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Diisononyl cyclohexane-1,2-dicarboxylate (DINCH) – Determination of DINCH metabolites in urine by LC-MS/MS

Biomonitoring Method – Translation of the German version from 2023

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Keywords

diisononyl cyclohexane-1,2-dicarboxylate; DINCH; MINCH; OH-MINCH; cx-MINCH; oxo-MINCH; plasticiser; biomonitoring; urine; LC-MS/MS

Abstract

The working group "Analyses in Biological Materials" of the German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method. The method described herein allows for the determination of cyclohexane-1,2-dicarboxylic acid monocarboxyisooctyl ester (cx-MINCH), cyclohexane-1,2-dicarboxylic acid monohydroxyisononyl ester (OH-MINCH), cyclohexane-1,2-dicarboxylic acid monooxoisononyl ester (oxo-MINCH), and cyclohexane-1,2-dicarboxylic acid monoisononyl ester (MINCH) as specific metabolites of diisononyl cyclohexane-1,2-dicarboxylate (DINCH) in urine. For the determination, urine samples buffered to pH 6.0 are mixed with deuterated internal standards (ISTDs), enzymatically hydrolysed, and then subjected to online preconcentration. By way of automatic column-switching, the analytes are then transferred onto the analytical column in back-flush mode, where they are chromatographically separated and, following negative ionisation, quantified by tandem mass spectrometry. Calibration is carried out using calibration standards prepared in pooled urine and processed in the same way as the samples to be analysed.

Citation Note:
Koch HM, Schütze A, Völkel W, Wöckner M. Diisononyl cyclohexane-1,2-dicarboxylate (DINCH) – Determination of DINCH metabolites in urine by LC-MS/MS. Biomonitoring Method – Translation of the German version from 2023. MAK Collect Occup Health Saf. 2023 Dec:Doc903. https://doi.org/10.34865/bi16641278eoj23_1or

Manuscript completed:
11 Apr 2013

Publication date:
20 Dec 2023

1 Characteristics of the method

Matrix	Urine
Analytical principle	Liquid chromatography with tandem mass spectrometry (LC-MS/MS)

Parameters and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
Diisononyl cyclohexane-1,2-dicarboxylate (DINCH)	166412-78-8	Cyclohexane-1,2-dicarboxylic acid monocarboxyisooctyl ester (cx-MINCH)	1637562-51-6
		Cyclohexane-1,2-dicarboxylic acid monohydroxyisononyl ester (OH-MINCH)	1637562-52-7
		Cyclohexane-1,2-dicarboxylic acid monooxoisononyl ester (oxo-MINCH)	1588520-62-0
		Cyclohexane-1,2-dicarboxylic acid monoisononyl ester (MINCH)	1889286-78-5

Reliability data

Cyclohexane-1,2-dicarboxylic acid monocarboxyisooctyl ester (cx-MINCH)

Within-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 2.76 µg or 12.7 µg cx-MINCH per litre of urine and n = 8 determinations	s_w = 6.79% or 1.95% u = 16.1% or 4.61%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 3.07 µg or 14.0 µg cx-MINCH per litre of urine and n = 8 determinations	s_w = 9.92% or 3.67% u = 23.5% or 15.8%
Accuracy:	Recovery (rel.) at a concentration of 1.94 µg or 9.7 µg cx-MINCH per litre of urine and n = 8 determinations	r = 97.9% or 97.4%
Detection limit:	0.025 µg cx-MINCH per litre of urine	
Quantitation limit:	0.05 µg cx-MINCH per litre of urine	

Cyclohexane-1,2-dicarboxylic acid monohydroxyisononyl ester (OH-MINCH)

Within-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 8.29 µg or 46.0 µg OH-MINCH per litre of urine and n = 8 determinations	s_w = 7.1% or 2.67% u = 16.8% or 6.31%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 9.49 µg or 45.6 µg OH-MINCH per litre of urine and n = 8 determinations	s_w = 6.56% or 3.63% u = 15.5% or 8.58%

Accuracy:	Recovery (rel.) at a concentration of 2.22 µg or 11.1 µg OH-MINCH per litre of urine and n = 8 determinations	r = 93.3% or 95%
Detection limit:	0.025 µg OH-MINCH per litre of urine	
Quantitation limit:	0.05 µg OH-MINCH per litre of urine	

Cyclohexane-1,2-dicarboxylic acid monooxoisononyl ester (oxo-MINCH)[a)]

Within-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 1.13 µg or 12.2 µg oxo-MINCH per litre of urine and n = 8 determinations	s_w = 1.43% or 1.84% u = 3.39% or 4.36%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 1.02 µg or 11.6 µg oxo-MINCH per litre of urine and n = 8 determinations	s_w = 15.0% or 8.94% u = 35.5% or 21.1%
Accuracy:	Recovery (rel.) at a concentration of 1 µg or 10 µg oxo-MINCH per litre of urine and n = 8 determinations	r = 91.7% or 99.7%
Detection limit:	0.05 µg oxo-MINCH per litre of urine	
Quantitation limit:	0.1 µg oxo-MINCH per litre of urine	

[a)] The validation data for the determination of oxo-MINCH in urine were collected by the external verifiers of the method.

