Sensitive monitoring of monoterpenene metabolites in human urine using two-step derivatisation and positive chemical ionisation-tandem mass spectrometry

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HIGHLIGHTS

• Sensitive monitoring of 10 metabolites of (R)-limonene, α-pinene, and Δ3-carene in human urine samples.
• Fast and simple sample preparation and derivatisation procedure using two-step silylation for unreactive tertiary hydroxyl groups.
• Synthesis of reference substances and isotopically labelled internal standards of (R)-limonene, α-pinene, and Δ3-carene metabolites.
• Study on (R)-limonene, α-pinene, and Δ3-carene metabolite background exposure of 36 occupationally unexposed volunteers.

ABSTRACT

A gas chromatographic–positive chemical ionisation-tandem mass spectrometric (GC–PCI-MS/MS) method for the simultaneous determination of 10 oxidative metabolites of the monoterpenoid hydrocarbons α-pinene, (R)-limonene, and Δ3-carene ((+)-3-carene) in human urine was developed and tested for the monoterpenene biomonitoring of the general population (n = 36). The method involves enzymatic cleavage of the glucuronides followed by solid-supported liquid–liquid extraction and derivatisation using a two-step reaction with N,O-bis(trimethylsilyl)-trifluoroacetamide and N-(trimethylsilyl)imidazole. The method proved to be both sensitive and reliable with detection limits ranging from 0.1 to 0.3 µg L \(^{-1}\). In contrast to the frequent and distinct quantities of (1S,2S,4R)-limonene-1,2-diol, the (1R,2R,4R)-stereoisomer could not be detected. The expected metabolite of (+)-3-carene, 3-carene-10-ol was not detected in any of the samples. All other metabolites were detected in almost all urine samples.

The procedure enables for the first time the analysis of trace levels of a broad spectrum of mono- and bicyclic monoterpenoid metabolites (alcohols, diols, and carboxylic acids) in human urine. This analytical procedure is a powerful tool for population studies as well as for the discovery of human metabolism and toxicokinetics of monoterpenes.

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1. Introduction

Monoterpenes are prevalent products of the biosphere [1–3]. Moreover, they are intensively used in household and consumer products [4]. Their ordinary purpose is to act as attracting fragrances, repellents, or as parts of disinfectant exudates in both
floral and faunal species. Because of their ubiquitous appearance and widespread use, the general population is exposed via food and indoor air as well as by dermal contact to monoterpene containing products, e.g., cosmetic and hygienic products [5–7]. The most abundant monoterpens in indoor air are α-pinene [αPN; (1S,5S)-(-)-isomer from European and (1R,5R)-(+) -α-pinene from North American pines], (R)-(+) -limonene (LMN), and Δ3-carene [CRN; (1S,6R)-(+) -3-carene] [6,8–10].

In humans, monoterpens are predominantly metabolised by cytochrome P450 monoxygenases (CYP), epoxide hydrolases, and dehydrogenases to mono- and dihydroxylated compounds (Fig. 1), as well as higher oxidised metabolites that are conjugated mainly to glucuronic acids [11]. In urine samples of individuals that are occupationally exposed to wood dust, cis-verbenol [rel-(1S,25,5S)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol, cVER] and trans-verbenol [rel-(1R,25,5R)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol, tVER] were detected as metabolites of αPN [9]. Besides verbenols, myrtenol [MYR; (1S,5R)-6,6-dimethylbicyclo[3.1.1]hept-3-en-2-ol, HO] and (+)-myrtenol has additionally been identified in human urine following ingestion of an αPN containing pharmaceutical product [12].

The main metabolites found in human urine after experimental exposure to LMN were limonene-8,9-diol (2-[(1R)-4-methyl-3-cyclohexen-1-yl]-1,2-propanediol, LMN-8,9-OH) and perilllic acid (PA; (4R)-(1-methylethenyl)-1-cyclohexene-1-carboxylic acid) [11,13]. In another study, in which LMN was applied in the form of orange-peel oil, limonene-1,2-diol (unknown stereochemistry) was identified as a further metabolite (see structures of (1S,25,4R)-1-methyl-4-(1-methylethenyl)cyclohexene-1,2-diol [(1S,25S)-LMN-1,2-OH] or (1R,2R,4R)-isomer [(1R,2R)-LMN-1,2-OH] in Fig. 1) [5]. Moreover, perillyl alcohol [POH; (R)-(4-(1-methylethenyl)-1-cyclohexen-1-methanol], and (+)-trans-carveol were determined in vitro after incubation of LMN with human liver microsomes (see Fig. 1 for the structures of (1S,5S)-2-methyl-5-(1-methylethenyl) -2-cyclohexen-1-ol [cCAR] and (1R,5S)-isomer [tCAR]) [14].

