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# Naphthalene – Determination of 1,2-dihydroxynaphthalene, 1-naphthol and 2-naphthol in urine by GC-MS/MS

## Biomonitoring Method – Translation of the German version from 2020

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## Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method.

This method allows the simultaneous determination of the naphthalene metabolites 1,2-dihydroxynaphthalene (1,2-DHN), 1-naphthol and 2-naphthol in urine in the presence of the antioxidant ascorbic acid. After adding isotope-labelled internal standards, the buffered urine samples are enzymatically hydrolysed to release the analytes from their conjugates. Subsequently, the analytes are purified and enriched using external solid-phase extraction. Following silylation, the analytes are separated by gas chromatography and then analysed by tandem mass spectrometry. Calibration standards are prepared in pooled urine and processed in the same way as the samples to be analysed. For 1,2-DHN, a glucuronide conjugate of 1,2-DHN is used for calibration.

# 1 Characteristics of the method

**Matrix** Urine

**Analytical principle** GC-MS/MS

**Parameters and their corresponding hazardous substances**

| Hazardous substance | CAS No. | Parameter | CAS No. |
|---|---|---|---|
| Naphthalene | 91-20-3 | 1,2-Dihydroxynaphthalene (1,2-DHN) | 574-00-5 |
| | | 1-Naphthol | 90-15-3 |
| | | 2-Naphthol | 135-19-3 |
| 1-Naphthyl methylcarbamate (carbaryl) | 63-25-2 | 1-Naphthol | 90-15-3 |

## Reliability data

### 1,2-Dihydroxynaphthalene (1,2-DHN)

| | | |
|---|---|---|
| Within-day precision: | Standard deviation (rel.) | $s_w$ = 4.5% or 4.2% |
| | Prognostic range | $u$ = 11.6% or 10.8% |
| | at a spiked concentration of 99 µg or 495 µg 1,2-DHN per litre of urine and n = 6 determinations | |
| Day-to-day precision: | Standard deviation (rel.) | $s_w$ = 4.1% or 2.1% |
| | Prognostic range | $u$ = 10.5% or 5.4% |
| | at a spiked concentration of 99 µg or 495 µg 1,2-DHN per litre of urine and n = 6 determinations | |
| Accuracy: | Recovery rate (rel.) | $r$ = 100%, 89%, or 100% |
| | at a nominal concentration of 6 µg, 120 µg, or 500 µg 1,2-DHN per litre of urine and n = 5 determinations | |
| Detection limit: | 0.2 µg 1,2-DHN per litre of urine | |
| Quantitation limit: | 0.5 µg 1,2-DHN per litre of urine | |

## 1-Naphthol

| | | |
|---|---|---|
| Within-day precision: | Standard deviation (rel.) | $s_w$ = 6.6% or 3.5% |
| | Prognostic range | $u$ = 17.0% or 9.0% |
| | at a spiked concentration of 97 µg or 484 µg 1-naphthol per litre of urine and n = 6 determinations | |
| Day-to-day precision: | Standard deviation (rel.) | $s_w$ = 4.9% or 3.9% |
| | Prognostic range | $u$ = 12.6% or 10.0% |
| | at a spiked concentration of 97 µg or 484 µg 1-naphthol per litre of urine and n = 6 determinations | |
| Accuracy: | Recovery rate (rel.) | $r$ = 101%, 90%, or 103% |
| | at a nominal concentration of 6 µg, 120 µg, or 500 µg 1-naphthol per litre of urine and n = 5 determinations | |
| Detection limit: | 0.1 µg 1-naphthol per litre of urine | |
| Quantitation limit: | 0.2 µg 1-naphthol per litre of urine | |

## 2-Naphthol

| | | |
|---|---|---|
| Within-day precision: | Standard deviation (rel.) | $s_w$ = 4.9% or 1.4% |
| | Prognostic range | $u$ = 12.6% or 3.6% |
| | at a spiked concentration of 103 µg or 515 µg 2-naphthol per litre of urine and n = 6 determinations | |
| Day-to-day precision: | Standard deviation (rel.) | $s_w$ = 4.6% or 2.7% |
| | Prognostic range | $u$ = 11.8% or 6.9% |
| | at a spiked concentration of 103 µg or 515 µg 2-naphthol per litre of urine and n = 6 determinations | |
| Accuracy: | Recovery rate (rel.) | $r$ = 96%, 92%, or 107% |
| | at a nominal concentration of 6 µg, 120 µg, or 500 µg 2-naphthol per litre of urine and n = 5 determinations | |
| Detection limit: | 0.1 µg 2-naphthol per litre of urine | |
| Quantitation limit: | 0.2 µg 2-naphthol per litre of urine | |

## 2 General information on naphthalene

Naphthalene belongs to the group of polycyclic aromatic hydrocarbons (PAH) and is a natural component of coal tar and crude oil. After purification, naphthalene is used, among other things, as a raw material in chemical syntheses; in the production of dyes, pyrotechnics, and mothballs; and as a pore-forming agent in the manufacture of high-porosity grinding wheels (EU 2003).

In addition, naphthalene is formed during the incomplete combustion of organic materials, which means that naphthalene is found ubiquitously in the environment. Exposure of the general population may occur by consumption of grilled or smoked foods and by inhaling cigarette smoke or combustion fumes. In industrial workplaces, such as in the chemical industry; in the production of grinding wheels; or in workplaces where tar, creosote, or bitumen are used, exposure levels in workers were found to be significantly higher than in the general population (Preuss et al. 2005; Sucker et al. 2017; Ziener 2012).

