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# Simultaneous determination of 1- and 2-naphthol in human urine using on-line clean-up column-switching liquid chromatography–fluorescence detection

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**Abstract**

We developed a new 3-D HPLC method for on-line clean-up and simultaneous quantification of two important naphthalene metabolites, 1-naphthol and 2-naphthol, in human urine. Except an enzymatic hydrolysis no further sample pre-treatment is necessary. The metabolites are stripped from urinary matrix by on-line extraction on a restricted access material pre-column (RAM RP-8), transferred in backflush mode onto a silica-based CN-(cyano)phase column for further purification from interfering substances. By another successive column switching step both analytes are transferred with a minimum of overlapping interferences onto a C12 bonded reversed phase column with trimethylsilyl endcapping where the final separation is carried out. The entire arrangement is software controlled. Eluting analytes are quantified by fluorescence detection (227/430 nm) after an external calibration. Within a total run time of 40 min we can selectively quantify both naphthols with detection limits in the lower ppb range (1.5 and 0.5 μg/l for 1- and 2-naphthol, respectively) with excellent reliability (ensured by precision, accuracy, matrix-independency and FIOH quality assurance program participation). First results on a collective of 53 occupationally non exposed subjects showed mean levels of 11.0 μg/l (1-naphthol) and 12.9 μg/l (2-naphthol). Among smokers ($n = 21$) a significantly elevated mean level of urinary naphthols was determined (1-naphthol: 19.2 μg/l and 2-naphthol: 23.7 μg/l) in comparison to non smokers ($n = 32$; 1-naphthol: 5.6 μg/l, 2-naphthol: 5.6 μg/l).


*Keyword:* Naphthol

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAH) represent a class of compounds to which humans are ubiquitously exposed from the environment, from cigarette smoke and especially at certain industrial workplaces [1–6]. Naphthalene is the most volatile PAH and in addition to its emission by all sorts of incomplete combustion processes it is also widespread as a basic substance in the chemical industry and in consumer products such as moth repellents or toilet bowl deodorants [7].

During a long-term inhalation study in the context of the US National Toxicology Program 2000 (NTP) [8], rats exhibited a significantly higher incidence of tumors in epithelial tissues of the respiratory tract even at the lowest level of exposure (50 mg naphthalene/$m^3$). Suitable human studies assessing the carcinogenic potential of naphthalene are currently not available but the results of the NTP 2000 lead to the conclusion that naphthalene is able to contribute considerably to human cancer risk. Thus, recently naphthalene was reclassified as possible human carcinogen by several national and international committees like IARC, DFG, EPA [9–15]. This new appraisal has consequences especially for those branches of industry which are concerned with naphthalene as raw material or inevitable contaminant in the production process.

In order to assess the individual naphthalene exposure ambient air monitoring is not sufficient. This is due to the fact, that naphthalene can not only be absorbed inhalatively but also percutaneously by skin contact. It is, therefore, necessary to draw attention to the individual internal burden. For biomonitoring purposes naphthols (hydroxynaphthalenes) excreted in urine seem to be suitable metabolites

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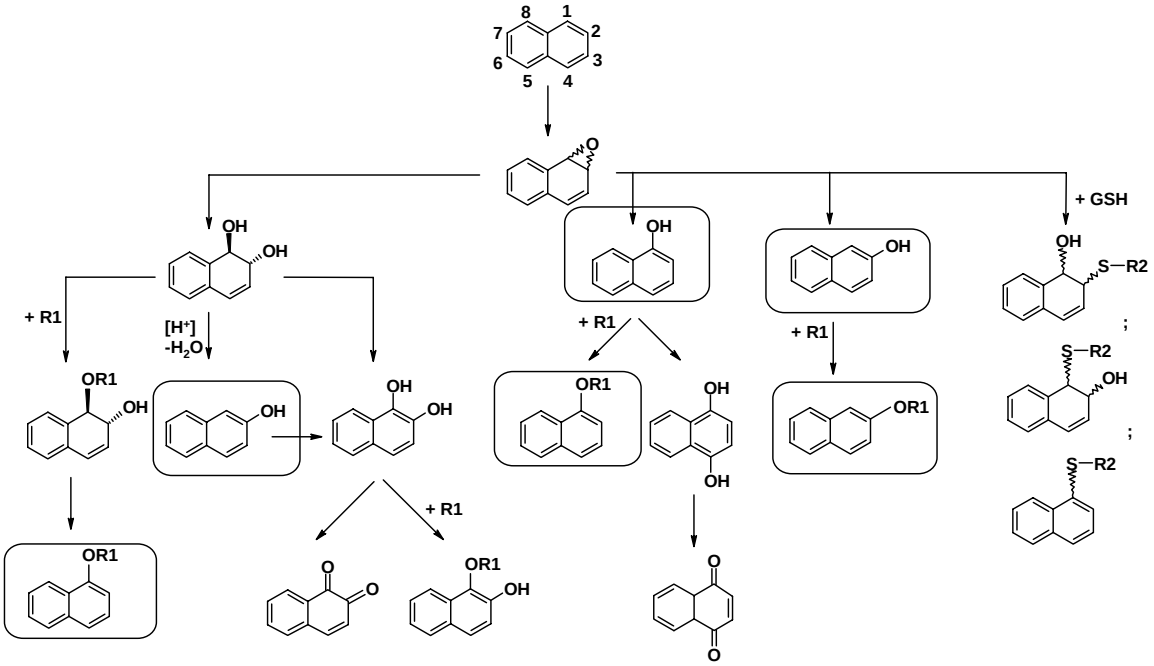


Fig. 1. Main metabolic pathways of naphthalene according to Preuss et al. [7] including the formation of 1- and 2-naphthol. R1: glucuronic acid/sulfate residue leading to conjugates 1-naphthylglucuronide/-sulfate, 2-naphthylglucuronide/-sulfate; GSH: glutathione-SH; R2: *N*-acetyl-L-cystein residue.