The human metabolism of CRN has been poorly explored. In vitro studies using human liver microsomes as well as recombinant human CYP 2B6 and CYP 2C19 isoenzymes identified two oxidative metabolites of CRN: Δ3-caren-10-ol [(1S,6R)-7,7-dimethylbicyclo[4.1.0]hept-3-ene-3-methanol, CRN-10-OH, Fig. 1] and Δ3-caren-epoxide [(1S,5S,5R,7R)-3,8,8-trimethyl-4-oxatricyclo[5.1.0.03,5]octane] [15].

In spite of the extensive use and consumption of monoterpens as well as exposure via indoor air, biomonitoring studies, which may reveal the exposure levels of the general population, are still missing. Only a few publications reported the assessment of

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**Fig. 1.** Proposed human in vivo metabolites of α-pinene: trans-verbenol (tVER), cis-verbenol (cVER), myrtenol (MYR); of (R)-limonene: cis-carveol (cCAR), trans-carveol (tCAR), perillyl alcohol (POH), perilllic acid (PA), limonene-1,2-diols (LMN-1,2-OH), limonene-8,9-diol (LMN-8,9-OH); as well as of Δ3-carene: 3-caren-10-ol (CRN-10-OH).
Fig. 2. Reference compounds and internal standards for identification and quantification of the human in vivo metabolites of α-pinene and (R)-(+)-limonene.

2. Experimental

2.1. Chemicals and consumables

The reference substances (S)-cis-verbenol [(1S,2S,5S)-cis-verbenol in Fig. 2; enantionic reference for cVER in Fig. 1], (1R)-(−)-myrtenol [(1R,5S)-myrtenol in Fig. 2; enantionic reference for MYR in Fig. 1], (−)-carveol [separable mixture of cis-(1R,5R)- and trans-(1S,5R)-isomers in Fig. 2; enantionic references for cCAR and tCAR in Fig. 1], (S)-(−)-perillyl alcohol [Fig. 2, enantionic reference for POH in Fig. 1], (S)-(−)-perillic acid [Fig. 2, enantionic reference for PA in Fig. 1], (1S,2S,4R),(-)-limonene-1,2-diol [(1S,2S)-LMN-1,2-OH in Fig. 1] each at highest purity available (≥96%), as well as (1R)-(−)-α-pinene, 13CD3], N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA, ≥98.5%), N-(trimethylsilyl) imidazole (TSIM, ≥98%), and trifluoroacetic acid (TFA, for HPLC, ≥99%) were purchased from Sigma–Aldrich (Steinheim, Germany). The internal standard (13CD2) substance (−)-D4-menthol [D4-MNT; 1,2,6,6-2H4-(−)-(1-methyl)-2-(1-methylethyl)-cyclohexanol; 98%] was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). D3-α-pinene [rel-(1R,5R)-2-(2H3-methyl)-6,6-dimethylbicyclo[3.1.1]hept-2-ene] was purchased from Aroma LAB AG (Freising, Germany). Acetonitrile (MeCN, >99%), dichloromethane (DCM, >99%), diethyl ether (DEE, dry, >99%), isooctane (>99%), glacial acetic acid (p.a.), aqueous hydrochloric acid (HCl, 25%, p.a.), sodium chloride (NaCl, p.a.), sodium hydroxide (NaOH, p.a.), and dry dried sodium sulphate (p.a.) were purchased from Merck KGaA (Darmstadt, Germany). β-Glucuronidase/arylsulphatase from Helix pomatia was supplied by Roche Diagnostics GmbH (Mannheim, Germany). Modified diatomaceous earth solid-supported liquid–liquid extraction (SSLLE) sorbent material (Isolute HM-N, bulk material) was obtained from Biotage AB (Uppsala, Sweden). Phosphomolybdic acid solution in ethanol (Sigma–Aldrich) was used as a developer of the TLC plates (TLC Silica gel 60 F254; VWR International, Darmstadt, Germany). All remaining chemicals were at highest purity available.

2.2. Synthesis of reference compounds and internal standards

(1R,2S,5R)-trans-verbenol (tVER in Fig. 1) and rel-(1R,2S,5R)-4-(2H3-Methyl)-6,6-dimethylbicyclo[3.1.1]hept-3-ene-2-ol (D3-tVER in Fig. 2) were prepared by oxidation of (1R)-(−)-α-pinene and D3-α-pinene with lead(IV) acetate in benzene and saponification of the corresponding acetates with KOH in methanol [23].

