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Quantification of biomarkers of environmental exposure to di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH) in urine via HPLC–MS/MS

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ABSTRACT

Di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH) is a major substitute for some high molecular weight phthalates that adversely affect reproductive function. Like for the phthalates a broad exposure of the population has to be expected. We postulated the DINCH monoester (MINCH) and secondary oxidized metabolites (OH-MINCH, cx-MINCH and oxo-MINCH) as human metabolites and possible biomarkers of DINCH exposure. We developed an on-line HPLC–MS/MS method for their determination in human urine. Identification was performed with authentic standard substances and quantification via isotope dilution. The analytical method is highly selective and sensitive with limits of quantification (LOQ) between 0.05 μg/l and 0.1 μg/l. In a pilot study with 22 volunteers from the general German population oxidized DINCH metabolites were found in above 80% of the samples. OH-MINCH was most abundant (mean 0.71 μg/l; maximum 3.69 μg/l) followed by cx-MINCH (0.61 μg/l; 2.82 μg/l) and oxo-MINCH (0.33 μg/l; 1.05 μg/l). All three oxidized metabolites correlated strongly among each other ($\rho \geq 0.76$). MINCH was detected in one sample only and has to be regarded a weak marker of exposure. With this analytical method we are able to perform human metabolism studies to provide metabolic conversion factors and to investigate the extent of DINCH exposure in the general population.

1. Introduction

Di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH) has been commercially introduced in 2002 as a substitute for high molecular weight phthalate plasticizers like di(2-ethylhexyl) phthalate (DEHP) or diisononyl phthalate (DINP) [1–3]. These phthalates have been under intensive scrutiny for reproductive toxicity and have been already banned from products like toys and childcare articles by the European Union (EU) [4–6]. According to the current toxicological data, DINCH is no endocrine disruptor and no reproductive toxicant; only at high dose levels in rats thyroid hyperplasia and signs of renal toxicity are observed [7]. DINCH is manufactured from DINP by catalytic hydrogenation of its aromatic ring. Of the two DINP types on the market (DINP1 and DINP2), for DINCH only DINP2 (BASF SE tradename Palatinol® N [8]) is used. The alkyl side-chains of DINCH are therefore the same as in DINP2. Thus, the alkyl side chain of DINCH consists of 9 carbon atoms, and not of 8–10 carbon atoms as in DINP1 [8]. The isononyl side-chain consists of approximately 10% n-nonyl, 35–40% methyloctyl, 40–45% dimethylheptyl and 5–10% methylethylhexyl isoforms [8]. Hydrogenation of the planar aromatic ring generates a non-planar cyclohexane-1,2-dicarboxylate moiety of DINCH with cis- and trans-configurations of the alkyl side chains [9]. The typical commercial product is composed of approximately 90% cis- and 10% trans-DINCH isomers [10].

As a high molecular weight phthalate substitute DINCH is utilized in various polyvinyl chloride (PVC) products [11]. In the EU DINCH has been approved for sensitive applications such as medical devices, toys and food packaging [12]. As a so-called external plasticizer, DINCH is not chemically bound to the polymer but only physically dissolved in it. Like the phthalates, DINCH can migrate of the polymers it is used in [13]. For the use of DINCH in food contact materials a maximum migration limit of 10 mg/dm^2 has been set by the EU [7]. As a consequence of the above we have to expect the general population to be exposed to DINCH via foodstuff or the use DINCH containing products.

Because of the structural relation between DINP2 and DINCH and our previous experiences in human DINP metabolism and method development [14,15] we postulated cyclohexane-1,2-dicarboxylic acid (CHDA), cyclohexane-1,2-dicarboxylic monoisononyl ester (MINCH) and especially the secondary oxidized metabolites cyclohexane-1,2-dicarboxylic mono hydroxyisononyl ester (OH-MINCH), cyclohexane-1,2-dicarboxylic mono carboxyisooctyl ester (cx-MINCH) and cyclohexane-1,2-dicarboxylic mono oxoisononyl ester (oxo-MINCH) to be putative human DINCH metabolites as well (Fig. 1). Therefore our postulated

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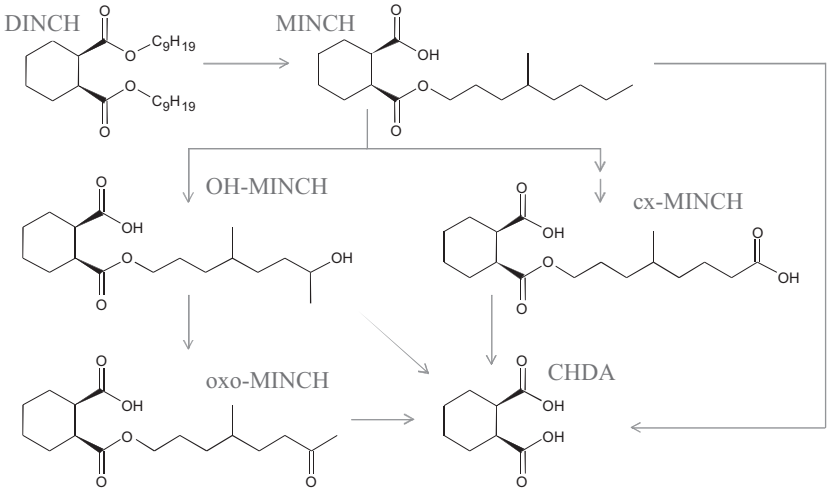