Naphthalene has been classified by the Commission in Carcinogen Category 2 and has been designated with an "H" (danger from percutaneous absorption). It has also been classified as a Category 3 B germ cell mutagen (DFG 2020). For biomonitoring, a biological reference value (BAR, *Biologischer Arbeitsstoff-Referenzwert*) of 35 µg/l urine for the sum of 1-naphthol and 2-naphthol (after hydrolysis) was derived for non-smokers. For a summary of the toxicological properties of naphthalene, please refer to the corresponding documentations of the Commission (Hartwig 2012; Klotz et al. 2018 a).

In humans, naphthalene is metabolised into two unstable, highly reactive epoxides, which are converted in further reaction steps into 1-naphthyl mercapturic acid and various monohydroxylated and dihydroxylated compounds or the corresponding conjugates with glucuronide or sulphate and are excreted in the urine (Preuss et al. 2003; Zobel et al. 2017, 2018) (see Figure 1). A subsequent secondary reaction of the hydroxylated metabolites may result in the formation of highly reactive naphthoquinones, which are considered to be the actual toxic agents (Bolton et al. 2000). Direct determination of naphthoquinones in urine is difficult due to their high reactivity. However, reaction products of these compounds with glutathione and proteins have already been identified (Ayala et al. 2015; Cho et al. 1994; Lin et al. 2014; Murty and Penning 1992; Tsuruda et al. 1995; Waidyanatha et al. 2002). 1,2-Dihydroxynaphthalene (1,2-DHN), a direct precursor of 1,2-naphthoquinone, has been shown in recent studies to be one of the major metabolites in human naphthalene metabolism and proved to be a sensitive and specific biomarker of exposure (Klotz et al. 2011, 2018 b; Wu et al. 2005). In addition, a higher diagnostic specifity has been shown for 1,2-DHN when compared to the frequently used naphthalene biomarkers 1-naphthol and 2-naphthol (Klotz et al. 2011, 2018 b). For the determination of 1-naphthol and 2-naphthol in urine, there are already analytical methods available that have been approved by the Commission (Hardt et al. 2010; Preuss et al. 2010).

