für Arzneimittel und Medizinprodukte) (2011): Ergebnisprotokoll für die 69. Routinesitzung nach § 63 AMG am 22. November 2011

Bizzari SN, Blagoev M, Kishi A (2009): Plasticizers. SRI Consulting. Chemical Economics Handbook Marketing Research Report
URL: www.sriconsulting.com

BUA (Beratergremium für umweltrelevante Altstoffe der GDCh) (Hrsg.) (1997): BUA-Stoffbericht 201: Diisobutylphthalat. ISBN 978-3-7776-0788-7, BUA-Stoffberichte, S. Hirzel Verlag

Bundesärztekammer (2008): Richtlinien der Bundesärztekammer zur Qualitätssicherung laboratoriumsmedizinischer Untersuchungen. *Dt. Ärztebl.* 105, A341–355

CDC (Centers for Disease Control and Prevention) (2012): Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables, September 2012.
URL: http://www.cdc.gov/exposurereport/

DFG (Deutsche Forschungsgemeinschaft) (2014): List of MAK and BAT Values 2014, Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Report No. 50, Wiley-VCH, Weinheim DOI: 10.1002/9783527682027

Dirven HAAM, Van Den Broek PHH, Peeters MCE, Peters JGP, Mennes WC, Blaauboer BJ, Noordhoek J, Jongeneelen FJ (1993): Effects of the peroxisome proliferator mono(2-ethylhexyl)phthalate in primary hepatocyte cultures derived from rat, guinea pig, rabbit and monkey. Relationship between interspecies differences in biotransformation and peroxisome proliferating potencies. *Biochem. Pharmacol.* 45, 2425–2434

ECB (European Commission – Joint Research Centre – Institute for Health and Consumer Protection – European Chemicals Bureau) (2003): European Union Risk Assessment Report – Dibutyl Phthalate. Series: 1st Priority List, Volume: 29, EUR 19840 EN (with addendum 2004) URL: www.dbp-facts.com/upload/documents/document30.pdf [accessed 5th of December 2012].

EFSA (European Food Safety Authority) (2005): Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Material in Contact with Food (AFC) on a request from the Commission related to Di-Butylphthalate (DBP) for use in food contact materials – Question N° EFSA-Q-2003-192. The EFSA Journal 242, 1–17

Elsisi AE, Carter DE, Sipes IG (1989): Dermal absorption of phthalate diesters in rats. *Fundam. Appl. Toxicol.* 12, 70–77

Göen Th, Dobler L, Koschorreck J, Müller J, Wiesmüller GA, Drexler H, Kolossa-Gehring M (2011): Trends of the internal phthalate exposure of young adults in Germany – follow-up of a retrospective human biomonitoring study. *Int. J. Hyg. Environ. Health* 215, 36–45

Hartwig A (ed) (2013): Di-n-butyl phthalate. The MAK-Collection for Occupational Health and Safety Part I: MAK Value Documentations, Volume 20, Wiley-VCH, Weinheim
DOI: 10.1002/3527600418.mb8474yole4813

Hubinger JC and Havery DC (2006): Analysis of consumer cosmetic products for phthalate esters. *J. Cosmet. Sci.* 57, 127–137

Itoh H, Yoshida K, Masunaga S (2005): Evaluation of the effect of governmental control of human exposure to two phthalates in Japan using a urinary biomarker approach. *Int. J. Hyg. Environ. Health* 208, 237–245

Kasper-Sonnenberg M, Koch HM, Wittsiepe J, Wilhelm M (2012): Levels of phthalate metabolites in urine among mother–child-pairs – Results from the Duisburg birth cohort study, Germany. *Int. J. Hyg. Environ. Health* 215, 373–382

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, et al. (2002): NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-n-butyl phthalate. *Reprod. Toxicol.* 16, 489–527

Koch HM, Drexler H, Angerer J (2003): An estimation of the daily intake of di(2-ethylhexyl) phthalate (DEHP) and other phthalates in the general population. *Int. J. Hyg. Environ. Health* 206, 77–83

Koch HM, Becker K, Wittassek M, Seiwert M, Angerer J, Kolossa-Gehring M (2007): Di-n-butylphthalate (DnBP) and Butylbenzylphthalate (BBzP) – urinary metabolite levels and estimated daily intakes: pilot study for the German Environmental Survey on children (GerES IV). *J. Expo. Sci. Environ. Epidemiol.* 17, 378–387

Koch HM, Angerer J, Bittner N, Trösken ER, Völkel W, Working group Analyses in Biological Materials: Di(2-ethylhexyl) phthalate (DEHP) metabolites (2-ethyl-5-hydroxyhexyl phthalate (5OH-MEPH), 2-ethyl-5-oxohexyl phthalate (5oxo-MEPH), mono(2-ethylhexyl) phthalate (MEHP)). In: Angerer J and Hartwig A (eds) (2007): The MAK-Collection for Occupational Health and Safety Part IV: Biomonitoring Methods, Vol. 11, Wiley-VCH, Weinheim
DOI: 10.1002/3527600418.bi11781e0011