[7]. Their metabolic formation is schematically shown in Fig. 1. Over the past 10 years several analytical methods for the determination of either 1-naphthol or 2-naphthol or even both have been developed. The techniques were mainly based on high-pressure liquid chromatography [16–21] as well as on gas chromatography [22–32]. Sample preparation procedures comprised enzymatic or acidic hydrolysis. Most of these methods (especially the GC based ones) required time consuming off-line clean-up procedures by liquid–liquid- or solid-phase extraction and derivatization steps. We, therefore, decided to develop a method for the simultaneous determination of 1- and 2-naphthol in a single analytical run with an integrated on-line sample preparation. By introducing an automated multidimensional on-line clean-up HPLC procedure, we hereby reduce time consumption as well as manpower. With our method and subsequent results we can provide insights into the actual naphthalene exposure of the general population as well as of occupationally naphthalene exposed subjects, e.g. in coking plants, in the production of refractories, graphite electrodes or aluminum as well as bitumen processing, paving, coal tar distillation etc.. The method represents a state-of-the-art analytical tool for risk assessment of naphthalene exposed subjects.

## 2. Experimental

### 2.1. Chemicals

Acetonitrile, methanol (both LiChrosolv, HPLC-grade), acetic acid (100%, p.a.), sodium hydroxide (p.a.), hydrochloric acid (37%, p.a.), 1-naphthol (p.a.) and 2-naphthol (p.a.) were purchased from Merck (Darmstadt, Germany). Water was purified by passing it through a Milli-Q treatment system (Millipore, Bedford, USA). β-Glucuronidase/Arylsulfatase from *helix pomatia* was purchased from Roche Biomedical (Mannheim, Germany). Specific activity of β-glucuronidase and arylsulfatase is ≈4.5 and 14 U/ml, respectively.

### 2.2. Apparatus

Liquid chromatography was carried out on a L-7000 Series HPLC apparatus from Merck-Hitachi (Darmstadt, Germany) consisting of one L-7250 auto sampler (equipped with a 5000 μl loop and a 5000 μl syringe), one L-7480 fluorescence detector (equipped with a xenon short arc lamp), one D-7000 interface and two L-7100 gradient pumps designated P2 and P3, respectively. Additionally we used a Merck L-7360 column oven as well as one Merck-Hitachi L-7110 isocratic pump designated P1. For the automated column switching technique we applied two six-port valves (Rheodyne LabPro from Rheodyne, Berkeley, CA, USA) designated V1 and V2. The entire arrangement was controlled by a D-7000 chromatography data station system (version 4.0) from Merck except the isocratic pump and the column oven, which were both operated independently.

### 2.3. HPLC columns

We used three different columns designated C1, C2 and C3 in this paper.

Table 1
Analytical procedure for the column switching arrangement shown in Fig. 2

| Time (min) | Valve position | | Event on column | | |
|---|---|---|---|---|---|
| | V1 | V2 | C1 | C2 | C3 |
| −10···0 | A | A | Initial pre-start period including eluent purging, column equilibration, detector and oven warm-up | | |
| Start = 0 | A | A | Sample injection RAM loading | Equilibration | Equilibration |
| 0.0···6.0 | A | A | On-line extraction | Equilibration | Equilibration |
| 6.1···7.4 | B | A | Backflush analyte transfer from C1 to C2 | | Equilibration |
| 7.5···8.9 | A | A | Forwardflush into waste | Analyte clean-up | Equilibration |
| 9.0···11.8 | A | B | Forwardflush into waste | Analyte transfer from C2 to C3 | |
| 11.9···11.9 | A | A | Forwardflush into waste | | |
| 12.0···27.9 | B | A | Backflush rinsing | Rinsing | Analysis |
| 28.0···35.0 | A | A | Equilibration | Equilibration | Rinsing |
| 35.1···40.0 | A | A | Equilibration in preparation for the next injection | | |

- *Column C1*: Alkyl diol silica based restricted access material (RAM)—LiChrospher® ADS RP-8, 25 mm × 4 mm i.d., 25 μm particle size from Merck (Darmstadt, Germany).
- *Column C2*: Silica-based CN-(cyano)phase column—Luna-CN, 150 mm × 4.6 mm i.d., 3 μm particle size from Phenomenex (Aschaffenburg, Germany).
- *Column C3:* RP12 bonded silica with trimethylsilyl endcapping—Synergi Max-RP, 150 mm × 4.6 mm i.d., 4 μm particle size from Phenomenex.