Fig. 1. Postulated DINCH metabolism in humans, displayed with a 4-methyl-1-octyl-sidechain.

metabolites are promising biomarkers for a human biomonitoring method [16,17]. A recent study tentatively identified these metabolites in the urine of rats after high oral dosages of DINCH [18]. We developed a selective and sensitive method for the determination of the potential DINCH biomarkers MINCH, OH-MINCH and cx-MINCH in urine based on authentic standards and isotope-labeled internal standards for isotope dilution quantification via high performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS). Oxo-MINCH was determined on semi quantitative basis in analogy to OH-MINCH. Therefore, in the collaboration project of the German Ministry of Environment (BMU) and the German Chemical Industry Association (VCI) DINCH was chosen as one of the first substances to develop a human biomonitoring method and to determine the exposure of the general population based on reliable urinary biomarkers of exposure [19].

2. Experimental

2.1. Chemicals

Hexamoll® DINCH (cyclohexane-1,2-dicarboxylic acid, diisononylester CAS No. 166412-78-8 (US only CAS No. 474919-59-0), purity ≥99.5%) was provided by BASF SE (Germany). DINP2 is used for the production of DINCH and therefore does only contain C9 moiety in the alkyl side chain. Cis/trans-cyclohexane-1,2-dicarboxylate-mono-4-methyloctyl ester (cis/trans MINCH), cis/trans-cyclohexane-1,2-dicarboxylate-mono-(7-carboxylate-4-methyl)heptyl ester (cis/trans cx-MINCH), cis/trans-cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl)octyl ester (cis/trans OH-MINCH) and there side-chain labeled analogous structures cis/trans-cyclohexane-1,2-dicarboxylate-mono-D2-4-methyloctyl ester (cis/trans D2-MINCH), cis/trans-cyclohexane-1,2-dicarboxylate-mono-D2-(7-carboxylate-4-methyl)heptyl ester (cis/trans D2-cx-MINCH), cis/trans-cyclohexane-1,2-dicarboxylate-mono-D4-(7-hydroxy-4-methyl)octyl ester (cis/trans D4-OH-MINCH) were synthesized by Dr. Belov, Max-Planck Institute for Biophysical Chemistry, Germany. All synthesized compounds had a purity ≥95% determined by ^{1}H NMR. The deuterated compounds had no detectable impurities of unlabeled and partially labeled compounds. Water and acetonitrile with a high purity (HPLC/MS grade), ammonium acetate p.a. and acetic acid (glacial, extra pure) were purchased from Merck, Darmstadt, Germany. K12 β-glucuronidase was purchased from Roche Biomedical, Mannheim, Germany.

2.2. Standard preparation

Standard stock solutions were prepared by dissolving approximately 10 mg MINCH, cx-MINCH and OH-MINCH weighed exactly in 10 ml acetonitrile. Stock solutions were stored at −20 °C in teflon capped glass vials until further use. For further analysis eight calibration standards were prepared by gradual dilution to final concentrations ranging from 0.05 μg/l to 60 μg/l. The internal stock solution was prepared by dissolving approximately 10 mg of each deuterated metabolite weighed exactly in acetonitrile. Volumetric flasks were used to adjust the concentration of the internal standard mixtures to 2 mg/l.

2.3. Sample collection and preparation

Urine samples were collected in 250 ml polyethylene containers and immediately stored at −20 °C. Before analysis, all samples were equilibrated to room temperature. Samples were vortex mixed prior to transferring 300 μl aliquots to 1.8 ml glass screw-cap vial. To each sample 100 μl 1 M ammonium acetate at pH 6.0, 10 μl internal standard solution and 6 μl β-glucuronidase (diluted 1:1 with 1 M ammonium acetate buffer at pH 6.0) were added. After incubation at 37 °C in a water bath for 2 h, 10 μl of acetic acid were added to adjust the pH value. All samples were frozen for at least 3 h to precipitate cryophobic proteins, thawed and centrifuged with 1900 × *g* for 10 min. The supernatant was transferred to a second same shaped vial. Urinary creatinine concentrations were determined according to Jaffe [20].

2.4. Calibration procedure and quantification

Calibration was performed with standard solutions in water. Each calibration sample was processed as the urine samples; the exact procedure is described in Section 2.3. Linear calibration curves with a 1/*x* weighting were obtained by plotting the quotients of peak area of DINCH metabolites and the specific internal standard as a function of concentration.