Cyclohexane-1,2-dicarboxylic acid monoisononyl ester (MINCH)

Within-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 1.47 µg or 6.6 µg MINCH per litre of urine and n = 8 determinations	s_w = 10.7% or 3.51% u = 25.2% or 8.30%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 1.49 µg or 6.52 µg MINCH per litre of urine and n = 8 determinations	s_w = 15.1% or 9.22% u = 35.8% or 21.8%
Accuracy:	Recovery (rel.) at a concentration of 2.01 µg or 10.0 µg MINCH per litre of urine and n = 8 determinations	r = 95% or 90.1%
Detection limit:	0.05 µg MINCH per litre of urine	
Quantitation limit:	0.1 µg MINCH per litre of urine	

2 General information on DINCH

Diisononyl cyclohexane-1,2-dicarboxylate (Hexamoll® DINCH, henceforth referred to as DINCH) is a plasticiser that has been developed as a substitute to high-molecular weight phthalate plasticisers, such as di(2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DINP), because of their reproductive toxicity (Crespo et al. 2007).

DINCH is manufactured by catalytic hydrogenation of the aromatic ring system of DINP, leading to a non-planar cyclohexane moiety. Currently, DINCH is only manufactured by catalytic hydrogenation of DINP2 with exactly nine

carbon atoms in the alkyl side chain. Through the hydrogenation, both a *cis*- and a *trans*-configuration become possible, whereby DINCH is comprised of 90 ± 10% *cis*-isomers and 10 ± 10% *trans*-isomers (NICNAS 2012).

DINCH is produced by BASF SE and has been on the market since 2002. It is permitted for use, for example, in medical devices, food contact materials, and toys (NICNAS 2012). Documentations for a MAK or BAT value for DINCH are currently not available from the MAK Commission (German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work area). The European Food Safety Authority (EFSA) established a Tolerable Daily Intake (TDI) of 1 mg/kg body weight/day. Due to the high fat content of certain food products, DINCH can be released from packaging material and ingested (EFSA 2006). In the general population, the intake of DINCH by way of packaged food products is the primary route of exposure (about 90%). The intake of polluted dust also plays a role in DINCH exposure (Giovanoulis et al. 2018). Furthermore, an intravenous exposure to the substance is also possible by infusions or transfusions, where DINCH can migrate from medical devices (Malarvannan et al. 2019). Children are additionally exposed to DINCH by oral contact with toys or an increased intake of dust (Weiss et al. 2018).

Following intake, DINCH is rapidly metabolised. Silva et al. (2012) detected a total of 15 metabolites (without accounting for stereoisomers) in urine of female Sprague–Dawley rats. Cyclohexane-1,2-dicarboxylic acid (CHDA) was identified as the primary metabolite. CHDA is the basic structure of DINCH, as well as of other substances which are also used as plasticisers (e.g. di(2-ethylhexyl) cyclohexane-1,2-dicarboxylate). For this reason, measured CHDA concentrations in human urine cannot be specifically attributed to DINCH exposure. In contrast to CHDA, the oxidised metabolites of DINCH are specific and thereby suitable biomarkers. The metabolism is comprised primarily of a first step of hydrolysis, through which the monoester MINCH is formed. MINCH is subsequently oxidised, whereby OH-MINCH and cx-MINCH are formed. In a following step, OH-MINCH is oxidised to oxo-MINCH. Furthermore, ring-hydroxylated monoesters and other degradation products, such as carboxylated metabolites with shortened alkyl side chains, are formed (Schütze et al. 2017). A simplified metabolism scheme of DINCH is depicted in Figure 1.

Following a single oral dose in humans, the oxidation products of MINCH were identified as specific biomarkers which are excreted with the urine. In this study, OH-MINCH represented about 10% of the administered dose, followed by cx-MINCH and oxo-MINCH (each about 2% of the dose). MINCH itself represented less than 1% of the dose. Within 48 hours, about 40% of the administered dose were excreted with the urine in the forms of both unspecific CHDA and oxidative DINCH metabolites. The secondarily oxidised metabolites exhibited half-lives of 10 to 18 hours (Koch et al. 2003).

In population studies in Germany from 2009 to 2017, DINCH metabolites could be detected in an increasing number of samples as well as in higher concentrations (Kasper-Sonnenberg et al. 2019; Schütze et al. 2014). From 2013 on, OH-MINCH was detected in all examined urine samples in concentrations above the quantitation limit. In 2017, the median values were determined to be 0.70 µg/l for OH-MINCH, 0.31 µg/l for oxo-MINCH, and 0.21 µg/l for cx-MINCH.

The Human Biomonitoring Commission (*HBM-Kommission*) of the German Environment Agency (*Umweltbundesamt*) derived HBM values for DINCH in 2014. An HBM-I value of 3000 µg/l for children and of 4500 µg/l for adults was derived for the sum of OH-MINCH and cx-MINCH (HBM-Kommission 2014).