2.3. Separation and identification of cis- and trans-carveols

Separation of the diastereomers cCAR and tCAR from the commercial (−)-carveol mixture (Fig. 2) was carried out using a Eurosorb 100 C18 HPLC column (20 mm × 250 mm; Knauer,
Berlin, Germany) and UV detection at 210 nm [24]. The mobile phase was MeCN/H2O with 0.1% TFA at a flow rate of 25 mL min\(^{-1}\) and a gradient of 20/80 to 50/50 in 25 min. Two peaks with retention times (\(t_R\)) of 26.3 and 27.0 min were resolved (ratio of peak areas 2:3). Both peak eluates were collected separately, cooled and neutralised with saturated aqueous NaHCO3, then solid NaCl was added to the fractions, and they were diluted with water and extracted several times with pentane, until cCAR and tCAR diastereomers could not be detected on TLC plates. Pentane solutions were washed with brine, dried (Na2SO4) and evaporated. The residues (ca. 2–5 mg) were dissolved in CDCl3, and their 1H NMR spectra (600 MHz) were recorded (see Supporting Information). The minor component of the commercial (−)-carveol mixture (with lower \(t_R\) in HPLC and higher GC-\(t_R\)) turned out to be trans-(1S,5R)-diastereomer (Fig. 2), and the major component-cis-(1R,5R)-isomer (with higher \(t_R\) in HPLC and lower GC-\(t_R\)).

2.4. Synthesis of dihydroxylated \((R)\)-(−)-limonene derivatives

Limonene-8,9-diol (2-[2-(1R,4S)-methyl-3-cyclohexen-1-yl]-1,2-propanediol, urotropenol, \((4R)\)-p-menth-1-en-8,9-diol; LMN-8,9-OH in Fig. 1) and \((4R)\)-acetyl-l-methylcyclohexene (limonaketone) were prepared from \((R)\)-(−)-limonene essentially as described by Dean et al. [25]. Limonaketone was distilled in Kugelrohr (b.p., 80 °C at 10 Torr) prior to use in a Wittig reaction. The \([9\text{-}13\text{C}]_2\text{H}_4\)\((R)\)-(−)-limonone was synthesised from limonaketone (550 mg, 4.0 mmol) and \([13\text{C}]_2\text{H}_4\)-methyl triphenylphosphonium iodide (2.0 g, 4.9 mmol) as described by Horst and Ryhlick (with slight modifications) [26]. Ylide was generated in dry THF (50 mL) at 0 °C using 1.6 M nBuLi in hexane (3.1 mL, 5.0 mmol). After the reaction was complete, THF was distilled from the reaction mixture using a Vigreux column at 400 mbar (bath tem. 60 °C), the residue was resuspended with pentane and filtered. The filtrate was passed through a short pad of silica gel (to remove Ph3PO). The filter cake was washed with pentane; fractions containing \([9\text{-}13\text{C}]_2\text{H}_4\)-(\(R)\)-(−)-limonene (TLC with pentane as eluent) were combined and evaporated in vacuo (200 mbar, RT to 45 °C) to yield 390 mg of the residue (with ca. 10% of hexane according to 1H NMR; yield 65%). The \([9\text{-}13\text{C}]_2\text{H}_4\)-(\(R)\)-(−)-limonene was used for the preparation of \(13\text{C}_2\)-LMN-8,9-OH (Fig. 2) according to the method of Dean et al. [25]. The \([9\text{-}13\text{C}]_2\text{H}_4\)-(\(R)\)-(−)-limonene was also converted into the diastereomeric mixture of cis- and trans-1,2-epoxides [27], which was not separated, but rather used directly for the synthesis of the individual \(1\text{C}_2\text{D}_2\)-(1R,2R,4R)-(−)-limonene-1,2-diol [(1R,2R)- and (1S,2S)-] and \(1\text{C}_2\text{D}_2\)-(1R,2R,4R)-(−)-limonene-1,2-diol [(1R,2R)- and (1S,2S)-] in Fig. 2. Under neutral conditions and in the presence of HgCl2, \((1\text{R},2\text{R},4\text{R})\)-limonene-1,2-diol is selectively formed in aqueous solutions from a diastereomeric mixture of 1,2-epoxides (reduction with NaBH4 complets the reaction) [28,29]. This procedure was applied for the preparation of \(1\text{C}_2\text{D}_2\)-(1R,2R,4R)-(−)-limonene-1,2-diol (Fig. 2). Another diastereomer of \(1\text{C}_2\text{D}_2\)-(1R,2R,4R)-(−)-limonene was isolated and used for the preparation of \(13\text{C}_2\text{D}_2\)-(1S,2S,4S)-(−)-limonene-1,2-diol, which turned out to be identical (in respect of \(t_R\)) to commercially available \((1\text{S},2\text{S},4\text{S})\)-(−)-limonene-1,2-diol (Sigma–Aldrich, Steinheim, Germany). For this, an acid-catalysed ring-opening reaction was used (diluted H2SO4 in aq. THF) [30].