2.5. Quality control

We prepared within laboratory control material from urine samples from an oral dosage study with 50 mg DINCH (IRB approval, reg. no. 3866-10). We pooled these urine samples from different volunteers to obtain control material with low and high concentrations for all selected DINCH metabolites (Q_{low} and Q_{high}). The

Table 1
LC program for chromatographic separation (binary pump), solvent A: water 99.95% + acetic acid 0.05%, solvent B: acetonitrile 99.95% + acetic acid 0.05%.

Total time (min)	Flow rate (μl/min)	Solvent A (%)	Solvent B (%)	Valve position
0.00	200	80	20	A
7.00	200	80	20	B
8.50	200	50	50	B
12.50	250	45	55	B
16.00	250	5	95	B
19.00	250	5	95	A
30.00	250	5	95	A
31.00	200	80	20	A
35.90	200	80	20	A

control urine was frozen, thawed and filtered three times before final use.

The reliability and precision of the method was determined by measuring Q_{low} and Q_{high} eight times in a row (intra-day precision) and at eight different days (day-to-day precision) (Table 3). Additionally we determined accuracy and precision by analyzing eight urine samples with varying creatinine concentrations between 0.5 g/l and 2.5 g/l. These samples were measured in native condition and spiked at two concentration levels with approximately 2 μg/l and 10 μg/l of authentic standards (Table 4).

2.6. High-performance liquid chromatography

For high performance liquid chromatography we used an Agilent Technologies LC 1200 instrument (Agilent autosampler G1329A, quaternary pump G1311A, binary pump G1312A and vacuum degasser G1379B and G1322A). In our two column assembly, previously described in [15,21,22], we used as first column a Capcell PAK 5u C18-MG-II for clean up and enrichment. Chromatographic separation was realized on a reversed phase C18 column (Atlantis dC18 2.1 × 150 mm; 3 μm). Two different solvent mixtures were used: solvent A 99.95% water and 0.05% acetic acid, solvent B 99.95% acetonitrile and 0.05% of acetic acid. 25 μl of the processed sample were injected with a constant flow of 1 ml/min 100% solvent A by the quaternary pump onto the PAK phase (valve position A, 0.00–7.00 min). After 7 min, the transfer of the analytes retained on the PAK phase was carried out by the binary pump after time programmed valve switching from the PAK phase in backflush mode onto the RP-C18 column (valve position B, 7.00–19.00 min). The gradient of the binary pump for transfer and chromatographic separation is given in Table 1. After 19 min, the switching valve was set back to position A and the PAK phase was flushed by the quaternary pump with 5% solvent A and 95% solvent B for 10 min and then reconditioned for 7 min with 100% solvent A.

2.7. Mass spectrometry

The mass spectrometric detection and quantification was performed on an AB Sciex 5500 QTrap tandem mass spectrometer. Ion source parameters were optimized manually depending on LC flow rate and eluent composition. We used negative ionization mode with an ion spray voltage (IS) of −4500 V. Nitrogen was used as nebulizer gas and turbo heater gas at 450 °C (ion source gas GS1 and GS2) and 50 psi for both. The curtain gas was adjusted to 35 psi. The fragmentation was performed using nitrogen at concentration level LOW (CAD). The entrance potential was set on −13 V.

Analyte specific parameters were determined with the automatic optimization mode of the analyst software from Perkin Elmer. For this purpose standard solutions were directly infused into the MS/MS with a constant flow. Based on the parent ion of each analyte fragmentations to two daughter ions were tuned in. The most intense fragment was used to calculate the analyte concentration (quantifier). The second fragmentation (qualifier) was used to confirm the results of the quantifier ion. MS/MS measurements were performed in scheduled multiple reaction monitoring (MRM) mode.

2.8. Study subjects

In a pilot human biomonitoring study we applied our method to spot urine samples from 22 individuals from the general German population (9 female, 13 male, ages between 23 and 57 years, median 44 years, collected in 2010). None of the individuals was occupationally exposed to DINCH. Creatinine concentrations varied between 0.1 g and 2.1 g creatinine per liter.

3. Results and discussion

3.1. General considerations

This new method is based on previously published on-line HPLC–MS/MS methods for phthalates [15,21,22]. The suitability of such fast on-line HPLC–MS/MS methods has been confirmed in a number of medium and large sized human biomonitoring studies [21,23–27]. The solvent gradient was adapted to the specific requirements of the DINCH metabolites. Special focus was on lowering the limit of quantification. Because of substitution process just having started, we expected exposures to DINCH to be lower than to the classical phthalates still in use. The biggest contribution to lowering the LOQ can be attributed to the high sensitivity of the mass spectrometer AB Sciex 5500 QTrap.

3.2. Mass spectrometry

As described in Section 2.7 we obtained the fragmentation patterns of MINCH, OH-MINCH, cx-MINCH and their deuterated analogues. In all cases the molecular ion, $[M-H]^-$, was used as the parent ion for specific mass transitions. The specific parent daughter combinations for each analyte, together with the instrument parameters, are shown in Table 2.