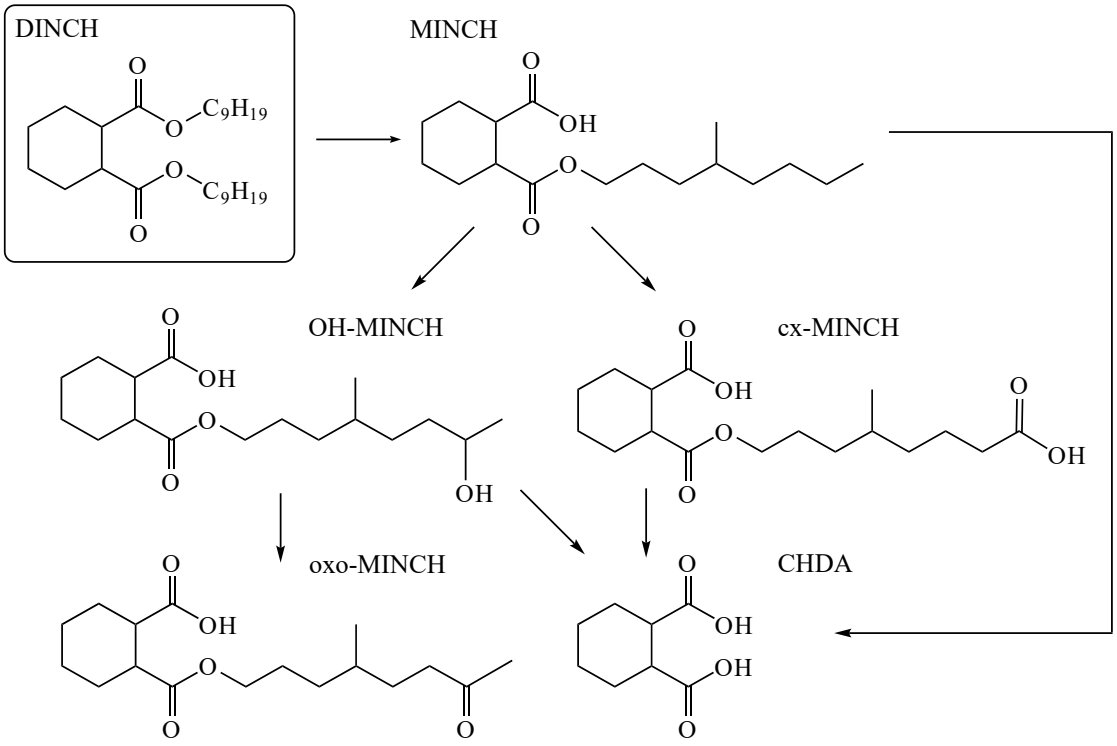


Fig. 1 Simplified metabolism scheme of DINCH (according to Koch et al. 2013); all metabolites depicted here are only examples for possible isomers

3 General principles

The method described herein allows for the determination of cx-MINCH, OH-MINCH, oxo-MINCH, and MINCH as specific metabolites of DINCH in urine. For the determination, urine samples buffered to pH 6.0 are mixed with deuterated internal standards (ISTDs), enzymatically hydrolysed, and then subjected to online preconcentration. By way of automatic column-switching, the analytes are then transferred in back-flush mode onto the analytical column, where they are chromatographically separated and, following negative ionisation, quantified by tandem mass spectrometry. Calibration is carried out using calibration standards prepared in pooled urine and processed in the same way as the samples to be analysed.

4 Equipment, chemicals, and solutions

4.1 Equipment

- HPLC system (e.g. Agilent 1200 Series with autosampler G1329A, quaternary pump G1311A, binary pump G1312A, and vacuum degasser G1322A or G1379B, Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany)
- Mass spectrometer (e.g. SCIEX QTRAP® 5500 with Turbo V™ Ion Source, AB SCIEX Germany GmbH, Darmstadt, Germany)

- Preconcentration column (e.g. Capcell Pak® C18 MG-II 100 Å, 5 µm, 25 mm × 4 mm, Phenomenex Ltd. Deutschland, Aschaffenburg, Germany)
- Analytical HPLC column (e.g. Atlantis dC18, 100 Å, 3 µm, 150 mm × 2.1 mm, Waters GmbH, Eschborn, Germany)
- Centrifuge (e.g. Megafuge™, Heraeus Deutschland GmbH & Co. KG, Hanau, Germany)
- Water bath (e.g. Gesellschaft für Labortechnik mbH, Burgwedel, Germany)
- 1.8-ml glass vials with screw caps and PTFE-lined septa (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)
- Glass pasteur pipettes (e.g. VWR International GmbH, Darmstadt, Germany)
- Various beakers, volumetric flasks, and graduated cylinders (e.g. SCHOTT AG, Mainz, Germany)
- pH meter with pH electrode (e.g. CG 842, SCHOTT AG, Mainz, Germany)
- Variable adjustable volume pipettes with 10-, 100- and 1000-µl pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- Urine cups (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be a minimum of *pro analysi* grade.

Reference standards

- Cyclohexane-1,2-dicarboxylic acid-mono-(7-carboxy-4-methyl)heptyl ester (cx-MINCH) (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- Cyclohexane-1,2-dicarboxylic acid-mono-(7-hydroxy-4-methyl)octyl ester (OH-MINCH) (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- Cyclohexane-1,2-dicarboxylic acid-mono-(7-oxo-4-methyl)octyl ester (oxo-MINCH) (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- Cyclohexane-1,2-dicarboxylic acid-mono-(4-methyl)octyl ester (MINCH) (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)

Internal standards

- Cyclohexane-1,2-dicarboxylic acid-mono-D_2-(7-carboxy-4-methyl)heptyl ester (D_2-cx-MINCH) (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- Cyclohexane-1,2-dicarboxylic acid-mono-D_4-(7-hydroxy-4-methyl)octyl ester (D_4-OH-MINCH) (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- Cyclohexane-1,2-dicarboxylic acid-mono-D_3-(7-oxo-4-methyl)octyl ester (D_3-oxo-MINCH) (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- Cyclohexane-1,2-dicarboxylic acid-mono-D_2-(4-methyl)octyl ester (D_2-MINCH) (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)

Other chemicals

- Acetonitril LiChrosolv® (e.g. No. 100030, Merck KGaA, Darmstadt, Germany)
- Ammonium acetate EMSURE® ACS (e.g. No. 101116, Merck KGaA, Darmstadt, Germany)
- Acetic acid (glacial) 100% (e.g. No. 100063, Merck KGaA, Darmstadt, Germany)