Appropriate guard columns were used for C2 and C3: SecurityGuard® cartridge kits from Phenomenex for Luna-CN and Synergi Max-RP, 4 mm × 3 mm i.d., each.

### 2.4. HPLC solvents

We used three different solvents (S1, S2, S3). All solvents were degassed prior to use by flushing through a stream of helium for 10 min. For details concerning the program of the gradient pumps see Tables 1 and 2.

- *Solvent S1*: A mixture of acetonitrile and water (3:97, v/v), which was slightly acidified by the addition of 100 μl acetic acid (100%) per liter. The isocratic pump P1 was solely operated with this mobile phase.
- *Solvent S2:* A mixture of acetonitrile and water (35:65, v/v). Both gradient pumps P2 and P3 were predominantly operated with this mobile phase for analyte transfer and chromatographic separation purposes.
- *Solvent S3:* A mixture of acetonitrile and water (80:20, v/v). Both gradient pumps were temporarily operated with this solvent running steep gradients on all three columns for rinsing purposes only.
- The auto sampler syringe washing solvent consisted of a mixture of methanol and water (50:50, v/v).

### 2.5. Urine sample collection and preparation

Urine samples were collected in 250 ml polyethylene bottles and frozen immediately (−18 to −25 °C) until analysis. In preparation for the analysis frozen urine samples were thawed, equilibrated to room temperature and then mixed to homogenize them thoroughly. Aliquots of 2 ml were transferred to 7 ml brown-glass screw-cap vials as quickly as possible to avoid sedimentation. Four milliliter of sodium acetate buffer solution (0.1 mol/l, pH 5.0) and 25 μl β-glucuronidase/arylsulfatase were added. The samples were mixed and thereafter incubated for 16 hours (overnight) at 37 °C in a water bath in the dark. After hydrolysis each sample was centrifuged at 1500 × *g* for 10 min. A total of 1.4 ml of the supernatant was transferred into 1.5 ml brown-glass screw-cap vials. Three hundred fifty microliters were then injected into the HPLC system for quantitative analysis. Urinary creatinine concentrations were determined according to Larsen [33].

Table 2
Analysis program of the gradient pumps P2 and P3

| Time (min) | Gradient pump P2 (%) | | Gradient pump P3 (%) | |
|---|---|---|---|---|
| | Solvent S2 | Solvent S3 | Solvent S2 | Solvent S3 |
| Start = 0.0 | 100 {0.5} | 0 | 100 {0.5} | 0 |
| 6.0···12.0 | 100 {1.0} | 0 | 100 {1.2} | 0 |
| 13.0···28.0 | 0 | 100 {1.0} | 100 {1.2} | 0 |
| 29.0···34.0 | 100 {1.0} | 0 | 0 | 100 {1.2} |
| 35.0···40.0 | 100 {0.5} | 0 | 100 {1.0} | 0 |

Flow rates are shown in cambered brackets. Flow rate variations are only due to economic and ecological purposes. S2: 35% aqueous solution of acetonitrile; S3: 80% aqueous solution of acetonitrile.

### 2.6. Standard preparation and calibration procedure

The stock solution for the native standards was prepared by dissolving 20 mg 1-naphthol and 20 mg 2-naphthol in 100 ml acetonitrile (200 mg/l). This stock solution was stored in the dark at −25 °C in a glass flask until further use. A total of 500 μl of this stock solution was placed in a 100 ml glass volumetric flask and diluted to the mark with water (1 mg/l). This solution served as the working solution for the preparation of six calibration standards with concentrations in the range from 3.0 to 360 μg/l by diluting with water. Unspiked water was used as a blank. These standard solutions including the blank were processed as described in Section 2.5. Linear calibration curves were obtained by plotting the quotients of the integrated peak areas as a function of the standard concentrations. These graphs were used to ascertain the unknown naphthol concentration in urine samples.

### 2.7. Quality assurance

To ensure the accuracy of the developed method we took part in the FIOH Quality Assurance Program for Organic Solvent Metabolites 1/2003 (Finish Institute of Occupational Health, Helsinki). Three different urine samples provided by the FIOH were analyzed as described in this paper. In addition to this external quality control measure we spiked pooled and filtered urine from laboratory staff (creatintine content: 0.85 g/l) with +15 μg ($Q_{low}$) and +120 μg ($Q_{high}$) naphthols per liter for the low and the high concentration range, respectively. This control material was divided into aliquots and stored at −25 °C in the dark. For quality assurance one low- and one high-concentration control sample was included in each of the following analytical series: Within-series imprecision was determined by preparing and analyzing these control materials eight times in a row. Between-day imprecision was determined by preparing and analyzing the control materials on eight different days during routine operation of the method. Furthermore, inaccuracy which might be due to the influence of the urinary matrix was determined using nine *different individual* urine samples with a creatinine content ranging from 0.27 to 2.34 g/l. The naphthol content of the non spiked specimens varied from subject to subject. These nine samples with a biological matrix as different as possible were spiked with two different concentrations ($U_{low}$ = +15 μg/l and $U_{high}$ = +120 μg/l), each. Spiked specimens and the same specimens without the addition of the naphthols were analyzed and the recovery of the spiked amount of 1- and 2-naphthol was determined as a measure of accuracy and independency of matrix influences.