Tentative fragment structures are given in Fig. 2. The predominant fragments of MINCH (parent ion *m/z* 270) and OH-MINCH (parent ion *m/z* 313) were *m/z* 153 and *m/z* 109, both fragments containing the cyclohexane moiety. D2-MINCH and D4-OH-MINCH, with deuterated side chains had the same fragmentation pattern. The most intense fragments of cx-MINCH and its deuterated analogue were *m/z* 173 (for cx-MINCH) and *m/z* 175 (for D2-cx-MINCH) representing the alkyl side chain, with and without the two deuterium labels. The quantifier ion (*m/z* 153) is identical in MINCH and OH-MINCH, again representing the cyclohexane moiety. This characteristic fragmentation pattern of MINCH and OH-MINCH showing fragments with the cyclohexane moiety and cx-MINCH showing major fragments containing the oxidized side chain are very similar to the fragmentation pattern previously observed for DINP and DEHP [15,21]. The cis and trans isomers of the DINCH metabolites did neither differ in fragmentation pattern nor response (data not shown).

3.3. HPLC–MS/MS quantification

The cis and trans isomers of the DINCH metabolites showed identical retention characteristics on several analytical columns tested (data not shown). Because of the same retention characteristics, same fragmentation patterns and responses we could not distinguish between the cis and trans isomers. All following results

Table 2
Scheduled MRM-parameters for parent and daughter ion pairs. The resolution for Q1 and Q3 was set to unit. MRM detections window was set to 180 s with a target scan time of 1 s. Settling time and MR pause was set to 5 ms.

Analyte	Q1 mass (Da)	Q3 mass (Da)	RT	DP	CE	CXP
cx-MINCH						
quan.	327	173	16.9	−115	−24	−11
qual.	327	153	16.9	−115	−30	−11
D2-cx-MINCH						
quan.	329	175	16.9	−115	−24	−13
qual.	329	153	16.9	−115	−32	−15
OH-MINCH						
quan.	313	153	17.4	−115	−24	−11
qual.	313	109	17.4	−115	−40	−9
D4-OH MINCH						
quan.	317	153	17.4	−115	−22	−13
qual.	317	109	17.4	−115	−40	−3
oxo-MINCH						
quan.	311	153	17.8	−115	−24	−11
qual.	311	109	17.8	−115	−40	−9
MINCH						
quan.	297	153	21	−130	−22	−11
qual.	297	109	21	−130	−40	−9
D2-MINCH						
quan.	299	153	21	−130	−20	−17
qual.	299	109	21	−130	−34	−5

quan, quantifier ion; qual, qualifier ion; RT, retention time (min); DP, declustering potential (V); CE, collision energy (V); CXP, collision exit potential (V).

represent the sum of the cis and the trans isomer. For quantification purposes we therefore used only one isomer.

Our analytical standard substances and the labeled internal standard substances derived from the 4-methyloctyl side chain of DINCH showed similar chromatographic properties and similar fragmentation patterns. Chromatographic peak shapes of the standards reflect the presence of a single isomer with a 4-methyloctyl side chain (see Fig. 3B). For native urine samples of individuals exposed to DINCH we expect to find metabolites and peaks of all possible DINCH alkyl side chain isomers. The presence of all these different isononyl isomers and the oxidative metabolites of these isononyl isomers is expected to show up in characteristic peak shapes in the chromatograms with a multitude of neighboring and superimposing peaks. These characteristic peak shapes (see Figs. 3A and 4) are therefore caused by the different isononyl isomers and also by different oxidation positions on the alkyl side chain (like the ω or $\omega - 1$ position). Based on the calibration curves of the three specific metabolites of the most abundant alky chain isomer (the 4-methyl octyl isomer) we quantified the sum of all other C9 alky chain isomers of MINCH and the alkyl chain isomers with oxidative functional groups: cx-MINCH and OH-MINCH. We have shown previously for DINP that the MS/MS fragmentation characteristics do not seem to depend on the branching position but on the type of the oxidative modification of the alkyl chain [15]. The carboxy metabolite favors the intact alkyl chain fragment, the hydroxy and oxo modified metabolites favor cyclohexane-ring containing fragments. To unambiguously distinguish between all possible different isomers solely by means of HPLC–MS/MS is hard to accomplish because of the high number of expected isomeric metabolites. Consequently we chose to integrate each m/z signal over the whole time range of elution of the DINCH metabolites. We are confident, based on the points mentioned above, that this approach represents the best possible estimate for the sum of the metabolites of DINCH excreted in urine.

Oxo-MINCH was determined semi-quantitatively by using the external calibration curve of OH-MINCH and D4-OH-MINCH as internal standard. CHDA can also be captured with this method and quantified via standard addition (not shown). However, because CHDA is not specific to DINCH (it is a possible metabolite of all substances with a cyclohexane-1,2-dicarboxylic acid moiety), we chose not to include CHDA in this method development of specific biomarkers of DINCH exposure.