- Methanol SupraSolv® (e.g. No. 106011, Merck KGaA, Darmstadt, Germany)
- *β*-Glucuronidase from *E. coli* K12 in 50% glycerol, 80 U/ml (e.g. No. 03708446103, Roche Diagnostics Deutschland GmbH, Mannheim, Germany)
- Ultra-pure water (according to ASTM Type 1)

4.3 Solutions

- Ammonium acetate buffer (1 mol/l, pH 6.0)
  Exactly 19.27 g of ammonium acetate are weighed into a beaker and dissolved in about 230 ml of ultra-pure water. Using a pH meter, the pH value of the solution is then adjusted to pH 6.0 with glacial acetic acid. The solution is transferred into a 250-ml volumetric flask and the flask is then made up to the mark with ultra-pure water. The buffer can be stored in the refrigerator at 4 °C for eight weeks.
- Eluent A
  In a graduated cylinder, 1 l of ultra-pure water is mixed with 500 µl of glacial acetic acid. The solution is thoroughly mixed prior to use.
- Eluent B
  In a graduated cylinder, 1 l of acetonitrile is mixed with 500 µl of glacial acetic acid. The solution is thoroughly mixed prior to use.

4.4 Internal standards (ISTDs)

- ISTD stock solutions (1000 mg/l)
  About 10 mg each of D_2-cx-MINCH, D_4-OH-MINCH, D_3-oxo-MINCH, and D_2-MINCH are weighed exactly into 10-ml volumetric flasks and dissolved in acetonitrile. The flasks are then made up to the mark with acetonitrile.
- ISTD spiking solutions (2 mg/l)
  200 µl of each ISTD stock solution are pipetted into a 100-ml volumetric flask. The flask is then made up to the mark with ultra-pure water.

The ISTD stock solutions can be stored in glass vials with screw caps and PTFE-lined septa at –20 °C for at least one year. The spiking solutions are also stored at –20 °C.

4.5 Calibration standards

- Stock solutions (1000 mg/l)
  About 10 mg each of cx-MINCH, OH-MINCH, oxo-MINCH, and MINCH are weighed exactly into 10-ml volumetric flasks and dissolved in acetonitrile. The flasks are then made up to the mark with acetonitrile.
- Spiking solution I (500 µg/l)
  5 µl of the stock solution are pipetted into a 10-ml volumetric flask. The flask is then made up to the mark with ultra-pure water.
- Spiking solution II (10 µg/l)
  200 µl of spiking solution I are pipetted into a 10-ml volumetric flask. The flask is then made up to the mark with ultra-pure water.

The stock solutions of the analytes can be aliquoted and stored in glass vials with screw caps and PTFE-lined septa at –20 °C for at least one year. The spiking solutions are also stored at –20 °C.

For the preparation of the calibration standards, the spiking solutions are diluted with ultra-pure water according to the pipetting scheme given in Table 1. Each calibration standard is aliquoted to 300 µl in 1.8-ml glass vials with screw caps and PTFE-lined septa and frozen at –20 °C. Under these conditions, the standards are stable for one year without analyte losses. The calibration standards are processed analogously to the samples as indicated in Section 5.

Tab. 1 Pipetting scheme for the preparation of calibration standards for the determination of DINCH metabolites in urine

Calibration standard	Spiking solution I [ml]	Spiking solution II [ml]	Ultra-pure water [ml]	Analyte concentration [µg/l]
1	-	0.2	ad 20	0.1
2	-	0.4		0.2
3	-	1.0		0.5
4	-	4.0		2
5	0.2	-		5
6	0.8	-		20
7	1.6	-		40
8	2.4	-		60

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine samples are collected in 250-ml urine cups and frozen until processing at –20 °C. Under these conditions, the urine is stable for at least one year without analyte losses.

5.2 Sample preparation

The urine sample is thawed overnight at room temperature. After the urine sample has reached room temperature, it is thoroughly mixed. Using a pipette, 300 µl of the urine sample are then transferred into a 1.8-ml glass vial with screw caps and PTFE-lined septum, followed by the addition of 100 µl of ammonium acetate buffer (1 mol/l, pH 6.0). Subsequently, 10 µl of the ISTD spiking solution and 6 µl of β-glucuronidase, which has been diluted to 1 : 1 with the ammonium acetate buffer, are added. The sample is thoroughly mixed and hydrolysed in a water bath for two hours at 37 °C. Afterwards, the sample is thoroughly mixed again. The sample is then mixed with 10 µl of acetic acid, frozen for at least three hours, thawed, and centrifuged for ten minutes at 1900 × *g*. The supernatant is applied for analysis by LC-MS/MS.

6 Operational parameters

Analytical determinations are carried out using a device configuration consisting of an HPLC system with a tandem mass spectrometer (LC-MS/MS).

6.1 High-performance liquid chromatography

Preconcentration column:	Material:	Octadecylsilane phase
	Length:	25 mm
	Inner diameter:	4 mm
	Column packing:	Capcell Pak® C18 MG-II 100 Å, 5 µm

Analytical column:	Material:	Octadecylsilane phase
	Length:	150 mm
	Inner diameter:	2.1 mm
	Column packing:	Atlantis dC18, 100 Å, 3 µm
Separation principle:	Reversed Phase	
Temperature:	Room temperature	
Injection volume:	25 µl	
Eluent:	A: Water + 0.05% acetic acid B: Acetonitrile + 0.05% acetic acid	
Gradient:	see Table 2 and 3	
Flow rates:	see Table 2 and 3	
Valve switching:	see Table 4	

All other parameters must be optimised according to the instructions of the corresponding manufacturer.