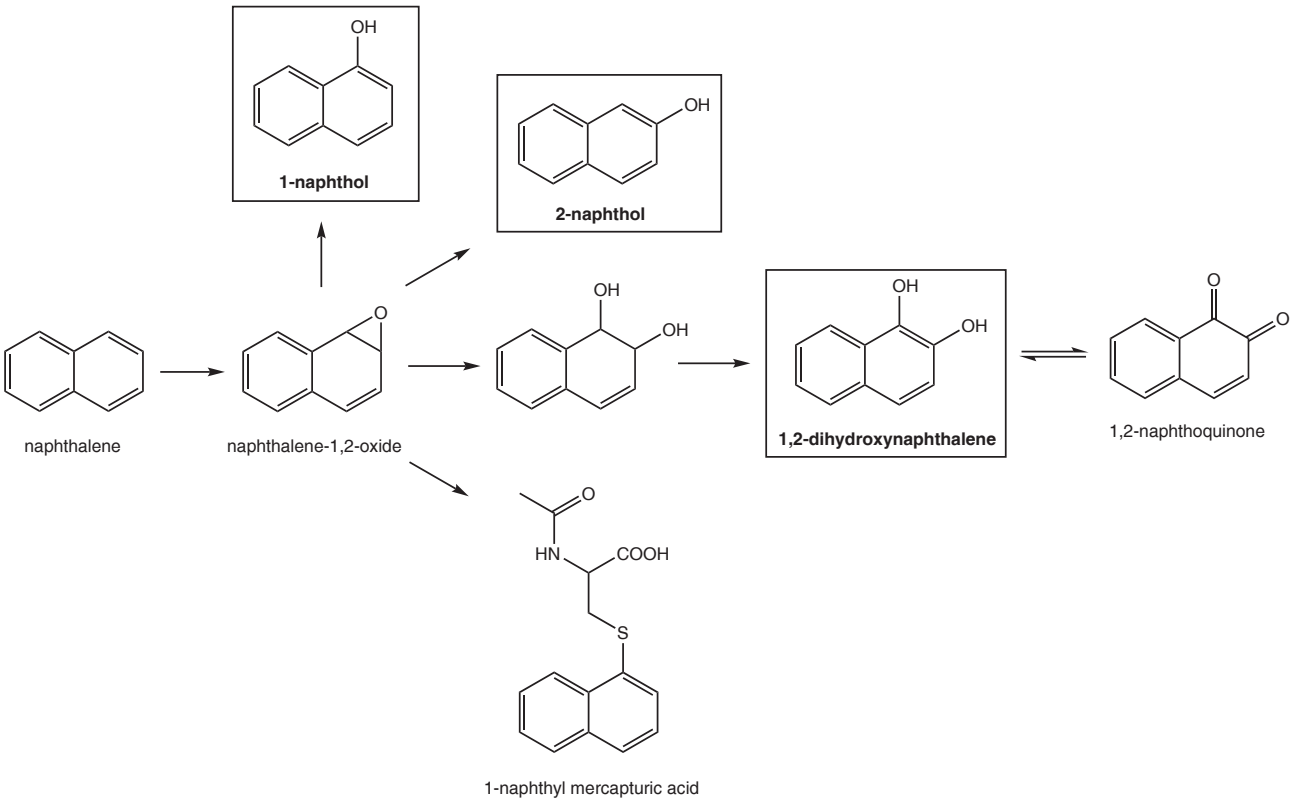


**Fig. 1** Schematic overview of naphthalene metabolism in humans (according to Zobel et al. 2017)

A large number of studies have been published on naphthol excretion in the urine of non-smokers and smokers not occupationally exposed to naphthalene (reviewed by Klotz et al. 2018 a). For non-smokers, concentration levels of < 30 µg/l and < 20 µg/l for 1-naphthol and 2-naphthol, respectively, were predominantly reported. For smokers, reported concentration levels of 1-naphthol and 2-naphthol in urine were < 45 µg/l and < 55 µg/l, respectively. Data on background exposure to 1,2-DHN are scarce. Wu et al. (2005) reported a geometric mean of 38.8 ± 2.3 µg/l urine for a collective of 21 Chinese office and hospital workers (18 non-smokers, three smokers). A control group of 29 individuals in Germany showed significantly lower levels. The median (range) for 20 non-smokers and for nine smokers in urine was 4.6 µg/l (< 1.0–19.3 µg/l) and 17.1 µg/l (1.9–62.0 µg/l), respectively (Klotz et al. 2011). Significantly higher exposure levels were observed for occupationally exposed persons in industrial workplaces. A study collective of nine workers at a wood-impregnation plant revealed urinary 1,2-DHN levels of up to 1500 µg/l with urinary levels of 1-naphthol and 2-naphthol of up to 150 µg/l each, respectively (Klotz et al. 2018 b). In another study, 32 naphthalene exposed workers of the abrasives industry were monitored, and urinary 1,2-DHN levels of up to 50 000 µg/l were reported with median levels in the range of 600 and 11 000 µg 1,2-DHN/l depending on the sampling time. The median urinary levels of 1-naphthol and 2-naphthol ranged between 20 and 400 µg/l each with maximum levels of 2700 and 1100 µg/l, respectively (Klotz et al. 2019).

When assessing exposure based on 1-naphthol excretion in the urine, it should be noted that 1-naphthol is also a metabolite of the insecticide carbaryl (Henschler 1973).

# 3 General principles

This method allows the simultaneous determination of the naphthalene metabolites 1,2-DHN, 1-naphthol, and 2-naphthol in urine in the presence of the antioxidant ascorbic acid. After adding isotope-labelled internal standards, the buffered urine samples are enzymatically hydrolysed to release the analytes from the conjugates. Subsequently, the analytes are purified and enriched using external solid-phase extraction. Following silylation, the analytes are separated by gas chromatography and then analysed by tandem mass spectrometry. Calibration standards are prepared in pooled urine and processed in the same way as the samples to be analysed. For 1,2-DHN, a glucuronide conjugate of 1,2-DHN is used for calibration.

# 4 Equipment, chemicals, and solutions

## 4.1 Equipment

- Gas chromatograph with tandem mass spectrometer (e.g. Agilent 7000 A, Agilent Technologies Deutschland GmbH, Waldbronn, Germany)
- Capillary gas chromatography column: stationary phase: 5%-phenyl-arylene-95%-dimethylpolysiloxane, length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 µm (e.g. Zebron® ZB 5 ms, Phenomenex Ltd. Deutschland, Aschaffenburg, Germany, No. 7HG-G010-11)
- Solid-phase extraction vacuum manifold (e.g. VacElut, Agilent Technologies Deutschland GmbH, Waldbronn, Germany)
- Solid-phase extraction columns, stationary phase: polystyrene-divinylbenzene copolymer (e.g. Isolute® 101, 100 mg, 3 ml, particle size 65 µm, pore diameter 100 Å, Biotage AB, Uppsala, Sweden, No. 101-0010-B)
- Centrifuge (e.g. Heraeus Deutschland GmbH & Co. KG, Hanau, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Vortex mixer (e.g. Scientific Industries, Inc., New York, USA)
- Incubator (e.g. Memmert GmbH & Co. KG, Schwabach, Germany)
- Water bath (e.g. Gesellschaft für Labortechnik mbH, Burgwedel, Germany)
- 8-ml amber glass vials with caps (e.g. LC Laborcenter GmbH & Co., Nuremberg, Germany)
- 13-ml plastic tubes with caps (e.g. VWR International GmbH, Darmstadt, Germany)
- Various pipettes and Multipettes® (e.g. Eppendorf AG, Hamburg, Germany)
- Various beakers and volumetric flasks (e.g. VWR International GmbH, Darmstadt, Germany)
- pH meter with pH electrode (e.g. Mettler-Toledo GmbH, Gießen, Germany)
- Ultrasonic bath (e.g. VWR International GmbH, Darmstadt, Germany)
- Nitrogen evaporator (e.g. Biotage AB, Uppsala, Sweden)
- 1.8-ml amber glass vials with crimp caps (e.g. Agilent Technologies Deutschland GmbH, Waldbronn, Germany)
- 200-µl micro inserts for the vials (e.g. Agilent Technologies Deutschland GmbH, Waldbronn, Germany)
- Urine-collection containers made of polyethylene (e.g. VWR International GmbH, Darmstadt, Germany)

## 4.2 Chemicals

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

### Reference standards

- 1-Naphthol, 99% (e.g. Sigma Aldrich GmbH, Steinheim, Germany, No. N1000)
- 2-Naphthol, 98% (e.g. Sigma Aldrich GmbH, Steinheim, Germany, No. 130109)
- 1,2-Dihydroxynaphthalene glucuronide × 1.5 triethylamine, 97% (custom synthesis, see Zobel et al. 2017)

### Internal standards

- $D_8$-1-Naphthol (1-OD-2,3,4,5,6,7,8-$D_7$-naphthalene), 98% (e.g. C/D/N Isotopes Inc., Quebec, Canada, No. D-2391)
- $D_7$-2-Naphthol (2-OH-1,3,4,5,6,7,8-$D_7$-naphthalene), 98% (e.g. C/D/N Isotopes Inc., Quebec, Canada, No. D-5051)
- $D_6$-1,2-Dihydroxynaphthalene glucuronide × 1.5 triethylamine, 95% (custom synthesis, see Zobel et al. 2017)