2.5. Synthesis of 3-carene-10-ol

CRN-10-OH (Fig. 1) was prepared from (−)-3-carene (Sigma) according to known methods [31,32]. For that, (−)-3-carene was epoxidized with 3-chloroperbenzoic acid and the product – trans-3,4-epoxy-3-carene – was subjected to the ring-opening reaction in the presence of diethylaluminum tetramethylpiperidide and gave (1R,3R,6S)-4-methylene-7,7-dimethylbicyclo-[4.1.0]heptan-3-ol, which, in turn, underwent an allylic rearrangement and oxidation to 3-carene-10-ol in 2 M aqueous chromic acid [31]. The reduction of 3-carene-10-ol with sodium borohydride in ethanol furnished 3-carene-10-ol [32].

2.6. Standard and reagent preparation

Stock solutions of 1 g L\(^{-1}\) were prepared by dissolving each reference substance in MeCN. Since LMN-8,9-OH is expected to occur in higher concentrations in the native samples, the calibration is divided into two concentration ranges: cVER, tVER, MYR, cCAR, tCAR, CRN-10-OH, POH, PA, and (1S,2S)-LMN-1,2-OH are calibrated from 0.2–100 μg L\(^{-1}\) and LMN-8,9-OH is calibrated from 10 to 1000 μg L\(^{-1}\). Therefore, three working solutions (I, II and III) were prepared by dilution of the stock solutions with water. Working solution I contained 40 mg L\(^{-1}\) of LMN-8,9-OH; working solution II contained 4 mg L\(^{-1}\) each of cVER, tVER, MYR, cCAR, tCAR, CRN-10-OH, POH, PA, (1S,2S)-LMN-1,2-OH, and LMN-8,9-OH; and 0.2 mg L\(^{-1}\) each of cVER, tVER, MYR, cCAR, tCAR, CRN-10-OH, POH, PA, and (1S,2S)-LMN-1,2-OH were contained in working solution III. The stock solutions of the ISTDs D\(_1\)-tVER, D\(_4\)-MNT, \(1\text{C}_2\text{D}_2\)-(1S,2S,4S)-LMN-1,2-OH, \(1\text{C}_2\text{D}_2\)-(1R,2R)-LMN-1,2-OH, and \(1\text{C}_2\text{D}_2\)-LMN-8,9-OH were prepared by dissolving the standard substances in MeCN. The stock solutions were then diluted with water to obtain a working solution containing 20 mg L\(^{-1}\) of each of the internal standards. All stock solutions were stored frozen at 18 °C under nitrogen protective gas, preserving the references from autoxidation. For the derivatisation process, a TSIM working solution containing 5% (v/v) TSIM in DCM was freshly prepared before each analytical series.

2.7. Calibration procedure

The calibration was carried out using 10 calibration levels prepared by spiking pooled urine with different volumes of working solutions I, II and III to achieve final concentrations of 0.2–100 μg L\(^{-1}\) each of cVER, tVER, MYR, cCAR, tCAR, CRN-10-OH, POH, PA, and (1S,2S)-LMN-1,2-OH, as well as 10–1000 μg L\(^{-1}\) of LMN-8,9-OH. Additionally, identical pooled urine was used as a blank matrix sample and was included into each analytical series. The calibration standards and blank samples were processed as described in Section 2.8. Linear calibration curves were obtained by plotting the quotients of the analytes’ peak areas to the peak areas of the corresponding labelled ISTD as a function of spiking concentration. Taking into account possible matrix blank values of the pooled urine, we applied the slope of the calibration graph for parameters that show notably urinary blank levels.