Tab. 2 Gradient program of the loading pump for the online preconcentration of the analytes

Time [min]	Flow rate [µl/min]	Eluent A [%]	Eluent B [%]
0.0	1000	100	0
7.1	1000	100	0
7.2	100	5	95
18.9	100	5	95
19.0	1000	5	95
29.0	1000	5	95
29.1	100	100	0
32.0	100	100	0
32.1	1000	100	0
35.9	1000	100	0

Tab. 3 Gradient program of the analytical pump for the chromatographic separation of the analytes

Time [min]	Flow rate [µl/min]	Eluent A [%]	Eluent B [%]
0.0	200	80	20
7.0	200	80	20
8.5	200	50	50
12.5	250	45	55
16.0	250	5	95
19.0	250	5	95
30.0	250	5	95
31.0	200	80	20
35.9	200	80	20

Tab. 4 Switching program of the six-port valve

Time [min]	Valve position
0.0	A
7.0	B
19.0	A
21.0	B
21.2	A
21.4	B
21.6	A
24.2	B
24.5	A

6.2 Tandem mass spectrometry

Ionisation mode:	Negative electrospray ionisation (ESI–)
Ion-spray voltage:	–4500 V
Nebuliser gas:	Nitrogen, 50 psi
Turbo-Heater gas:	Nitrogen, 50 psi, 450 °C
Curtain gas:	Nitrogen, 35 psi
Q1 resolution:	unit
Q3 resolution:	low
Settling time:	0.005 s
MR pause:	0.005 s
Collision gas:	Nitrogen; low instrument units
Scan time:	1 s
Parameter-specific settings:	see Table 5

The quantitation of the analytes is carried out using the primary mass transition (quantifier), while the qualitative confirmation of the results is carried out using the second mass transition (qualifier).

Tab. 5 Parameter-specific settings and retention times for the determination of DINCH metabolites

Substance	Retention time [min]	Parent ion [*m/z*]	Product ion [*m/z*]	Collision energy [V]
cx-MINCH	16.9	327	173[a)]	–24
			153[b)]	–30
D_2-cx-MINCH	16.9	329	175[a)]	–24
			135[b)]	–32
OH-MINCH	17.4	313	153[a)]	–24
			109[b)]	–40
D_4-OH-MINCH	17.4	317	153[a)]	–22
			109[b)]	–40

Tab. 5 *(continued)*

Substance	Retention time [min]	Parent ion [*m/z*]	Product ion [*m/z*]	Collision energy [V]
oxo-MINCH	17.8	311	153[a)]	–24
			109[b)]	–40
D_3-oxo-MINCH	17.8	314	153[a)]	–24
			109[b)]	–38
MINCH	21.0	297	153[a)]	–22
			109[b)]	–40
D_2-MINCH	21.0	299	153[a)]	–20
			109[b)]	–34

[a)] Quantifier
[b)] Qualifier

All parameters must be individually adjusted by the user following the process of preparatory performance inspection of the MS/MS system. The parameters specified above are only intended as cursory guidance.

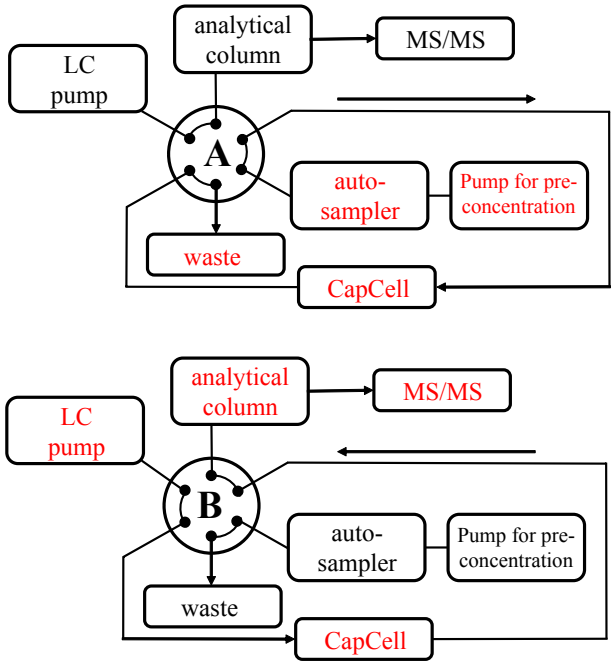
7 Analytical determination

For the analytical determination of the urine samples processed according to Section 5.2, an aliquot of 25 µl of the sample solution is injected into the LC-MS/MS system via the autosampler.

In valve position A, the analytes are concentrated on the Capcell Pak® column using the quaternary pump at a flow rate of 1 ml/min. After the preconcentration of the analytes and the separation of the matrix components, the analytes are transferred to the analytical column in valve positon B in back flush mode (see Figure 2). After online preconcentration, chromatographic separation, and determination via MS/MS, the analytes are identified by their retention times as well as their characteristic ion transitions (see Table 5). Ultra-pure water is processed and analysed in every analytical run as a reagent blank, as well as two quality-control samples.

The retention times given in Table 5 can only serve as a point of reference. The user of the method must ensure proper separation performance of the analytical column as well as the resulting retention behaviour of the analytes.

Figure 3 shows a representative chromatogram of a native urine sample. Due to the high number of possible isomers, the peak forms of the analytes are clearly different from the peak forms of the relevant ISTDs.