3.4. Detection limit and quantification limit

The limits of detection (LOD), defined as a signal to noise ratio of three for the quantifier ions were estimated to be 0.025 μg/l for cx-MINCH and OH-MINCH and 0.05 μg/l for MINCH. The limits of quantification (LOQ) defined as a signal to noise ratio of six were estimated to be 0.05 μg/l for cx-MINCH and OH-MINCH and 0.1 μg/l for MINCH. The LOQ values proved to be sufficiently low to detect the environmental background exposure of the general population.

3.5. Reliability of the HPLC–MS/MS method

To determine the reliability of this method, we prepared quality control urines from different individual urine samples with different native concentrations of DINCH metabolites. The low concentration quality control (Q_{low}) was prepared to reflect the expected exposure level of the general population (concentrations for the different metabolites between 1.47 μg/l and 9.49 μg/l). Q_{high} was prepared to reflect possible occupational exposure levels (concentrations between 6.6 μg/l and 45.99 μg/l). All metabolite concentrations of the Q samples were within the linear calibration range of the analytical method.

Q_{high} and Q_{low} were analyzed eight times in a row. The intra-day relative standard deviation (RSD) is between 10.7% and 6.8% for Q_{low}; 2% and 3.5% for Q_{high}. The between day precision was performed by measuring the quality controls on eight different days. For Q_{low}, the RSDs were below 10%, except for MINCH (15%). The RSD's for Q_{high} ranged between 3.6% and 3.7% for the oxidized metabolites and 9.2% for MINCH (Table 3).

Table 3
Precision test of our method, with self made quality control samples. All quality controls (Q_{low}, Q_{high}) contain native DINCH metabolites with a broad spectrum of concentrations to simulate different exposure levels.

	MINCH		OH-MINCH		cx-MINCH	
	Q_{low}	Q_{high}	Q_{low}	Q_{high}	Q_{low}	Q_{high}
Intra-day series ($n = 8$)						
Measured conc. (μg/L)	1.47	6.60	8.29	45.99	2.76	12.71
RSD (%)	10.67	3.51	7.10	2.67	6.79	1.95
Between day ($n = 8$)						
Measured conc. (μg/L)	1.49	6.52	9.49	45.64	3.07	13.96
RSD (%)	15.13	9.22	6.56	3.63	9.92	3.67