2.8. Sample preparation (standard procedure)

Aliquots of urine samples were stored frozen at −18 °C under nitrogen protective gas. Initially, urine samples were thawed, equilibrated to room temperature (RT), and vortex-mixed. For hydrolysis, an aliquot of 4 mL urine was transferred into a glass vial containing 10 μL of the ISTD working solution and 400 μL of sodium acetate buffer (1 mol L\(^{-1}\), pH 5.0). Subsequently, 10 μL of β-glucuronidase/arylsulfatase was added, briefly vortex-mixed, and then the sample was incubated for a minimum of 3 h at 37 °C. To assess possible sample contamination from defiled materials or reagents, every sample preparation series contained a blank sample, consisting of all used reagents and purified water instead of urine. After the hydrolysis step, the sample was acidified with 50 μL of aqueous HCl (25%) and instantly transferred to a glass column containing a PTFE-frit and 3.4 ± 0.1 g of isolute HM-N. To ensure complete absorption of the aqueous phase to the porous diatomaceous earth material, the sample was allowed to seep in for 10 min. Afterwards, the drenched stationary phase was extracted two times with 4 mL of DCM and one time with 6 mL of DCM, including an
The sample preparation process was optimised with regard to the appropriate solvent and sample pH for SSLLE. Identical pooled urine samples spiked with 1 mg L\(^{-1}\) of each of the analytes were transferred to Isolute HM–N SSLLE columns and subsequently extracted with 16 mL each of DCM and DEE, divided into 4 mL portions and extraction periods of 10 min. To study the influence of pH value on the extraction yield of the pH sensitive limonene metabolite PA, the procedure was, additionally, tested under strongly (pH <2.0) and weakly (pH 5.0) acidic conditions. To establish pH <2.0, spiked urine samples (1 mg L\(^{-1}\) of each of the analytes), which had previously been adjusted to pH 5.0 with sodium acetate buffer to simulate hydrolysis conditions, were acidified with 50 µL of aqueous HCl (25%) and the pH value was checked using a pH indicator prior to DCM extraction. Afterwards, the silylated extracts were measured using GC–PCImS. Relative extraction yield correlated with the respective pH value and solvent is expressed as the quotient of absolute peak area of the analytes versus corresponding highest analyte peak area (Fig. 4). Furthermore, the optimum DCM volume used throughout the SSLLE process was evaluated. Therefore, identical pooled urine samples spiked with 1 mg L\(^{-1}\) of all of the analytes were transferred to SSLLE columns and subsequently extracted with increasing volumes of DCM (6–16 mL), divided into 2 mL portions. Relative yield of analyte extraction (calculated as the quotient of absolute analyte peak area related to the corresponding highest peak area) was plotted against the corresponding DCM volume (Fig. 5).

The applicability of BSTFA in combination with TSIM for derivatisation was initially checked by analysis of identical reaction mixtures containing 1 mg L\(^{-1}\) of each of the analytes in DCM and 10% (v/v) of BSTFA either with or without addition of 1% (v/v) TSIM. These reaction mixtures were allowed to react at varying incubation conditions, i.e., two different temperatures [RT and 70 °C (data not shown)] and incubation times ranging from 10 min up to 24 h, and subsequently were analysed by GC–PCImS. Mean relative peak area values, based on the respective highest peak area obtained, were plotted against reaction time as degree of derivatisation (Fig. 6).

The sample’s initial temperature was held at 90 °C for 1 min, then raised to a rate of 6 °C min\(^{-1}\) to 135 °C and subsequently raised to a rate of 15 °C min\(^{-1}\) to 170 °C. From this temperature, it was raised by 40 °C min\(^{-1}\) to 300 °C, remaining at this level for 4 min. The temperature of the transfer line was set to 280 °C. The ion source was operating at 250 °C in positive chemical ionisation mode with methane reagent gas (purity 5.5, Linde, München, Germany). The mass selective detector was adjusted to 150 °C quadrupole temperature. Collision gas (nitrogen) and collision cell quench gas (helium) were set to flow rates of 1.5 mL min\(^{-1}\) and 2.25 mL min\(^{-1}\), respectively. For detection, the mass spectrometer was operating in the multiple reaction monitoring mode (MRM). To establish optimum MRM operating conditions, standard solutions of each analyte and IS were determined separately. Two mass transitions of the most sensitive precursor ions were optimised regarding product ions and corresponding collision induced dissociation (CID) energies for every analyte. The mass transition with the higher intensity or lower noise was used as the quantifier, the second as the qualifier ion. Optimised MRM parameters are summarised in Table 1. The chemical structures of the silylated analytes and their corresponding proposed precursor and product ions, formed during PCI-MS/MS, are shown in Fig. 3.
Table 1
Retention times and MRM-specific parameters of the trimethylsilylated analytes and IS
ds.

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<th>Analyte</th>
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<th>Quantifier ion [( m/z )]</th>
<th>CID [V]</th>
<th>Qualifier ion [( m/z )]</th>
<th>CID [V]</th>
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<td>225</td>
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<td>10</td>
<td>299</td>
<td>135</td>
<td>10</td>
<td>299</td>
<td>135</td>
</tr>
</tbody>
</table>

\( t_R \): retention time; CID: collision induced dissociation; Q1: quadrupole 1; Q3: quadrupole 3.

* Predicted mass transitions and \( t_R \) based on data gained from \( ^{13} \)CD<sub>2</sub>-(1R,2R)-LMN-1,2-OH reference.