Valve position A: Extraction and preconcentration of the analytes on the polar CapCell phase

Valve position B: Elution of the target analytes from the polar CapCell phase; separation on the analytical column; MS/MS-detection

Fig. 2 Outline of the setup of the LC-MS/MS system and illustration of switching options A and B of the six-port valve

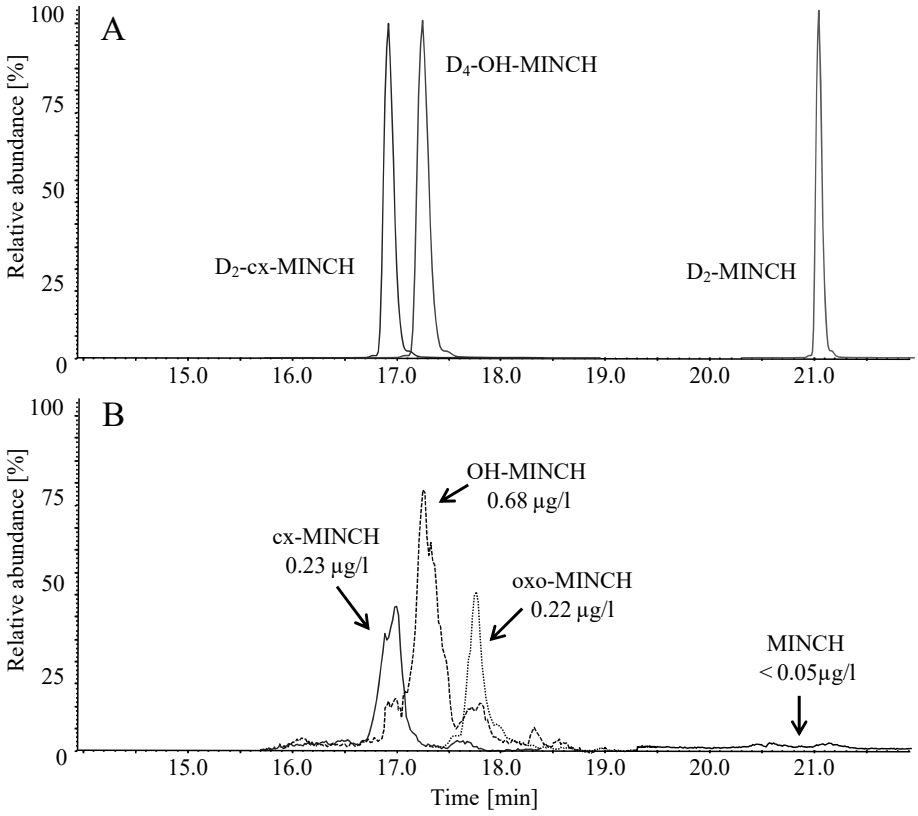


Fig. 3 Chromatograms of a native urine sample with analyte concentration levels of 0.23 µg/l of cx-MINCH, 0.68 µg/l of OH-MINCH, 0.22 µg/l of oxo-MINCH, and < 0.05 µg/l of MINCH

8 Calibration

The calibration standards, described in Section 4.5, are processed (see Section 5) and analysed by LC-MS/MS (see Section 6 and 7) analogously to the samples. The calibration curves are generated by plotting the quotients of the peak area of the analyte and the corresponding ISTD against the spiked concentrations. The calibration curves are linear in the range from 0.1 µg/l up to 80 µg/l. Figure 4 depicts representative calibration curves for cx-MINCH, OH-MINCH, oxo-MINCH as well as MINCH.