### 2.8. Analytical procedure

Fig. 2 shows a schematic diagram of the whole assembly. The timetables of the automated switching procedure as well as the analysis program of the gradient pumps are given in Tables 1 and 2. All steps were controlled by a chromatography data station system except the isocratic pump. Appropriate guard columns were placed in front of the analytical columns C2 and C3 to extend their lifetimes. During routine measurements these guard columns were replaced after approximately 100 injections. In order to protect the RAM pre-column C1 a replaceable filter element was also integrated into the system, which was changed after around 100 injections.

The primary isocratic pump (P1) was used to load the sample (350 μl aliquot) on the RAM precolumn (C1) using solvent S1 (acidified 3% acetonitrile) under a constant flow rate of 1.0 ml/min for 6 min at room temperature. After this

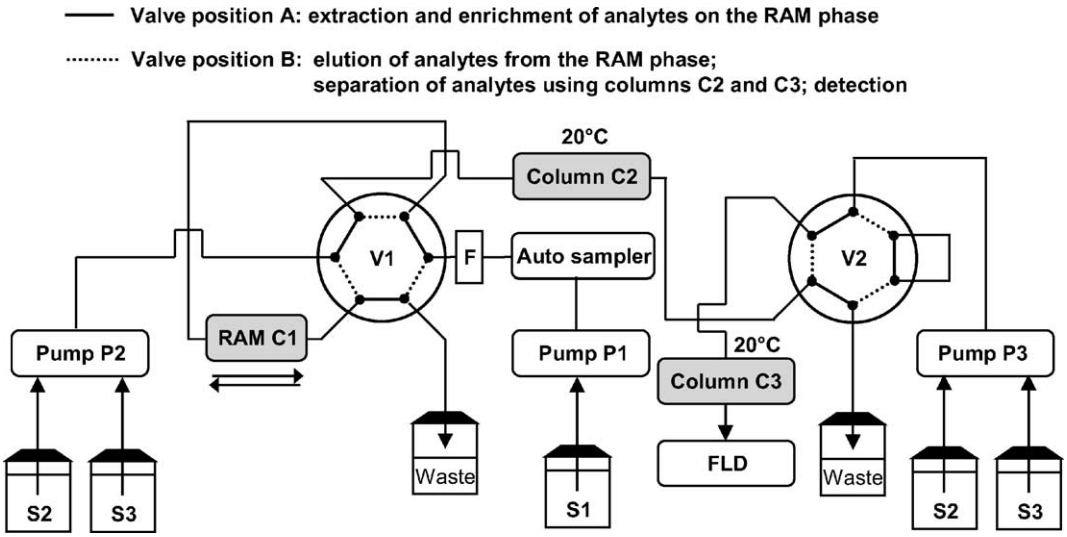


Fig. 2. Automated column switching on-line clean-up system for the determination of 1- and 2-naphthol in human urine. S1: 3% acidified aqueous acetonitrile; S2: 35% aqueous acetonitrile; S3: 80% aqueous acetonitrile. RAM C1: Restricted access material precolumn LiChrospher ADS RP-8 (25 mm × 4 mm, 25 μm); C2: Luna-CN (150 mm × 4.6 mm, 3 μm); C3: Synergi Max-RP (150 mm × 4.6 mm, 4 μm). V1, V2: software controlled 6-port-valves. FLD: fluorescence detector set to 227/430 nm; F: replaceable filter element.

on-line sample extraction and enrichment step the analytes were transferred in backflush mode onto the cyano phase column (C2) using mobile phase S2 (35% aqueous acetonitrile) with a constant flow rate of 1.0 ml/min. C2 was set to 20 °C in the column oven. This first column switching step was accomplished by the software controlled six-port valve V1. The transfer step as well as the preliminary separation on this cyano phase column was carried out using isocratic elution. Short before the analytes elute from C2 another column switch was accomplished by the second software controlled six-port valve V2 and the analytes were hereby transferred from C2 to C3 ("heart-cut" technique). The final chromatographic separation was carried out on column C3 which was also located in the oven at 20 °C using isocratic elution with mobile phase S2 and a flow rate of 1.2 ml/min. All columns were rinsed as soon as the column switches were completed using steep gradients increasing the organic solvent to 80% aqueous acetonitrile (S3) within one minute. Eighty percent were maintained for 15 and 5 min on C2 and C3, respectively. Thereafter both analytical columns were re-equilibrated with S2. Column C1 was rinsed in backflush mode simultaneously with C2 and re-equilibrated with S1 in preparation for the next sample injection.

In order to minimize solvent consumption, expenses and toxic waste the flow rates on C2 and C3 were temporarily reduced by the software program during the re-equilibration periods to a minimum of 0.5 ml/min.

The fluorescence detector was set to an excitation wavelength of 227 nm and an emission wavelength of 430 nm. The photomultiplier voltage was set to medium.

### 2.9. Study subjects

In a pilot study, we investigated 53 spot urine samples from volunteer office employees who were occupationally not exposed to PAHs. Their urine samples were collected at the end of an 8–10 h working day. Twenty-one of them were smokers and 32 non smokers. The entire collective was aged between 16 and 58 years and urinary creatinine levels were in the range from 0.16 to 2.79 g/l. Furthermore, we investigated 18 workers occupationally exposed to PAHs at their workplaces in a coal tar distillation plant (data not shown in detail). Spot urine samples of these workers were collected at the end of an eight hours working shift.