![Fig. 3. Structural formulae of the silylated analytes as well as of the proposed structures of their corresponding precursor (quantifier ion and qualifier ion) and product ions formed during positive chemical ionisation (PCI) and collision induces dissociation (CID, MS/MS experiment).](image-url)
to monoterpens. The subjects were at the median age of 40 years (range: 19–69 years) and composed of females and males. The samples were stored frozen at −18 °C under nitrogen protective gas until analysis. Sample and data collection was approved by the local ethics committee of the University of Erlangen-Nuremberg (Re-No. 4457, 16.06.2011).

3. Results and discussion

3.1. Internal and reference standards

The commercially available compounds (S)-cis-verbenol [(15S,25S)-cis-verbenol, (1R)-(-)-myrtenol [(1R,5S)-myrtenol], (−)-carveol [separable mixture of cis-(1R,5R)- and trans-(15S,5R)-isomers], (S)(−)-perillyl alcohol, (S)(−)-perillic acid (all structures in Fig. 2), and (15S,25R)-(−)-limonene-1,2-diol (Δ15S,25S)-LMN-1,2-OH in Fig. 1) were used as reference substances for the monoterpene metabolites cVER, MYR, cCAR, POH, PA and (15S,25S)-LMN-1,2-OH (Fig. 1), respectively. Remarkably, all of them, except (15S,25R)-(−)-limonene-1,2-diol (Δ15S,25S)-LMN-1,2-OH, are enantiomers of the natural metabolites. As “enantiomeric standards”, they possess the same retention times as natural metabolites. We also prepared trans-verbenol (tVER in Fig. 1) from (+)-α-pinene, its Δ13-analogue (Δ13-tVER in Fig. 2) from Δ13-(+)-α-pinene, limonene-8,9-diol (LMN-8,9-OH in Fig. 1) from (R)-(−)-limonene, and its Δ13CD2-analogue (Δ13CD2-LMN-8,9-OH in Fig. 2) as well as two diastereomeric Δ13CD2-limonene-1,2-diols from Δ13CD2-(R)-(−)-limonene [(1S,25S,4R)- and (1R,25R,4R)-isomers in Fig. 2].

3.2. Sample preparation and derivatisation

Due to the volatility and chemical diversity of the monoterpeneoid analytes, we developed a relatively unspecific but low-loss and highly concentrating extraction procedure. For this purpose, we selected solid–supported liquid–liquid extraction (SSSLE) because of its flexibility regarding varying functional groups. Moreover, SSSLE provides reasonably clean and highly concentrated dry extracts, as well as low evaporation loss during the concentration step. Due to their immiscibility with water, but high eluting power of mid-polar hydrocarbons, DCM and DEE were tested as possible extraction solvents. Additionally, we varied pH to optimise the extraction efficiency. We assessed the influence of low pH on the extraction yield of the pH sensitive LMN metabolite PA by extracting samples at pH ≤2.0 and pH 5.0. The results of these experiments showed highest extraction efficiency when urine samples were extracted with DCM at pH ≤2.0. At pH 5.0, PA (pKa = 4.94 ± 0.40) [34] was partly deprotonated, which retards extraction in organic solvents. In comparison to DCM, DEE exhibits much lower and insufficient elution power (Fig. 4).

Subsequently, the required total DCM extraction volume was tested using volumes from 6 mL to 16 mL in 2 mL portions. This experiment showed that a total volume of 14 mL DCM is appropriate for maximum extraction of all analytes from a 4 mL urine sample (Fig. 5).

For optimisation of the derivatisation process, we added the strong trimethylsilyl donor TSIM to support BSTFA in silylation of the unreactive tertiary hydroxyl groups of the diol metabolites LMN-1,2-OH and LMN-8,9-OH. Since BSTFA, in contrast to TSIM, exhibits good dissolving properties and produces highly volatile reaction by-products, which do not influence the gas chromatographic separation, we used a surplus of BSTFA as a base derivatisation reagent and tested various amounts of TSIM for accelerating the derivatisation reaction. The results showed that even small amounts of TSIM (<1%) in the reaction mixture notably accelerated the reaction. The silylation of LMN-1,2-OH and LMN-8,9-OH without TSIM did not reach maximal yield after more than 20 h (data not shown), whereas it was achieved after 10 min when 1% TSIM was added (Fig. 6).

3.3. GC–MS/MS conditions

A gas chromatographic temperature programme, which enabled the separation of all 10 analytes within an optimum runtime of 18 min, was elaborated. Despite the short runtime, the programme enables baseline separation of the diastereomers cVER and tVER, cCAR and tCAR as well as (15S,25S)-LMN-1,2-OH and (1R,25R)-LMN-1,2-OH, LMN-8,9-OH and (8R,25R)-LMN-8,9-OH could only be separated, albeit poorly, under runtime optimised conditions. Therefore, a slightly shouldered peak is observed for LMN-8,9-OH. However, separation of these isomers is feasible by applying longer runtimes and lower temperature gradients if desired. Fig. 8 displays the ion traces of the analytes from a real human urine sample to prove the applicability of the method. Like in all samples analysed so far, the suggested metabolites CRN-10-OH and (1R,25R)-LMN-1,2-OH are below the detection limit in this general example (Fig. 8).