### Other chemicals

- Bis(trimethylsilyl)acetamide with 3–5% trimethylchlorosilane (BSA+TMCS) (e.g. Sigma Aldrich GmbH, Steinheim, Germany, No. 15256)
- Methanol for gas chromatography (e.g. Merck KGaA, Darmstadt, Germany, No. 100837)
- Toluene for gas chromatography (e.g. Merck KGaA, Darmstadt, Germany, No. 100849)
- Acetonitrile, anhydrous, 99.8% (e.g. Sigma Aldrich GmbH, Steinheim, Germany, No. 271004)
- Acetic acid, 100% (e.g. Merck KGaA, Darmstadt, Germany, No. 100063)
- Sodium hydroxide, pellets (e.g. Merck KGaA, Darmstadt, Germany, No. 106469)
- Ascorbic acid, ≥ 99% (e.g. Carl Roth GmbH + Co. KG, Karlsruhe, Germany, No. 3525.3)
- $\beta$-Glucuronidase (5.5 U/ml)/arylsulfatase (2.6 U/ml), isolated from *Helix pomatia* (e.g. Roche Diagnostics GmbH, Mannheim, Germany, No. BGALA-RO)
- Ultra-pure water (e.g. via MilliPore® technology)
- Helium 5.0 (e.g. Linde GmbH, Pullach, Germany)

## 4.3 Solutions

- Sodium hydroxide solution (1 mol/l)

  Exactly 40.0 g of sodium hydroxide pellets are weighed into a 1000-ml volumetric flask and dissolved in ultra-pure water. The flask is then made up to the mark with ultra-pure water.

- Sodium acetate buffer (0.1 mol/l, pH 5)

  Approximately 800 ml of ultra-pure water are placed in a beaker and 11.5 ml of acetic acid are added. Using a pH meter, the pH is adjusted to 5 by adding the sodium hydroxide solution (1 mol/l) under constant stirring. After transferring the solution to a 2-l volumetric flask, the latter is made up to the mark with ultra-pure water.

- Aqueous methanol solution (8% methanol, v/v)

  Approximately 50 ml of ultra-pure water are placed in a 100-ml volumetric flask and 8 ml of methanol are added. The flask is then made up to the mark with ultra-pure water.

The solutions are stored in glass bottles at room temperature and are stable for at least six months under these conditions.

- Ascorbic acid solution (250 g/l)

  Exactly 2.50 g of ascorbic acid are weighed into a 10-ml volumetric flask and approximately 7 ml of ultra-pure water are added. The solid material is dissolved by careful warming and by using an ultrasonic bath. The flask is then made up to the mark with ultra-pure water. The solution must be prepared freshly every workday.

## 4.4 Internal standards (ISTD)

- ISTD stock solutions (approx. 100 mg/l)

  Approximately 0.5 mg of the isotope-labelled standard substances $D_8$-1-naphthol and $D_7$-2-naphthol as well as 1.5 mg of the trimethylamine salt of $D_6$-1,2-dihydroxynaphthalene glucuronide are each weighed exactly into a 5-ml volumetric flask and dissolved in methanol. The flasks are then made up to the mark with methanol.

- ISTD spiking solution

  350 µl of each ISTD stock solution are pipetted into a 10-ml volumetric flask, which is then made up with ultra-pure water. The analyte concentration of the ISTD in the spiking solution is approximately 3.5 mg/l each.

The ISTD solutions are stored in the dark at −20 °C in amber screw-cap vials with Teflon-lined caps. Under these conditions, they are stable for at least six months.

## 4.5 Calibration standards

- Stock solutions (400 mg/l)

  4 mg each of the standard substances of 1-naphthol and 2-naphthol and, taking into account the mass ratio to glucuronide and triethylamine, 12.5 mg 1,2-DHN are weighed exactly into a 10-ml volumetric flask each and dissolved in methanol. The flasks are then made up to the mark with methanol.

- Working solution (100 mg/l)

  2.5 ml of each stock solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with methanol.

- Spiking solution 1 (SpS 1, 10 mg/l)

  1 ml of the working solution is pipetted into a 10-ml volumetric flask, which is then made up to the mark with ultra-pure water.

- Spiking solution 2 (SpS 2, 1 mg/l)

  0.1 ml of the working solution is pipetted into a 10-ml volumetric flask, which is then made up to the mark with ultra-pure water.

The standard solutions are stored in the dark at −20 °C in amber screw-cap glass vials with Teflon-lined caps. Under these conditions, they are stable for at least six months.

The calibration standards are prepared in pooled urine. The pooled urine is prepared using a mixture of individual urine samples from non-smokers not occupationally exposed to naphthalene or other polycyclic aromatic hydrocarbons. The pooled urine is stored at −20 °C and is passed through a fluted filter prior to use.

To prepare the calibration standards, the aqueous spiking solutions are mixed with pooled urine yielding a final sample volume of 2 ml according to the pipetting scheme given in Table 1. The calibration solutions are processed in the same way as the samples to be analysed as described in Section 5. Alternatively, larger amounts of the calibration solutions can be prepared and aliquoted to 2 ml each. The calibration solutions are stored in screw-cap glass vials with Teflon-lined caps at −20 °C. Under these conditions, they are stable for at least six months.