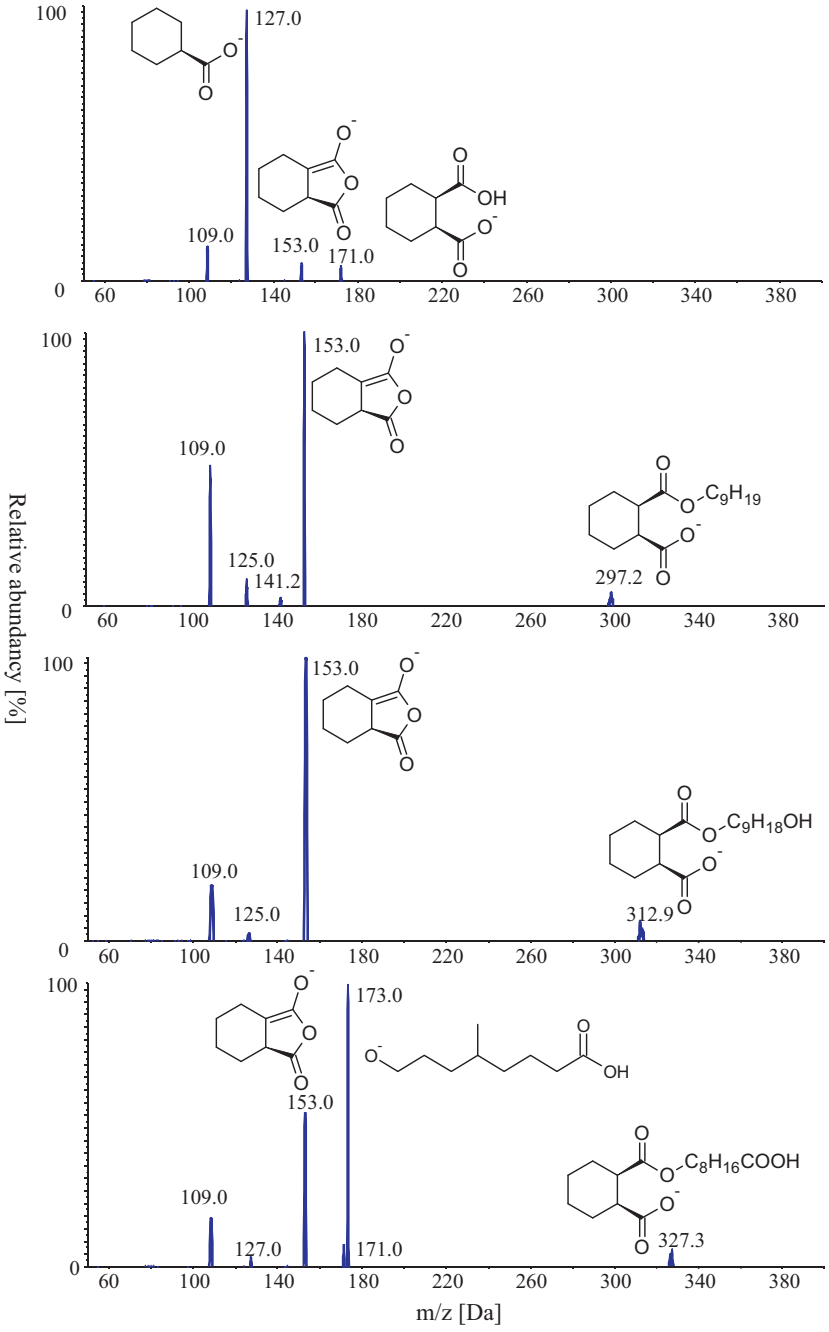

Fig. 2. ESI-negative Q3 mass spectra of CHDA, MINCH, cx-MINCH and OH-MINCH.

Table 4
Calculated accuracy and precision data for our HPLC–MS/MS method from eight different urine samples with varying creatinine levels. Moreover, with unequal native DINCH body burdens and addition of specific concentrations.

	MINCH		OH-MINCH		cx-MINCH	
Spiked conc. (μg/L)	2.01	10.03	2.22	11.1	1.94	9.7
Native conc. measured (μg/L)	<LOQ (<LOQ–0.08)		0.57 (<LOQ–3.11)		0.44 (<LOQ-2.37)	
Native and spiked conc. measured (μg/L)	1.92 (1.83–1.99)	9.05 (8.48–9.75)	2.64 (2.15–4.97)	11.11 (10.1–13.3)	2.34 (1.98–4.07)	9.88 (9.19–11.0)
Spiked conc. calculated (μg/L)						
Mean	1.91	9.04	2.07	10.55	1.90	9.44
Range	1.83–1.99	8.48–9.75	1.86–2.22	9.73–10.97	1.7–2.09	8.63–10.26
RSD (%)	2.69	4.35	6.42	4.07	6.18	5.56
Accuracy (%)	95.0 (91.2–99.2)	90.1 (84.5–97)	93.3 (83.8–100.2)	95.0 (87.6–98.9)	97.9 (87.6–107.7)	97.4 (89.0–105.8)

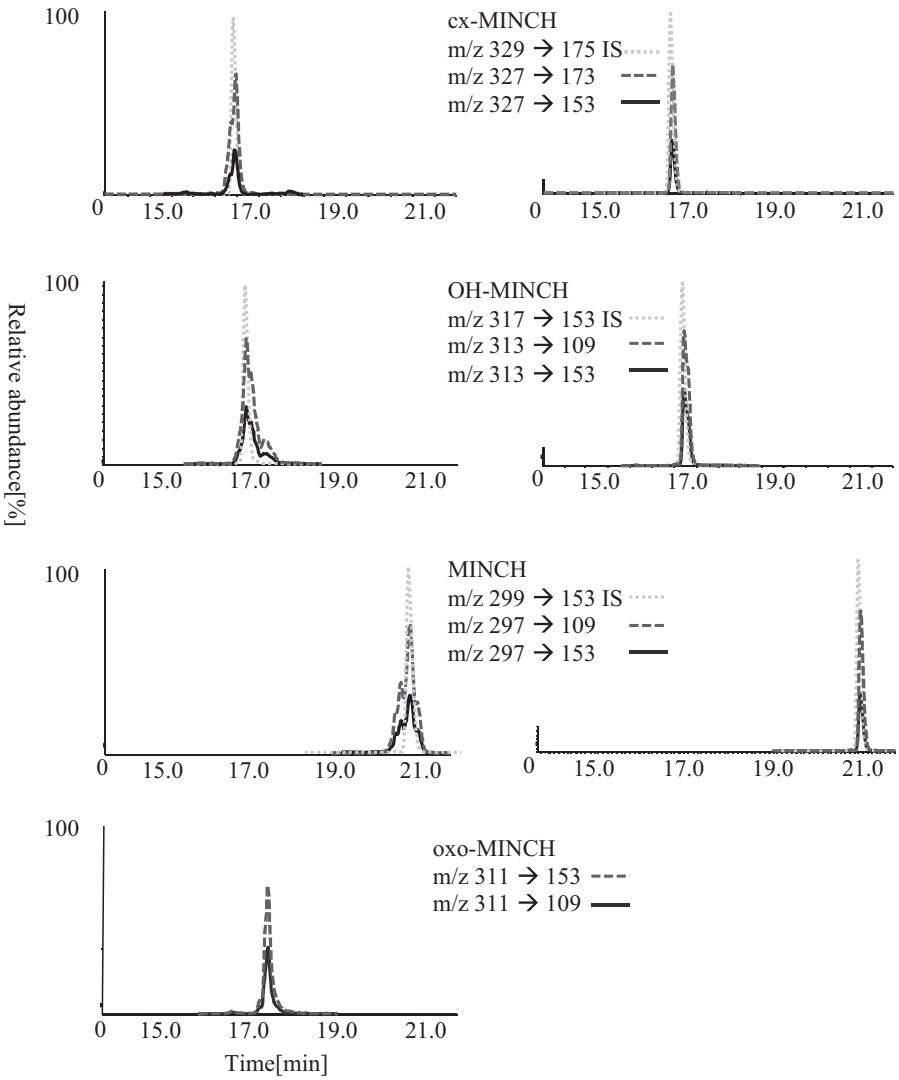

Fig. 3. Extracted ion chromatograms (XIC). On the left side, analyte peaks in a native urine sample (A); on the right side, peaks of the 4-methyloctyl derived analytical standards (60 μg/l) (B).