## 3. Results and discussion

### 3.1. General considerations

In order to allow a biological monitoring of occupational and environmental exposure to naphthalene—a possible human carcinogenic PAH—the aim of our work was to develop an analytical method to quantify 1- and 2-naphthol simultaneously in human urine samples. Today's routine analysis requirements made us reduce the sample pre-treatment to a minimum. Furthermore, previously reported renal elimination figures for the general population lead to the demand of detection limits below 10 μg/l [7,34]. The detection of naphthols by mass spectrometry is hampered due to the lack of specific fragmentation patterns, as experiments on a LC-MS/MS-System showed. On the other hand the insufficient fluorescence of the naphthols—as already reported by [35]—forces one to apply sophisticated sample purification procedures to ensure specificity in this low concentration range. In the past, we already applied a tailor made pre-column (copper phthalocyanine) for the determination of three and more ring PAH metabolites [36–38]. Unfortunately, naphthols does not interact with this special affinity material. In order to overcome this situation, we applied novel HPLC on-line extraction and column switching techniques combined with fluorescence detection. The applied RAM phase enabled us to extract the analytes on-line out of the matrix in order to transfer them directly onto successive analytical columns to further separate the analytes from that part of the urinary matrix that is retained on the RAM phase. Minimizing off-line sample preparation and manpower we simultaneously increase the precision of the method so that internal standardization is dispensable.

### 3.2. Enzymatic hydrolysis

Naphthalene metabolites are mainly excreted in urine as glucuronide and sulfate conjugates. Deconjugation of PAH metabolites of the glucuronic and the sulfuric acid residue is commonly carried out by enzymatic hydrolysis with β-glucuronidase/arylsulfatase. This procedure proved to be effective and practicable. Thus we adopted the sample preparation and hydrolysis conditions according to the procedure described elsewhere [39] and modified them slightly.

### 3.3. Liquid chromatography

We applied a 3-D state-of-the-art liquid chromatography column switching method. This technique enabled us to discard interfering urinary matrix which is initially eluted from the primary RAM column. The column selected for this purpose was a LiChrospher® ADS RP-8 (Alkyl-Diol-Silica), a restricted access material which is a special reversed phase sorbent for LC-integrated on-line sample extraction [40]. Two superposing chromatographic processes are responsible for this effect: on the one hand macromolecules with a molecular weight larger than 15,000 Dal are not able to penetrate the pores and elute with the void volume of the column (size exclusion chromatography). On the other hand analytes migrating into the pores are retained by reversed phase interactions with the bonded C8-phase on the internal surface of the pores.

The isocratic solvent was optimized to a slightly acidified 3% aqueous solution of acetonitrile with 1.0 ml/min flow rate ensuring maximum clean-up on the RAM phase and no analyte losses. Acidification of the solvent was used to suppress the ionization of the naphthols into naphtholat ions which

are not expected to be entirely retained by the RAM. In order to precipitate suspended particles, the hydrolyzed urine samples were centrifuged prior to HPLC injection. This procedure considerably expands the lifetime of the RAM phase, which can not be protected by a guard column due to the backflush arrangement. The precipitation proved not to influence the precision and accuracy of the method as shown by high recovery rates with spiked individual specimens (creatinine range 0.27–2.34 g/l) and with spiked quality control urines. Backflush means a change in flow direction for the first transfer step which caused the analytes to refocus on the secondary column (C2) thus increasing sensitivity. By transferring the analytes from the secondary column (C2) onto the final column (C3) with a minimum of overlapping interferences the whole procedure includes on-line sample extraction and fractionation as well as subsequent chromatographic separation of the analytes all in one.

The secondary column C2 was optimized to lead to very sharp analyte peaks to obtain a time slot for the heart-cut transfer as tight as possible. The retention time was optimized as constant and as short as possible. All this boiled down to a silica based cyano phase column operated in reversed phase mode using eluent S2 (35% aqueous acetonitrile) with 1.0 ml/min at 20 °C.

The desire for high peak resolution and sufficient separation from residual matrix components mounted in a silica based C12 bonded reversed phase column with trimethylsilyl endcapping as tertiary column using the same mobile phase S2 with 1.2 ml/min. This column was also set to 20 °C in the same column oven in order to stabilize peak retention times and to improve separation. It had to be taken into account that the back pressure of all three columns must not exceed the recommended pressure limit (approximately 300 bar) during the transfer steps. This restricted the free choice of different tertiary columns with respect to particle size and column dimensions. The chosen column served as good compromise with respect to peak resolution, total analysis time and back pressure.

Washing of the RAM phase and the analytical columns was performed by the gradient pumps providing a steep gradient up to 80% of acetonitrile. The application of two gradient pumps reduced the required time for these rinsing steps significantly, since the RAM phase as well as C2 can be rinsed directly after the transferring steps, while the ongoing analysis takes place on the tertiary column C3.

This 3-D column switching technique was carried out by software controlled six-port switching valves to automate the procedure and to ensure high reproducibility. The application of three different phases causes a highly selective sample clean-up, separation and detection (size exclusion → reversed phase RP-8 → CN-phase → reversed phase RP-12).