**Tab. 1** Pipetting scheme for the preparation of calibration standards used to determine naphthalene metabolites in urine

| Calibration standard | Volume of spiking solution [µl] | | Pooled urine [µl] | Spiked concentration per analyte [µg/l] |
|---|---|---|---|---|
| | SpS 1 | SpS 2 | | |
| 0 | – | – | 2000 | 0 |
| 1 | – | 10 | 1990 | 5 |
| 2 | – | 20 | 1980 | 10 |
| 3 | 20 | – | 1980 | 100 |
| 4 | 50 | – | 1950 | 250 |
| 5 | 100 | – | 1900 | 500 |
| 6 | 150 | – | 1850 | 750 |
| 7 | 200 | – | 1800 | 1000 |

# 5 Specimen collection and sample preparation

## 5.1 Specimen collection

The urine samples are collected in sealable polyethylene containers and stored at −20 °C until sample preparation.

## 5.2 Sample preparation

Prior to analysis, the urine samples are thawed to room temperature and thoroughly mixed. Exactly 150 µl of the freshly prepared ascorbic acid solution and 50 µl of the ISTD spiking solution are added to a 2-ml aliquot of urine in an 8-ml amber glass screw-cap vial. Subsequently, 1 ml of the sodium acetate buffer is added and the sample is thoroughly mixed using a vortex mixer. For hydrolysis, 20 µl of $\beta$-glucuronidase/arylsulfatase are then added. The sample is again thoroughly mixed (ten seconds on the vortex mixer) and then hydrolysed at 37 °C in a water bath for 16 hours. Following hydrolysis, the sample is thoroughly mixed once more (ten seconds on the vortex mixer) and centrifuged at 1600 × $g$ for ten minutes.

The analytes are enriched by solid-phase extraction (SPE) using Isolute® 101 cartridges. The cartridge is conditioned on a SPE vacuum manifold by washing it twice with 1.5 ml methanol and then equilibrating it twice with 1.5 ml of ultra-pure water and 2 ml of sodium acetate buffer each time. Then, 3 ml of the supernatant of the hydrolysed and centrifuged sample solution are loaded. The sample should not be loaded by applying a vacuum. The cartridge is then washed in succession with 3 ml of sodium acetate buffer, twice with 1.5 ml each of ultra-pure water, and 2 ml of the aqueous methanol solution. The cartridge is dried for approximately five minutes by applying a vacuum and is

then placed in a 13-ml plastic tube and centrifuged at 1600 × *g* for ten minutes to remove any residual liquid. After the cartridge is again dried for ten minutes on the SPE vacuum manifold, the analytes are eluted into a 1.8-ml amber glass vial by loading acetonitrile onto the cartridge three times, 0.5 ml each time. The eluate is then evaporated to dryness under a stream of nitrogen. For complete removal of residual water, the residue is reconstituted in 200 µl of acetonitrile and again evaporated to dryness under a stream of nitrogen.

For derivatisation, 10 µl of the derivatisation reagent BSA+TMCS and 200 µl toluene are added to the residue. The vial is sealed, thoroughly mixed (ten seconds on the vortex mixer), and then treated for ten minutes in an ultrasonic bath. The sample is then incubated for one hour at 70 °C in an incubator. Afterwards, the sample solution is transferred into a 200-µl micro insert and injected into the GC-MS/MS system for analysis.

# 6 Operational parameters

Analysis is performed using a gas chromatograph coupled with a tandem mass spectrometer (GC-MS/MS system).

## 6.1 Gas chromatography

| | | |
|---|---|---|
| Capillary column: | Stationary phase: | Zebron® ZB 5 ms |
| | Length: | 30 m |
| | Inner diameter: | 0.25 mm |
| | Film thickness: | 0.25 µm |
| Temperatures: | Column: | Initial temperature 100 °C, 1 min isothermal, increase at a rate of 10 °C/min to 120 °C, then increase at a rate of 5 °C/min to 195 °C, then increase at a rate of 30 °C/min to 300 °C, 15 min at final temperature |
| | Injector: | 280 °C |
| | Transfer line: | 300 °C |
| Carrier gas: | Helium 5.0 | |
| Flow rate: | 1.8 ml/min, constant | |
| Inlet liner: | Glass wool, deactivated | |
| Injection: | 1 µl, splitless | |

## 6.2 Tandem mass spectrometry

| | |
|---|---|
| Ionisation mode: | Electron ionisation (EI) |
| Ionisation energy: | 70 eV |
| Source temperature: | 230 °C |
| Solvent delay: | 10 min |
| Detection mode: | MRM |
| Parameter-specific settings: | see Table 2 |

All settings of the ion source as well as the MRM parameters are instrument-specific and must be adjusted individually by the user. The parameters given are therefore only intended as cursory guidance.

# 7 Analytical determination

1 µl each of the samples, prepared as described in Section 5, is injected into the GC-MS/MS system. Identification of the analytes is based on the specific mass transitions and retention times (see Table 2). The retention times given in Table 2 are intended only as a rough guide. Users must ensure proper separation performance of the column and any subsequent influence on the resulting retention behaviour of the analytes. A reagent blank (ultra-pure water instead of the urine sample) is included in each analytical run. As an example, Figure 2 shows chromatograms of a processed urine sample from a worker occupationally exposed to naphthalene.