The use of pulsed splitless injection mode allows large injection volumes without loss of chromatographic performance. In
Fig. 6. Time dependent relative increase in peak area in relation to the peak area of the corresponding entirely silylated peak under varying reaction conditions (n = 2): 1 mg L\(^{-1}\) analyte in DCM + 10% BSTFA with and without 1% TSIM at RT: (a) sum of cVER, tVER, MYR, cCAR, tCAR, CRN-10-OH, POH, PA; (b) LMN-1,2-OH; (c) LMN-8,9-OH.

Fig. 7. GC–PCI-MS/MS chromatogram of 40 \(\mu\)g L\(^{-1}\) of each of the reference standards in hydrolysed urine (extracted ion chromatogram; EIC).

Fig. 8. GC–PCI-MS/MS chromatogram of a hydrolysed urine sample from a human who was exposed to LMN by inhalation (extracted ion chromatogram; EIC).
combination with tandem mass spectrometry, this injection technique results in elevated peak areas without raising the chemical noise level. Therefore, we were able to lower the LOD values by pulsed splitless injection of 3 μL of extract without the need of sophisticated solvent venting or cooled injection techniques.

Additionally, we applied methane positive chemical ionisation of the analytes, since pilot experiments revealed that the monoterpene metabolites, even if silylated, are instable under electron ionisation conditions. This means that the analytes showed mass spectra with many low-mass fragments and just scarce molecular ions ([M]+; shown exemplarily for POH in Fig. 9a). These strong fragmentation patterns are disadvantageous for tandem mass spectrometry, which needs to be focused on abundant and characteristic precursor ions. In contrast, positive chemical ionisation leads predominantly to characteristic, stable, and copious analyte precursor ions: m/z 223 ([M–H]+), m/z 209 ([M–15]+), and m/z 135 ([M–89]+) for monohydroxy metabolites (shown exemplarily for POH in Fig. 9b; proposed precursor ion structures shown in Fig. 3) as well as m/z 299 ([M–15]+), m/z 225 ([M–89]+), and m/z 135 ([M–2×89]+) for dihydroxy metabolites (Table 1; Fig. 3). These ions were well suited for tandem mass spectrometry. Hence, the analyte and ISId trimethylsilyl derivatives were each registered by two specific precursor ions, which underwent collision induced dissociation (CID) forming characteristic product ions. The most abundant or lowest noise mass transition was used as a quantifier, the second as a qualifier ion (Table 1; Fig. 3).

### 3.4. Reliability of the method

Linearity of the calibration graphs was demonstrated for all analytes within the observed calibration ranges: 0.2–100 μg L⁻¹ each of cVER, tVER, MYR, cCAR, tCAR, CRN-10-OH, POH, PA, and (15S)-LMN-1,2-OH, and 10–1000 μg L⁻¹ of LMN-8,9-OH. Since it was hardly feasible to obtain a blank urine matrix without any traces of monoterpene metabolites, we applied the slope of the calibration graphs for the quantitation of parameters with high blank values, i.e., tVER, MYR, PA, (15S)-LMN-1,2-OH, and LMN-8,9-OH. Intraday precision was determined by sequential analysis of Q_{low} and Q_{high} material and ranged from 1 to 19% (Q_{low}) and 1 to 6% (Q_{high}) for all analytes (Table 2). Comparable results were generally found for interday repeatability, which ranged from 3 to 17% (Q_{low}) and 1 to 6% (Q_{high}). Optimum accuracy values around 100% were achieved for all analytes, presumably due to the use of structurally matching or closely related isotopically labelled ISld for most of the analytes (Table 2).

The LOD levels in urine matrix, calculated using DIN 32 645 [33], were found to range between 0.1 and 0.3 μg L⁻¹. Hence, the corresponding LOQ levels ranged from 0.2 to 1.1 μg L⁻¹. The highest LOD of 1.1 μg L⁻¹ was calculated for PA, which is the only parameter that is not closely related to one of the ISld because of its carboxyl group.