### 3.4. Fluorescence detection

Generally speaking the natural fluorescence of 1-naphthol as well as 2-naphthol is poor in comparison with higher molecular PAHs [35]. Previously published HPLC-FLD methods used several different wavelengths most of which we found to be inadequate for a simultaneous determination of 1- and 2-naphthol in the concentration range we were aiming at. So our experiments led to a wavelength combination which enabled us to detect both metabolites with sufficient fluorescence yield and without changing the wavelengths during the run. We checked the selected wavelength with two different fluorescence detectors to exclude technical induced imprecision in the wavelength alignment. Yet with the detector set to an excitation and emission wavelength of 227 and 430 nm, respectively, 2-naphthol showed approximately twice the higher response than 1-naphthol did. One have to keep in mind that the transmissibility of the HPLC eluents (water and acetonitrile) is already decreased to approximately 80% at an excitation wavelength of 227 nm. The chromatographic baseline noise level is comparatively high due to this fact. This, however does only affect the detection in very low concentration ranges below 10 μg/l. Furthermore the baseline reacts sensitive to changes in the eluent composition. For this reason we decided to apply an isocratic HPLC mode to avoid such baseline driftings.

### 3.5. Calibration graphs

For peak identification purposes urine samples were spiked separately with 1-naphthol and thereafter with 2-naphthol. The same procedure was initially applied to the standard solutions. Subsequently the retention times served as identification parameters of both analytes. Software assisted peak area integration in conjunction with the calibration procedure was used for the quantitative assessment which was not influenced by urinary matrix in terms of slope and linearity. We ensured this by comparing aqueous and urinary standard solutions in a double determination on two different days. By means of linear regression in the concentration range between 0 and 360 μg/l we obtained in pooled urine: $y_{1\text{-naphthol}} = 106x + 225$ ($r = 0.99998$) and $y_{2\text{-naphthol}} = 149x + 671$ ($r = 0.99996$). Water spiked with the same standard concentrations gave nearly the same slopes: $y_{1\text{-naphthol}} = 92x - 176$ ($r = 0.99989$) and $y_{2\text{-naphthol}} = 150x$–15 ($r = 0.99996$). Reagent blanks were found to contain no traces of naphthols. Autonomy from the urinary matrix was also demonstrated by determining the naphthols out of nine spiked individual urines with a wide range of creatinine content (0.27–2.34 g/l) as described in Section 2.7. Therefore, calibration graphs were obtained by analyzing aqueous standard solutions prepared as described in Section 2.5. Real urine samples with concentrations above the highest calibration standard have to be diluted to fit the calibration range.

### 3.6. Reliability

To ensure the accuracy of the method we analyzed urine samples of three different concentrations in the framework of

Table 3
Results of the determination of 1- and 2-naphthol in human urine in the framework of the Quality Assurance Program for Organic Solvent Metabolites 1/2003 supported by the Finish Institute of Occupational Health (FIOH, Helsinki)

| | 1-Naphthol | | | 2-Naphthol | | |
|---|---|---|---|---|---|---|
| | Sample A | Sample B | Sample C | Sample A | Sample B | Sample C |
| Target value (μg/l) | 44 | 579 | 611 | 16 | 682 | 698 |
| Recovery (%) | 120 | 100 | 104 | 95 | 89 | 95 |

the Quality Assurance Program for Organic Solvent Metabolites 1/2003 supported by the Finish Institute of Occupational Health (FIOH, Helsinki). The samples were analyzed on three different days in double determinations. Briefly, all relative recovery rates were in the range between 89 and 120% (Table 3).

In addition to this external quality assurance measure, accuracy was also checked by recovery experiments with self-made pooled quality control urine (0.85 g creatinine/l). $Q_{low}$ was spiked with 15 μg/l, representing about 10-times the limit-of-detection (LOD). $Q_{high}$ was spiked with 120 μg/l, representing about 100 times the LOD. We applied those investigations as strong acceptance criteria (Table 4).

Besides this extensive testing during method development quality control during routine operation was accomplished by preparing and analyzing aliquots of a pooled urine of known naphthol content within every analytical series. Furthermore, new standard dilutions were prepared and analyzed for calibration purposes prior to each series.

- *Within-series imprecision and accuracy*: In order to assess the within-series imprecision $Q_{low}$ and $Q_{high}$ were prepared and analyzed eight times in a row. Relative standard deviations (R.S.D.) were in the range from 2.0 to 7.2% for both concentrations and both parameters demonstrating very good reproducibility over the whole concentration range. Mean relative recovery rates obtained with this method were in the range from 90.5 to 106.5% representing excellent accuracy, again (Table 4).
- *Between-day imprecision and accuracy*: The between-day imprecision was determined on eight different days during routine operation of the method. Relative standard deviations for both parameters at the low and the high concentration level were between 4.2 and 12.1%. Mean relative recovery rates obtained during this test ranged from 93.3