**Tab. 2** Parameter-specific settings and retention times of the analytes

| Analyte | Retention time [min] | Ion traces [*m/z*] | | Collision energy [V] |
|---|---|---|---|---|
| | | Precursor ion (Q1) | Product ion (Q3) | |
| 1-Naphthol | 11.1 | 201 | 185 | 10 |
| | | 201 | 145[a)] | 12 |
| | | 216 | 201[b)] | 7 |
| $D_8$-1-Naphthol | 11.0 | 208 | 191 | 14 |
| | | 208 | 151[a)] | 14 |
| | | 223 | 208[b)] | 7 |
| 2-Naphthol | 11.6 | 201 | 145 | 12 |
| | | 201 | 185[a)] | 10 |
| | | 216 | 201[b)] | 7 |
| $D_7$-2-Naphthol | 11.5 | 208 | 151 | 14 |
| | | 208 | 191[a)] | 14 |
| | | 223 | 208[b)] | 7 |
| 1,2-DHN | 16.3 | 304 | 216 | 16 |
| | | 216 | 201[a)] | 10 |
| $D_6$-1,2-DHN | 16.2 | 310 | 222 | 16 |
| | | 222 | 207[a)] | 12 |

[a)] qualifier
[b)] alternative ion trace, used as a quantifier during external verification

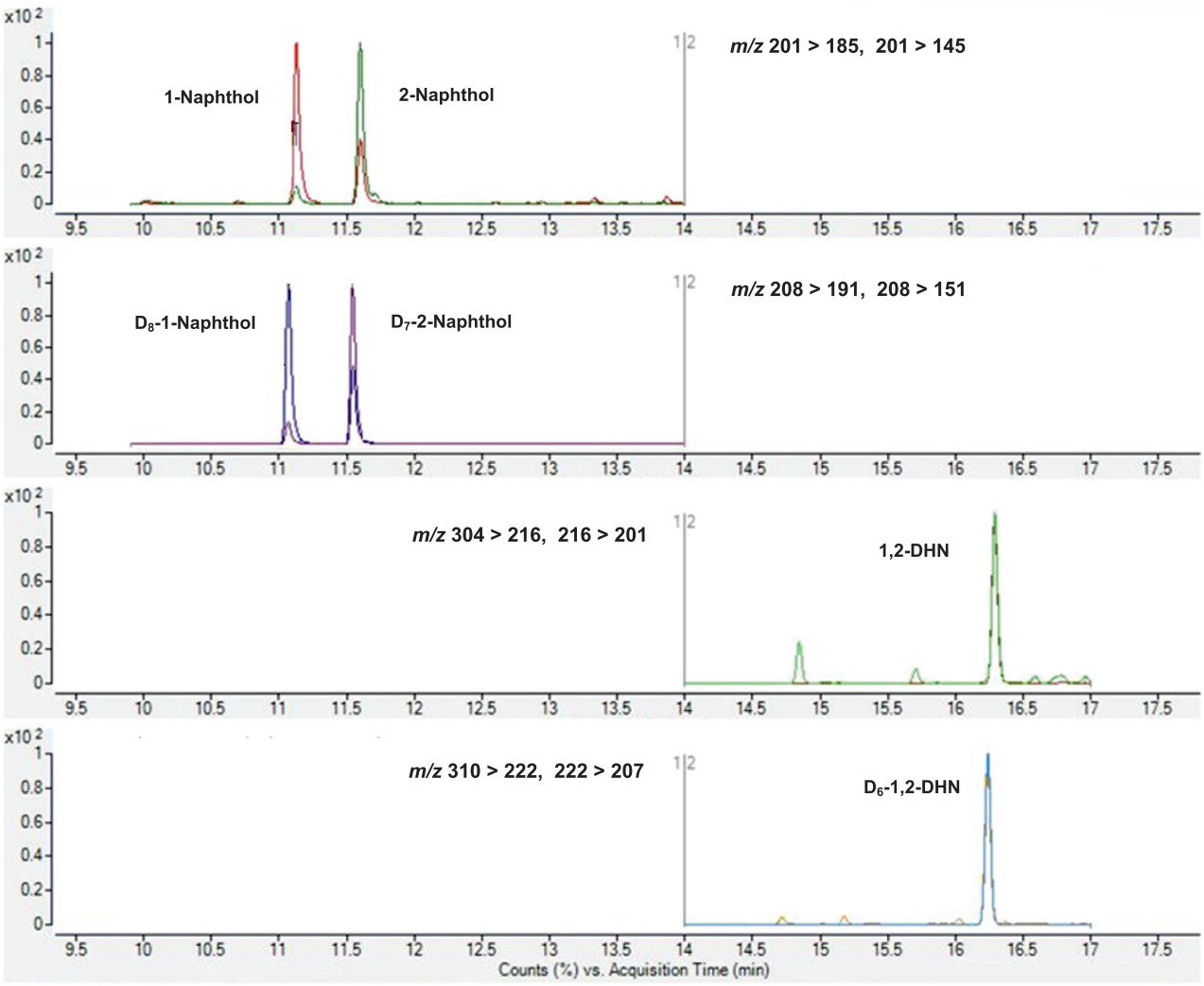


**Fig. 2** GC-MS/MS chromatograms of a processed urine sample from a worker occupationally exposed to naphthalene (analyte levels determined: 1-naphthol: 30.2 µg/l, 2-naphthol: 22.5 µg/l and 1,2-DHN: 260 µg/l)

## 8 Calibration

The calibration standards, prepared as described in Section 4.5, are processed in the same way as the samples to be analysed (cf. Section 5) and analysed by GC-MS/MS (cf. Sections 6 and 7). Calibration graphs are obtained by plotting the quotients of the peak areas of the analyte and the corresponding isotope-labelled internal standard against the spiked concentration of the calibration standard. The calibration curve is linear under the described analytical conditions in the concentration range from 5 to 1000 µg/l with a correlation coefficient of $r \geq 0.995$ for all analytes. Figures 3 to 5 show calibration curves for the analytes in pooled urine, as an example.