The accuracy of the method was calculated by measuring eight native urine samples spiked with the specific metabolite standards (derived from the 4-methyloctyl isomer). The native urine samples were chosen to reflect the broad spectrum of urinary matrix with creatinine levels between 0.5 μg/l and 2.5 g/l creatinine. All of the eight urine samples contained native oxidized metabolites in concentrations between <LOQ and 3.11 μg/l. Each sample was analyzed in native condition (non-spiked) and spiked with two different concentrations of DINCH metabolites (approximately 2 μg/l and 10 μg/l). Over all samples and over both spiked metabolite concentrations, the mean calculated accuracy were in the range from 90.1% to 97.9% (see Table 4). The RSDs calculated from the spiked metabolite concentrations were between 2.7% and 6.4% for all parameters. Taking into account that both the urinary matrix and the native level of DINCH metabolites differed considerably in these eight urine samples, these results are highly comparable to the intra-day and between day precisions. Also, calibration curves in urine and water showed no influence of the matrix (data not shown).

3.6. Results of biological monitoring

In the pilot study with 22 volunteers occupationally not exposed to DINCH we found DINCH metabolites in nearly all samples analyzed (86%). The maximum levels in all samples investigated were 3.69 μg/l for OH-MINCH, 2.82 μg/l for cx-MINCH, 0.41 μg/l for MINCH and 1.05 μg/l oxo-MINCH (semi-quantitative). OH-MINCH was the predominant DINCH metabolite in the urine samples. Only 3 samples were below the LOQ for OH-MINCH. MINCH proved to be a very weak biomarker of DINCH exposure with only one urine sample above the LOQ and a MINCH level of 0.41 μg/l. The means of the oxidized metabolites were between 0.33 μg/l and 0.71 μg/l (see Table 5). Statistical analysis (SPSS 19.0) confirmed strong correlations between all oxidized metabolites (see Fig. 5). Due to the low detection rate of MINCH no correlation between MINCH and other measured metabolites are shown. These findings are very similar to our previous findings on DINP metabolism and DINP metabolites in the general population [14,15]. Like for DINP we have

Table 5
Human biomonitoring study with 22 volunteers.

	cx-MINCH	OH-MINCH	MINCH	oxo-MINCH
Median (μg/l)	0.23	0.36	<LOQ	0.22
Mean (μg/l)	0.61	0.71	<LOQ	0.33
Range (μg/l)	<LOQ–2.82	<LOQ–3.69	<LOQ–0.41	<LOQ–1.05
Detection rate (%)	82	86	5	73

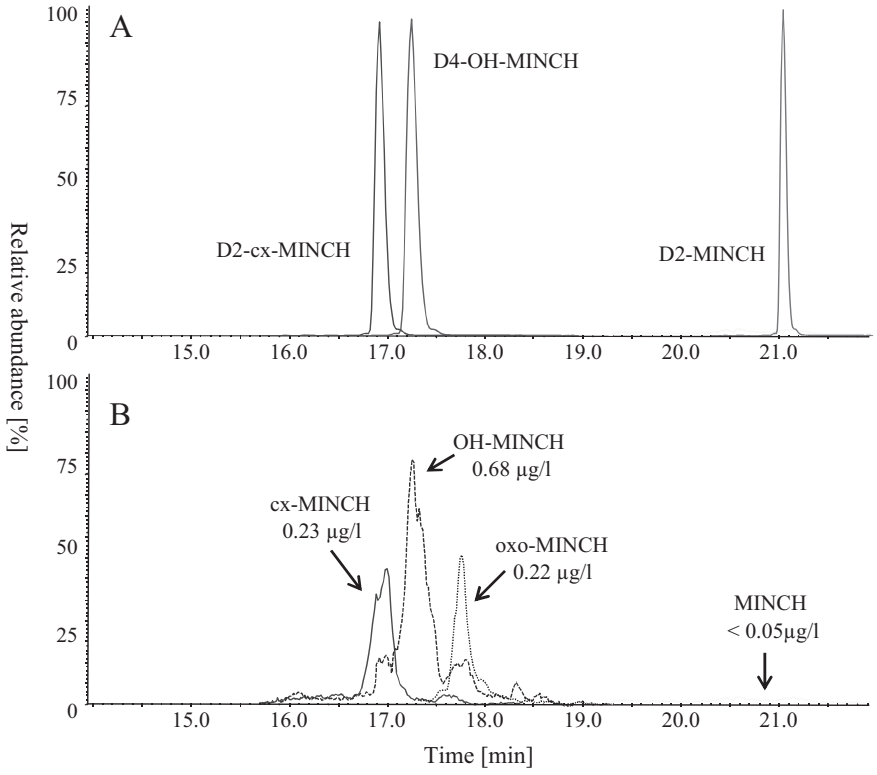

Fig. 4. Total ion chromatogram (TIC) of a typical urine sample with internal standards (A) and DINCH metabolites with concentrations in range of the mean values of the pilot study (B).