Table 4
Imprecision and relative recovery of the determination of 1- and 2-naphthol in human urine

| | Imprecision | | Relative recovery | |
|---|---|---|---|---|
| | $Q_{low}$ (15 μg/l), R.S.D. (%), $n = 8$ | $Q_{high}$ (120 μg/l), R.S.D. (%), $n = 8$ | $Q_{low}$ (15 μg/l) range (mean) (%) $n = 8$ | $Q_{high}$ (120 μg/l) range (mean) (%) $n = 8$ |
| Within-series | | | | |
| 1-Naphthol | 7.2 | 2.0 | 79.6–98.3 (90.5) | 96.3–101.6 (99.4) |
| 2-Naphthol | 5.4 | 2.4 | 94.2–111.9 (103.4) | 103.0–110.6 (106.5) |
| | $Q_{low}$ (15 μg/l), R.S.D. (%), $n = 8$ | $Q_{high}$ (120 μg/l), R.S.D. (%), $n = 8$ | $Q_{low}$ (15 μg/l) range (mean) (%) $n = 8$ | $Q_{high}$ (120 μg/l) range (mean) (%) $n = 8$ |
| Between-day | | | | |
| 1-Naphthol | 8.9 | 11.0 | 80.8–108.4 (96.1) | 71.2–100.6 (93.3) |
| 2-Naphthol | 4.2 | 12.1 | 102.6–115.2 (107.2) | 81.3–121.5 (102.9) |
| | $U_{low}$ (15 μg/l), R.S.D. (%), $n = 9$ | $U_{high}$ (120 μg/l), R.S.D. (%), $n = 9$ | $Q_{low}$ (15 μg/l) range (mean) (%) $n = 9$ | $Q_{high}$ (120 μg/l) range (mean) (%) $n = 9$ |
| Interindividual | | | | |
| 1-Naphthol | 8.9 | 9.7 | 82.8–108.2 (96.0) | 90.6–121.1 (102.0) |
| 2-Naphthol | 7.6 | 5.8 | 95.3–122.5 (106.6) | 95.0–118.3 (107.9) |

Table 5
Results of biological monitoring of 1- and 2-naphthol in urine samples from occupational non exposed office employees

| | Overall ($n = 53$) | | Non smoker ($n = 32$) | | | Smokers ($n = 21$) | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | Median | Mean | Median | Range ($n <$ LOD) | Mean | Median | Range (% < LOD) |
| 1-Naphthol (μg/l) | 11.0 | 4.0 | 5.6 | 2.0 | <LOD–69.3 (34%) | 19.2 | 19.0 | <LOD–48.5 (5%) |
| 2-Naphthol (μg/l) | 12.9 | 7.0 | 5.6 | 4.0 | <LOD–30.3 (9%) | 23.7 | 19.1 | 2.7–73.3 (0%) |
| 1 + 2 Naphthol [μg/l] | 23.6 | 10.4 | 11.0 | 6.1 | 1.0–78.6 | 42.9 | 38.2 | 4.3–100.8 |