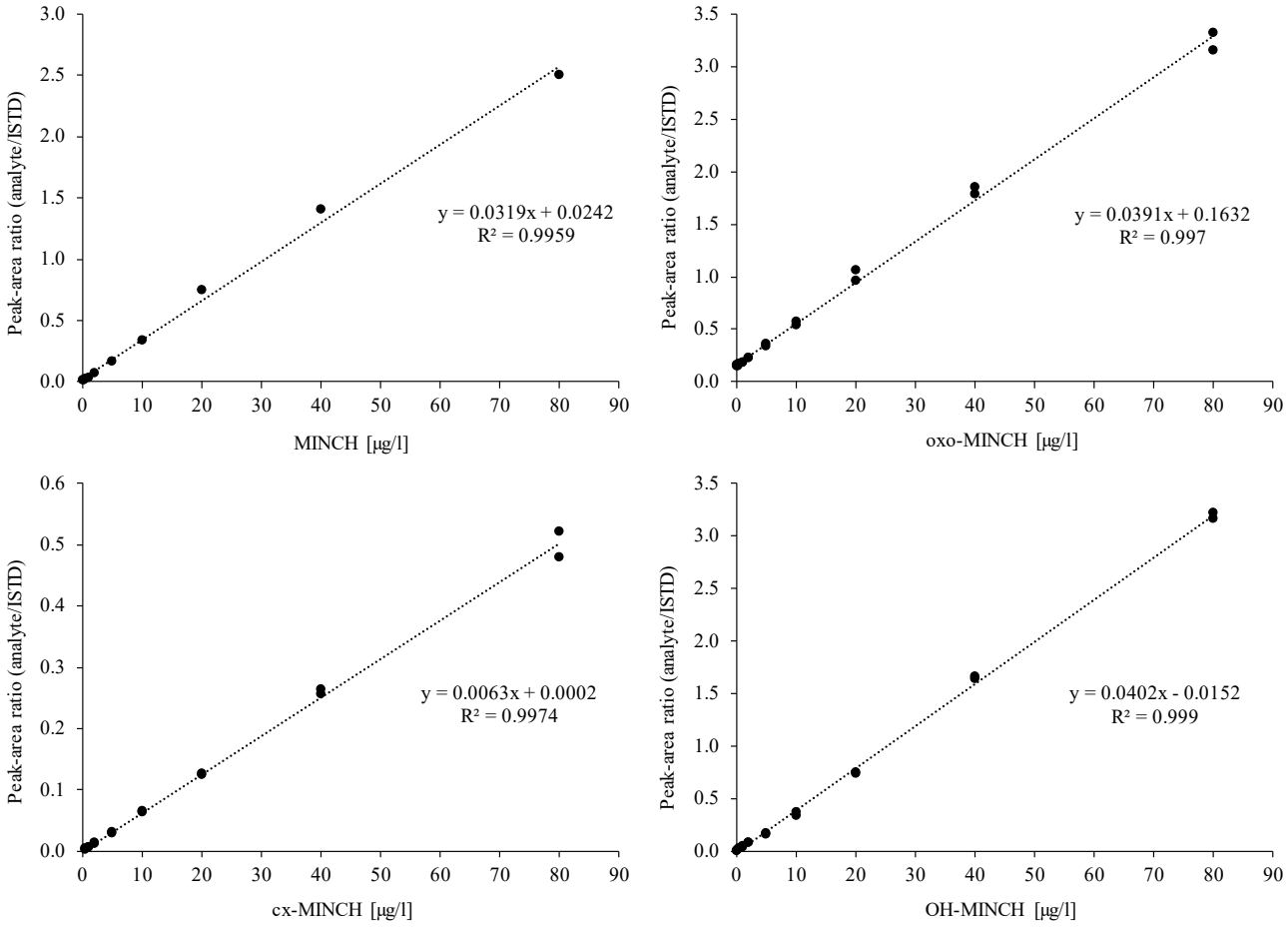


Fig. 4 Representative calibration curves for the determination of cx-MINCH, OH-MINCH, oxo-MINCH as well as MINCH

9 Calculation of the analytical results

The analyte concentration of a sample is determined by calculating the quotient of the peak area of the respective analytes and the peak area of the relevant ISTD, and applying this quotient to the calibration function according to Section 8. The analyte concentration thereby calculated is given in µg/l. Any reagent blank values must be subtracted from the analytical results.

10 Standardisation 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 published by the Commission (Bader et al. 2010; Bundesärztekammer 2014).

For quality control, every analytical run includes the determination of at least two quality-control samples with low (Q_{low}) and high (Q_{high}) analyte concentrations, which contain known concentrations of the analytes. Since commercial materials are not available, these must be prepared by the user by spiking pooled urine. The spiked concentration should lie within the expected concentration range. The nominal values and the tolerance ranges of the quality-control materials are determined in a pre-analytical period (one analysis of the control material each on ten different days) (Bader et al. 2010).

11 Evaluation of the method

The reliability of this method was confirmed by comprehensive validation as well as by replication and verification in a second, independent laboratory.

11.1 Precision

Within-day precision

Within-day precision for the analytes cx-MINCH, OH-MINCH, and MINCH was ascertained using the quality-control materials Q_{low} and Q_{high} (see Section 10). For this purpose, the Q_{low} and Q_{high} materials were each processed and analysed eight times in parallel. For oxo-MINCH, within-day precision was determined using a urine sample spiked with 1.13 µg/l or 12.2 µg/l of the analyte, processed, and analysed eight times in parallel. The results of these analyses are summarised in Table 6.

Tab. 6 Within-day precision for the determination of DINCH metabolites in urine (n = 8)

Analyte	Concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
cx-MINCH	2.76	6.79	16.1
	12.7	1.95	4.61
OH-MINCH	8.29	7.1	16.8
	46.0	2.67	6.31
oxo-MINCH[a)]	1.13	1.43	3.39
	12.2	1.84	4.36
MINCH	1.47	10.7	25.2
	6.6	3.51	8.30

[a)] These data were collected by the external verifiers of the method (see Section 12).

Day-to-day precision

Day-to-day precision for the analytes cx-MINCH, OH-MINCH, and MINCH was determined by processing and analysing the Q_{low} and Q_{high} samples on eight different days. For oxo-MINCH, day-to-day precision was ascertained using a urine sample spiked with 1.02 µg/l or 11.6 µg/l of the analyte; these samples were then processed and analysed on eight consecutive workdays. The results are summarised in Table 7.

Tab. 7 Day-to-day precision for the determination of DINCH metabolites in urine (n = 8)

Analyte	Concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
cx-MINCH	3.07	9.92	23.5
	14.0	3.67	15.8
OH-MINCH	9.49	6.56	15.5
	45.6	3.63	8.58
oxo-MINCH[a)]	1.02	15.0	35.5
	11.6	8.94	21.1
MINCH	1.49	15.1	35.8
	6.52	9.22	21.8

[a)] These data were collected by the external verifiers of the method (see Section 12).

11.2 Accuracy

For the analytes cx-MINCH, OH-MINCH, and MINCH, the accuracy of the method and the influence of possible matrix effects were checked by analysing eight individual urine samples with creatinine levels ranging from 0.5 to 2.5 g/l. For oxo-MINCH, eight urine samples with creatinine concentrations between 0.49 and 2.37 g/l were used. The urine samples were spiked with the analytes at two concentration levels. Both the spiked and unspiked urine samples were processed and analysed. The calculation of the relative recovery was carried out using the concentrations quantified in the spiked urine samples, subtracting any background levels of the analytes. The results of these determinations are presented in Table 8.