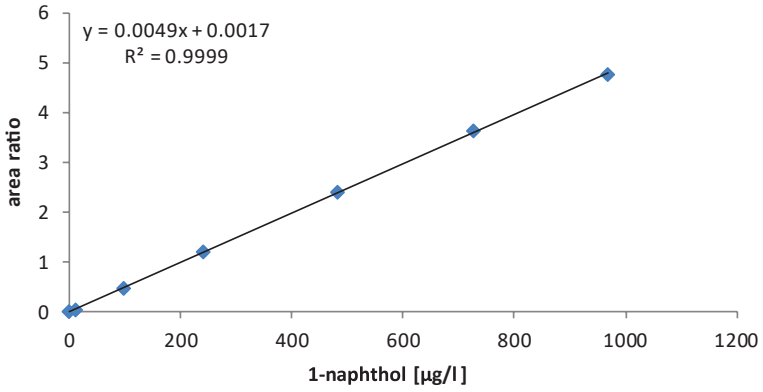


**Fig. 3** Calibration curve for 1-naphthol in pooled urine in the concentration range between 5 and 1000 µg/l

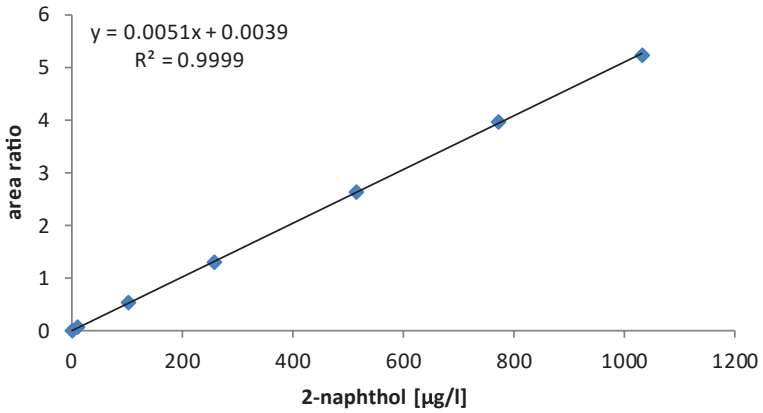


**Fig. 4** Calibration curve for 2-naphthol in pooled urine in the concentration range between 5 and 1000 µg/l

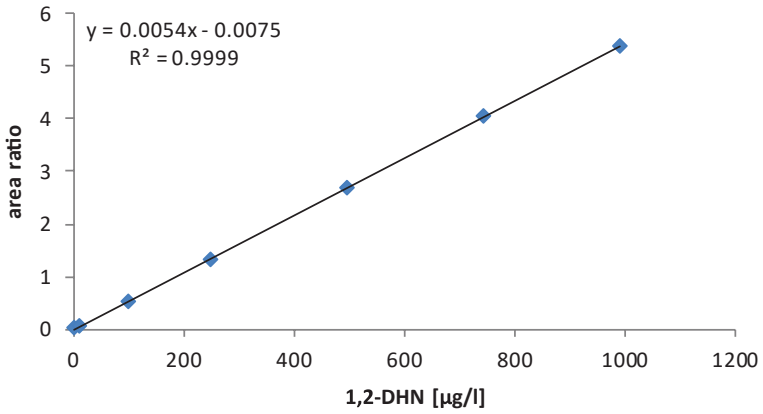


**Fig. 5** Calibration curve for 1,2-DHN in pooled urine in the concentration range between 5 and 1000 µg/l

# 9 Calculation of the analytical results

The analyte concentration in a urine sample is calculated by dividing the peak area of the analyte by the peak area of the corresponding ISTD. The quotient thus obtained is entered into the respective calibration equation according to Section 8 to give the respective analyte concentration in µg/l urine. If the analytical result is above the calibration range, the sample in question is diluted with ultra-pure water, reprocessed, and newly analysed.

# 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 of the MAK Collection for Occupational Health and Safety (Bader et al. 2010; Bundesärztekammer 2014). To check precision, at least two quality-control samples with known low ($Q_{low}$) and high ($Q_{high}$) analyte concentration levels are analysed within each analytical run. As control material is not commercially available, it must be prepared in the laboratory by spiking pooled urine of non-smokers with the analytes. The analyte concentration in the quality-control material should be within the relevant concentration range (e.g. 100 µg/l and 500 µg/l). Aliquots of these samples are stored at −20 °C and are included in each analytical run as quality-control samples. The nominal value and the tolerance ranges of the quality-control material are determined in a pre-analytical period (one analysis of each control material on ten different days) (Bader et al. 2010).

# 11 Evaluation of the method

The reliability of the method was verified by comprehensive validation as well as by implementation and replication of the method in a second, independent laboratory.

## 11.1 Precision

Within-day precision was determined using the quality control material $Q_{low}$ and $Q_{high}$ (see Section 10). To this end, the $Q_{low}$ and $Q_{high}$ material was processed and analysed six times in parallel. The obtained within-day precision data are presented in Table 3.

**Tab. 3** Within-day precision for the determination of naphthalene metabolites in urine (n = 6)

| Analyte | Determined level [µg/l] | Standard deviation (rel.) $s_w$ [%] | Prognostic range $u$ [%] |
|---|---|---|---|
| 1,2-DHN | 102 | 4.5 | 11.6 |
| | 507 | 4.2 | 10.8 |
| 1-Naphthol | 94.6 | 6.6 | 17.0 |
| | 457 | 3.5 | 9.0 |
| 2-Naphthol | 100 | 4.9 | 12.6 |
| | 495 | 1.4 | 3.6 |

Day-to-day precision was determined by processing and analysing the $Q_{low}$ and $Q_{high}$ samples on six different days. As part of external verification, day-to-day precision was also determined at a lower concentration level. The obtained day-to-day precision data are presented in Table 4.