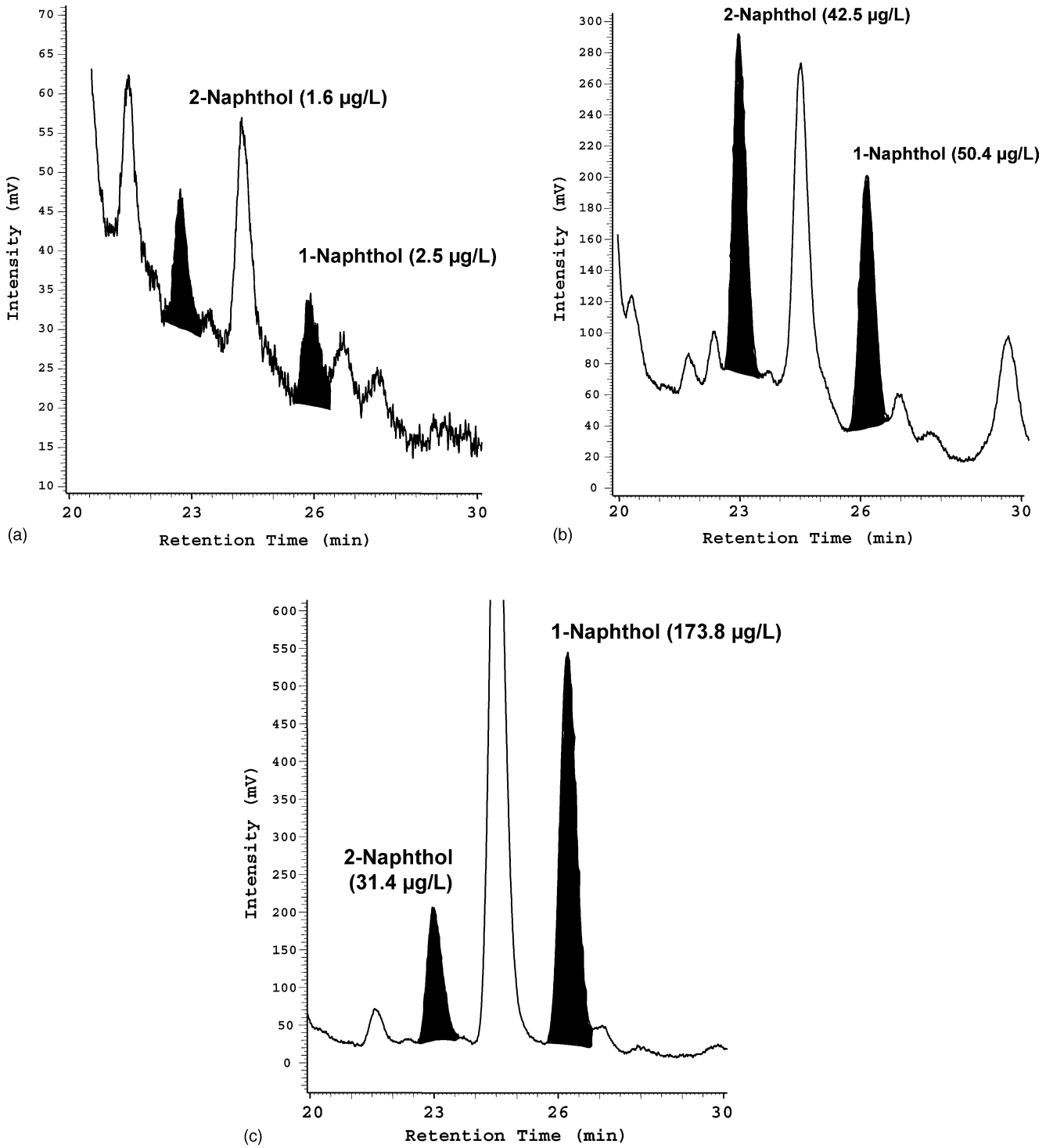


Fig. 3. Chromatograms of three processed individual urine samples obtained with the presented method: (a) non smoking subject; (b) heavily smoking subject; (c) non smoking worker from a coal tar distillation plant. Creatinine concentrations were (a) 1.30 g/l; (b) 1.83 g/l; (c) 1.09 g/l.

to 107.2% (Table 4). These data show very good reproducibility and accuracy.

- *Interindividual matrix dependent reliability*: Furthermore, nine individual urine specimens reflecting a composition as different as possible (creatinine content from 0.27 to 2.34 g/l) were spiked with the naphthols ($U_{low}$: +15 µg/l and $U_{high}$: +120 µg/l). By determining the unspiked and the two spiked samples of each individual urine and subsequent calculation of the recovery rates we were able to exclude a possible influence of the complex biological matrix on the analytical result. Mean relative recoveries were obtained for $U_{low}$ and $U_{high}$ in the range between

96.0 and 102.0% for 1-naphthol and between 106.6 and 107.9% for 2-naphthol. These results are well within the limits of recovery experiments indicating that there are no matrix interferences.

In summary, these results show that the application of an internal standard is not necessary due to the automated on-line procedure.

*3.6.1. Limit-of-detection (LOD)*

The LOD, defined as a signal-to-noise ratio of three were estimated to be 1.5 μg/l (1-naphthol) and 0.5 μg/l (2-naphthol) for a 350 μl sample injection.

*3.6.2. Sources of error*

The developed LC/LC/LC-FLD method has proven to be robust and reliable as described above. The lifetime of the primary RAM phase has exceeded 250 injections during method development and no deterioration of quality control data has been observed. The retention times of the analyte peaks were highly stable under the conditions described above. It is highly recommended to use a column oven for the secondary and the tertiary column (C2, C3) to make the retention times less sensitive to temperature changes. The accuracy and reproducibility of all analyte transfer steps were ensured in the temperature range between 20 and 30 °C. Varying retention times within these limits will not lead to analyte losses. The best separation of both analyte peaks were obtained at 20 °C. As a rule, peak resolution gets worse with increasing temperature on C3.

*3.7. Results of biological monitoring*

Chromatograms of two extremely different individual urine samples of one non smoker and one heavily smoking subject obtained with the presented method are shown in Fig. 3a and b. Table 5 shows the results of biological monitoring of 53 spot urine samples of office employees occupationally not exposed to PAHs. Smokers exhibited a significantly increased naphthol excretion in comparison to non smokers. The naphthol excretion of occupationally non exposed subjects is hereby demonstrated to be in good accordance with previously reported mean levels for the general population ranging from 1.3 to 130.0 μg/l (1-naphthol) and from 1.1 to 17.2 μg/l (2-naphthol) [7]. It seems reasonable to apply the sum of both urinary naphthols (1 + 2) as a measure of naphthalene exposure. In doing so, smokers (mean: 42.9 μg/l) exhibited a naphthol excretion which is 4-times higher than that of nonsmokers (11.0 μg/l).

Besides this, the method described in this paper has already been applied to around 250 individual urine samples among workers employed in coking plants, graphite electrode and fire proof material manufacture, coal tar distillation and bitumen processing [1,41].

Fig. 3c shows a urine sample of a non smoking worker from a coal tar distillation plant who was exposed to naphthalene at his workplace (personal ambient naphthalene concentration: 258.4 μg/m$^3$).

## 4. Conclusions

We hereby present an analytical method that offers a sensitive, selective and simultaneous quantification of 1- and 2-naphthol at concentration levels down to 0.5 μg/l of enzymatic hydrolyzed human urine. The proposed column switching technique is automated by software control and it does not exceed 40 min of total run time. The sample preparation is reduced to a minimum by excluding any off-line extraction and enrichment steps. A great number of samples can easily be prepared for one analytical series. The proposed method is, therefore, attractive for routine analysis and sensitive enough to determine environmental as well as occupational naphthalene exposure.

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