Tab. 8 Mean relative recovery for the determination of DINCH metabolites in urine (n = 8)

Analyte	Background level [µg/l]	Spiked concentration [µg/l]	Mean rel. recovery r [%]	Range [%]
cx-MINCH	< LOQ–2.37	1.94	97.9	87.6–108
		9.7	97.4	89.0–106
OH-MINCH	< LOQ–3.11	2.22	93.3	83.8–100
		11.1	95	87.6–98.9
oxo-MINCH[a)]	–	1.00	91.7	78.0–99.4
		10.0	99.7	93.9–105
MINCH	< LOQ–0.08	2.01	95	91.2–99.2
		10.0	90.1	84.5–97

LOQ: limit of quantitation
[a)] These data were collected by the external verifiers of the method (see Section 12).

11.3 Limits of detection and quantitation

For the analytes cx-MINCH, OH-MINCH, and MINCH, the determination of the detection limits was ascertained from the threefold signal-to-noise ratio. The quantitation limits were similarly calculated from the sixfold signal-to-noise ratio. The detection and quantitation limits of oxo-MINCH were computed using the signal-to-noise ratio multiplied by a factor of either 15 or 30.

The values calculated for the determination of cx-MINCH, OH-MINCH, oxo-MINCH, and MINCH are presented in Table 9.

Tab. 9 Limits of detection and quantitation for the determination of DINCH metabolites in urine

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
cx-MINCH	0.025	0.05
OH-MINCH	0.025	0.05
oxo-MINCH[a)]	0.05	0.1
MINCH	0.05	0.1

[a)] These data were collected by the external verifiers of the method (see Section 12).

11.4 Sources of error

DINCH, which is applied as a plasticiser, can be contaminated by the monoester MINCH. For this reason, even though DINCH is not considered a ubiquitously widespread environmental chemical, laboratory equipment, glassware, and all other materials used should be inspected for MINCH blank values. In contrast, the quantitation of the secondary metabolites cx-MINCH, OH-MINCH, and oxo-MINCH is not at risk of contamination.

The Capcell Pak® phase used for online preconcentration proves to be exceptionally robust despite the direct injection of urine. In order to extend the lifetime of the analytical column, a precolumn can be used. It is additionally recommended to place a 5-µm particle filter between the autosampler and the six-port valve. If a pressure increase arises in the system, it can be averted by extending the flush time following sample injection with a maximum flow rate of 1.5 ml/min.

12 Discussion of the method

The biomonitoring method presented herein was developed within the framework of the cooperative project between the *Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit* (Federal Ministry of the Environment, Nature Conservation and Nuclear Safety; BMU) and the *Verband der chemischen Industrie* (German Chemical Industry Association; VCI) for the promotion of human biomonitoring and allows for the selective and sensitive determination of environmental and occupational DINCH exposures. The method presented can be rapidly performed due to its relatively simple workup and short runtime. The validation data confirm the high reproducibility, accuracy, and sensitivity of the method, which results, among other things, from the usage of structurally identical isotope-labelled ISTDs.

As DINCH metabolites are only found in small concentrations in the urine of the general population, the lowest concentrations of the calibration standards should be primarily guided by the instrument-specific quantitation limit. The quantitation limits of 0.05 µg/l (cx-MINCH and OH-MINCH) or 0.1 µg/l (oxo-MINCH and MINCH), determined within the course of method development and external verification, are sufficient to quantify even the background levels in the urine of the general population. The median of the measured concentrations in the urine of the general population lies in the range of 0.2–0.3 µg/l. Higher concentrations, as a result of occupational exposure, could only be roughly estimated until now. It is therefore recommended to establish a calibration range between 0.1 µg/l and 60 µg/l.

At the time of method development, there were neither reference material nor a deuterated ISTD available for oxo-MINCH. For this reason, the determination of oxo-MINCH was performed semiquantitatively by relating the peak areas of oxo-MINCH to those of D_4-OH-MINCH. The calculated quotients were evaluated using the calibration function of OH-MINCH to calculate the respective concentration of oxo-MINCH. The external verifiers of the method had oxo-MINCH and D_3-oxo-MINCH synthesised and included these in a comprehensive validation of the method. The validation data for oxo-MINCH, as collected by the external verifiers of the method, were adopted into the manuscript.

As a general rule, other metabolites can be included in this method. For example, hexyl-cx-MINCH is, in addition to the metabolites analysed herein, another primary metabolite. In contrast, ring-hydroxylated MINCH metabolites, as well as further carboxy metabolites with shortened alkyl chains, play a much smaller role. The unspecific primary

metabolite CHDA can be separately determined using standard addition. For this purpose, the samples are analysed both before and after the addition of 10 µg or 50 µg CHDA per litre. For this determination, it is important to ensure that all samples exhibit the same volumes as well as nearly identical ion concentrations.

For the preparation of the eluents, formic acid may be used in place of acetic acid.

Instruments used HPLC system (Agilent 1200 Series) consisting of autosampler G1329A, quaternary pump G1311A, binary pump G1312A, and vacuum degasser G1322A or G1379B (Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany); mass spectrometer SCIEX QTRAP® 5500 with Turbo V™ Ion Source (AB SCIEX Germany GmbH, Darmstadt, Germany)

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (www.dfg.de/mak/conflicts_interest) ensure that the content and conclusions of the publication are strictly science-based.

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