**Tab. 4** Day-to-day precision for the determination of naphthalene metabolites in urine (n = 6)

| Analyte | Determined level [µg/l] | Standard deviation (rel.) $s_w$ [%] | Prognostic range $u$ [%] |
|---|---|---|---|
| 1,2-DHN | 7.0 | 9.4 | 24.2 |
| | 100 | 4.1 | 10.5 |
| | 490 | 2.1 | 5.4 |
| 1-Naphthol | 4.9 | 9.9 | 25.4 |
| | 96.4 | 4.9 | 12.6 |
| | 457 | 3.9 | 10.0 |
| 2-Naphthol | 6.6 | 11.1 | 28.6 |
| | 103 | 4.6 | 11.8 |
| | 504 | 2.7 | 6.9 |

## 11.2 Accuracy

In order to check the accuracy of the method and to examine the impact of possible matrix effects, five individual urine samples with creatinine levels ranging from 0.3 to 2.6 g/l were spiked with concentration levels of about 6, 120 and 500 µg each of 1,2-DHN, 1-naphthol, and 2-naphthol per litre of urine. Both unspiked and spiked urine samples were processed and analysed. The relative recovery rates were calculated on the basis of the determined concentration levels in the spiked urine samples, subtracting any background levels of the analytes. The mean relative recovery rates are presented in Table 5.

**Tab. 5** Mean relative recovery rates for the determination of naphthalene metabolites in individual urine samples (n = 5)

| Analyte | Spiked concentration [µg/l] | Mean relative recovery [%] | Range [%] |
|---|---|---|---|
| 1,2-DHN | 6 | 100 | 95–105 |
| | 120 | 89 | 87–96 |
| | 500 | 100 | 98–103 |
| 1-Naphthol | 6 | 101 | 95–111 |
| | 120 | 90 | 86–97 |
| | 500 | 103 | 100–106 |
| 2-Naphthol | 6 | 96 | 93–100 |
| | 120 | 92 | 88–97 |
| | 500 | 107 | 99–112 |

## 11.3 Absolute recovery

To determine the process-related losses of the method, pooled urine was spiked with about 100 µg/l of each analyte, processed, and analysed (n = 4). In addition, unspiked pooled urine samples were processed and spiked with the analytes only after enzymatic hydrolysis, solid-phase extraction, and evaporation under a stream of nitrogen, then derivatised and analysed. By comparing the absolute peak areas without taking the internal standards into account, the process-related losses were determined for each analyte. With absolute recovery rates of 93 to 97%, solid-phase extraction proved to be highly efficient with only minimal process-related analyte losses.

## 11.4 Limits of detection and quantitation

The limits of detection and quantitation of the described analytical method were determined using the blank-value method according to DIN 32645 (DIN 2008) by calculating the statistical variance of three determinations from ten blank urine samples. The results are presented in Table 6.

**Tab. 6** Limits of detection and quantitation in urine

| Analyte | Detection limit [µg/l] | Quantitation limit [µg/l] |
|---|---|---|
| 1,2-DHN | 0.2 | 0.5 |
| 1-Naphthol | 0.1 | 0.2 |
| 2-Naphthol | 0.1 | 0.2 |

## 11.5 Sources of error

A quenching effect may occur in urine samples with a high matrix load, which suppresses the ionisation of the analytes and thus, in some cases, yields detection limits that are higher than those indicated. The matrix effects are efficiently compensated for by use of the isotope-labelled internal standards.

Generally, there is a risk of oxidative degradation of the analytes to be considered in the analysis of hydroxylated PAH – especially for vicinal dihydroxy PAH. Such degradation can, however, be significantly reduced by adding antioxidants such as ascorbic acid.

# 12 Discussion of the method

The analytical method presented above allows the simultaneous determination of the naphthalene metabolites 1,2-DHN, 1-naphthol, and 2-naphthol in human urine samples.

The validation data show high reproducibility, accuracy, and sensitivity of the method, which is achieved by the use of isotope-labelled internal standard substances as well as efficient sample preparation and derivatisation. The use of a glucuronide conjugate of 1,2-DHN as a stable reference substance helped to avoid the difficulties described in an earlier method, which were caused by the oxidative instability of free 1,2-DHN used as a reference substance (Klotz et al. 2011). Glucuronidated 1,2-DHN is not only more stable in solution, but it is also less sensitive to degradation during storage as a solid compared to free 1,2-DHN. The use of the glucuronide conjugate of 1,2-DHN as reference material thus considerably facilitates the practical implementation of the method and thus contributes significantly to obtain reliable results.

When processed according to the method described above, 1,2-DHN glucuronide is hydrolysed. Hence, the method enables the determination of 1,2-DHN as the sum of free and conjugated 1,2-DHN.

As part of external method verification, experiments were carried out in parallel with the alternative SPE material Bond Elut PPL (column dimension 100 mg/3 ml by Agilent Technologies Deutschland GmbH, Waldbronn, Germany), yielding very similar results. It was also shown that for extended analytical runs, recalibration of the method can be done by way of single-point calibration provided that a complete calibration curve was created previously.

Overall, the method is highly sensitive and linear over a wide concentration range (up to 1000 µg/l), making it suitable for application in both environmental medicine and occupational health.

**Instruments used** GC-MS/MS Agilent 7000A Series Triple Quadrupole System, 7890A gas chromatograph with AS7693 autosampler, split/splitless injector and mass-selective detector 7000A Triple Quadrupole (all from Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

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