Koch HM and Calafat AM (2009): Human body burdens of chemicals used in plastic manufacture. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 364, 2063–2078

Koch HM, Christensen KLY, Harth V, Lorber M, Brüning Th (2012): Di-n-butyl phthalate (DnBP) and diisobutyl phthalate (DiBP) metabolism in a human volunteer after single oral doses. *Arch. Toxicol.* 86, 1829–1839

ROTE LISTE® (Arzneimittelverzeichnis für Deutschland (einschließlich EU-Zulassungen und bestimmter Medizinprodukte)) (2012): Rote Liste® Service GmbH, Frankfurt/Main.
URL: www.rote-liste.de

SCCP (Scientific Committee on Consumer Products) (2007): Opinion on Phthalates in Cosmetic Products. 11th plenary meeting on 21 March 2007. DG SANCO-European Commission, Health & Consumer Protection Directorate-General

Sears JK and Darby JR (1982): The technology of plasticizers. SPE Monograph Series, Wiley, New York, pp. 1166

Wittassek M, Wiesmüller GA, Koch HM, Eckard R, Dobler L, Müller J, Angerer J, Schlüter C (2007): Internal phthalate exposure over the last two decades – A retrospective human biomonitoring study Int. *J. Hyg. Environ. Health* 210, 319–333

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11 Appendix

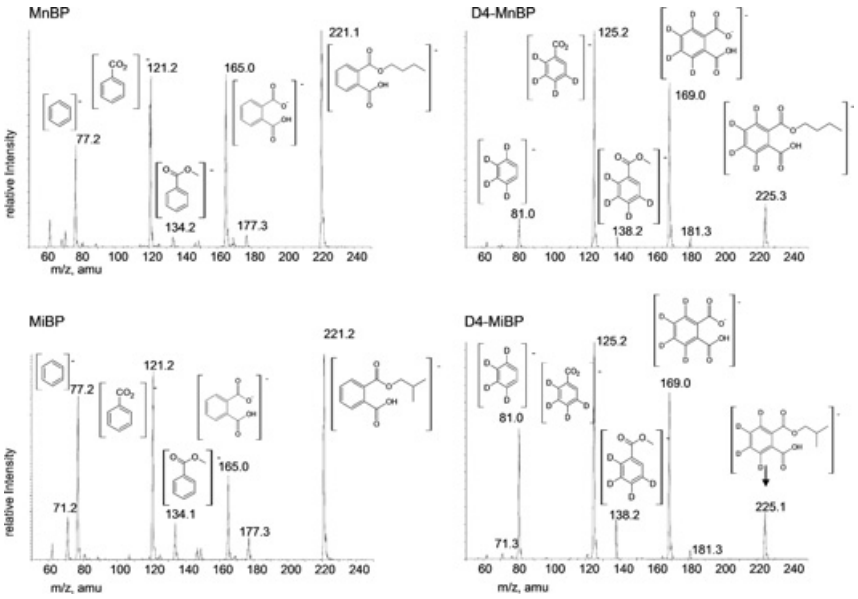

Figure 2 ESI negative Q1 mass spectra for MnBP, d_4-MnBP, MiBP and d_4-MiBP with the corresponding fragment structures.

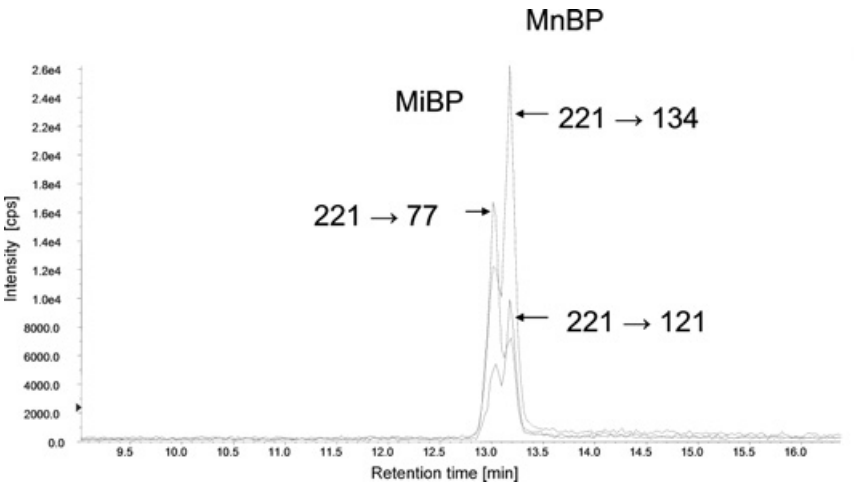

Figure 3 Chromatogram of a processed urine sample (creatinine: 1.32 g/L) showing all three transitions of the analytes MiBP and MnBP. The determined concentrations were as follows: MiBP 36,7 µg/L and MnBP 59.3 µg/L.