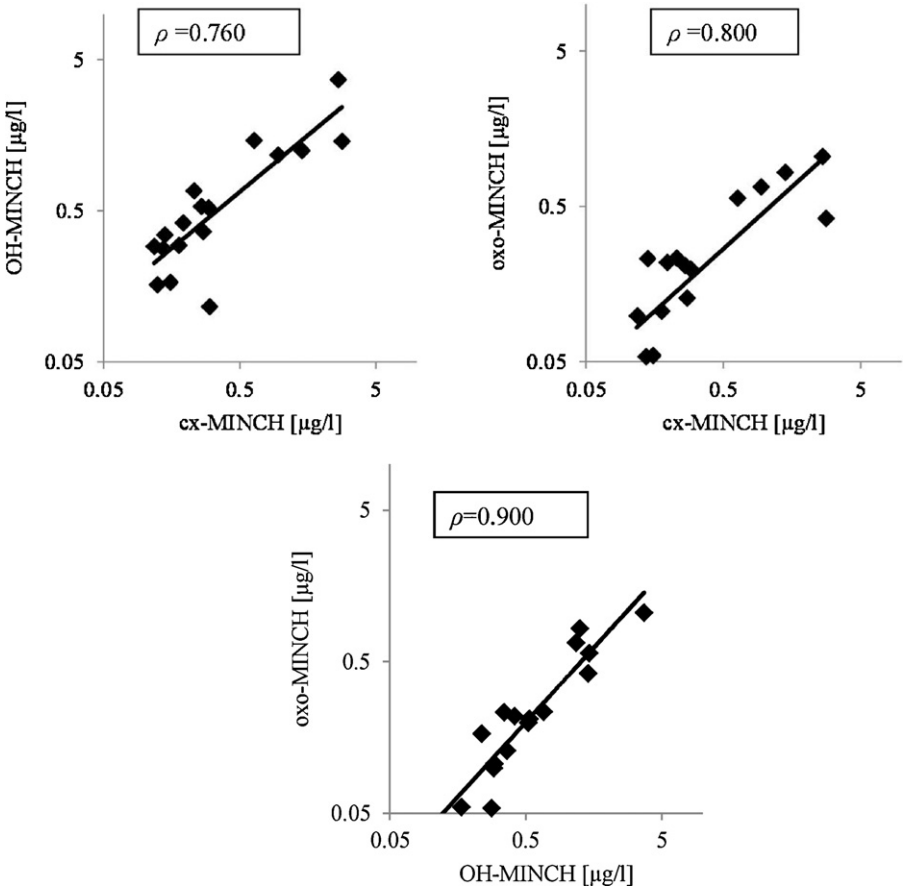

Fig. 5. Correlations of the oxidized DINCH metabolites in the urine samples of the pilot study (ρ = Spearman's rank correlation coefficient).

shown the oxidized DINCH metabolites to be perfectly applicable to monitor DINCH exposure in the general population at environmental exposure levels.

4. Conclusions

In the last decade adverse health effects of phthalates have been thoroughly investigated and discussed. For DINP and DEHP (and some other phthalates) extensive risk assessments have been performed to calculate the potential health risk for men [28–33]. Human biomonitoring data proved vital in these risk assessments as an integral measure of exposure (capturing all possible sources and routes of exposure).

DINCH is considered to be a relatively safe substitute for DINP and DEHP [18]. With the method presented above we provide the basic tool to determine the DINCH exposure of the general population. We have shown that after the market introduction of DINCH in 2002, DINCH in the meantime reached the investigated population. Urinary metabolites of DINCH can be determined in the majority of urine samples in our pilot study (detection rate over 80%). With an increase in DINCH production we also expect an increase DINCH body burdens in the next years. With the analytical method provided here we will be able to follow this possible trend and provide valuable information on the extent of DINCH exposure in the general population that can be used in future exposure and risk assessments.

Up to now, the knowledge on DINCH metabolism is limited to studies in animals. Human metabolism studies as well as long term studies in humans are missing. We will use the method provided above to quantitatively investigate human metabolism and urinary elimination kinetics of DINCH metabolites after oral dosage. With such a metabolism study we will provide valuable conversion factors to extrapolate from urinary metabolite levels to the oral dose (of the general population or subjects occupationally exposed to DINCH). DINCH doses calculated this way can be compared to oral doses of the phthalates or health benchmarks derived from animal studies performed on DINCH. This way we can significantly contribute to a sound and reliable exposure and risk assessment for the human population ubiquitously exposed to DINCH.

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