Matrix effects needed to be checked, since urine is a very complex and mutable fluid that may rapidly change its composition due to varying reasons, even within one subject. The creatinine level of a urine sample, therefore, is a good measure for its overall matrix load. To rule out potential matrix effects that may interfere the analysis method, we determined relative analyte recovery rates in 10 urine samples with increasing creatinine levels ranging from 0.4 to 2.5 mg L⁻¹. The results showed that there are mean relative recoveries of 105% (range: 91–116%) for cVER, tVER, MYR, cCAR, tCAR, CRN-10-OH, and POH at 10 μg L⁻¹; of 110% (range: 105–115%) for (15S)-LMN-1,2-OH and PA at 40 μg L⁻¹; and of 106% for LMN-8,9-OH at 100 μg L⁻¹. Additionally, no significant correlation between the increasing creatinine levels and the analyte recovery rates could be detected (Pearson’s correlation test). Therefore, distinct matrix effects can be excluded.

The stability of the reference compounds in aqueous medium could be verified over a period of 2 months. Within this period, no significant decrease of analyte concentration in the quality control materials was detected.

### 3.5. Method application and background exposure data

To check and prove the performance and requirements of the developed method with respect to native biological samples, the procedure was applied to urine samples of occupationally non-exposed individuals of the general population. The results showed that the procedure enables the detection of metabolites of αPN

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**Table 2**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOD [μg L⁻¹]</th>
<th>LOQ [μg L⁻¹]</th>
<th>Q_{low} [μg L⁻¹]</th>
<th>Q_{high} [μg L⁻¹]</th>
<th>Precision [%]</th>
<th>Repeatability [%]</th>
<th>Accuracy [%] (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cVER</td>
<td>0.1</td>
<td>0.3</td>
<td>1.9</td>
<td>19.2</td>
<td>8</td>
<td>4</td>
<td>100–105</td>
</tr>
<tr>
<td>tVER</td>
<td>0.1</td>
<td>0.5</td>
<td>2.2</td>
<td>21.6</td>
<td>7</td>
<td>2</td>
<td>98–110</td>
</tr>
<tr>
<td>MYR</td>
<td>0.2</td>
<td>0.6</td>
<td>2.4</td>
<td>23.6</td>
<td>9</td>
<td>6</td>
<td>91–100</td>
</tr>
<tr>
<td>cCAR</td>
<td>0.1</td>
<td>0.3</td>
<td>2.4</td>
<td>23.6</td>
<td>9</td>
<td>4</td>
<td>93–101</td>
</tr>
<tr>
<td>CRN-10-OH</td>
<td>0.1</td>
<td>0.4</td>
<td>1.6</td>
<td>15.8</td>
<td>12</td>
<td>1</td>
<td>93–101</td>
</tr>
<tr>
<td>POH</td>
<td>0.1</td>
<td>0.2</td>
<td>1.9</td>
<td>19.0</td>
<td>11</td>
<td>3</td>
<td>90–95</td>
</tr>
<tr>
<td>(15S)-LMN-1,2-OH</td>
<td>0.1</td>
<td>0.4</td>
<td>2.1</td>
<td>21.4</td>
<td>8</td>
<td>1</td>
<td>90–97</td>
</tr>
<tr>
<td>PA</td>
<td>0.3</td>
<td>1.1</td>
<td>2.1</td>
<td>21.4</td>
<td>19</td>
<td>2</td>
<td>90–94</td>
</tr>
<tr>
<td>LMN-8,9-OH</td>
<td>0.1</td>
<td>0.5</td>
<td>2.0</td>
<td>19.6</td>
<td>3</td>
<td>1</td>
<td>100–101</td>
</tr>
</tbody>
</table>

* LOD and LOQ calculated according to DIN 32 645 [33].
Table 3
Biomonitoring data of monoterpene metabolites in urine from occupationally non-exposed volunteers of the common population (n = 36).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>&gt;LOD [%]</th>
<th>Median [µg.L⁻¹]</th>
<th>Range [µg.L⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>cVER</td>
<td>97</td>
<td>10.3</td>
<td>&lt;0.1 to 120.9</td>
</tr>
<tr>
<td>iVER</td>
<td>100</td>
<td>16.2</td>
<td>3.6 to 242.7</td>
</tr>
<tr>
<td>MYR</td>
<td>97</td>
<td>33.8</td>
<td>0.2 to 2434.9</td>
</tr>
<tr>
<td>cCAR</td>
<td>100</td>
<td>6.6</td>
<td>&lt;0.1 to 63.4</td>
</tr>
<tr>
<td>tCAR</td>
<td>92</td>
<td>6.3</td>
<td>&lt;0.1 to 214.0</td>
</tr>
<tr>
<td>POH</td>
<td>94</td>
<td>2.0</td>
<td>&lt;0.1 to 38.4</td>
</tr>
<tr>
<td>(1S,2S)-LMN-1,2-OH</td>
<td>89</td>
<td>63.0</td>
<td>&lt;0.1 to 744.0</td>
</tr>
<tr>
<td>(1R,2R)-LMN-1,2-OH</td>
<td>0</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>92</td>
<td>47.8</td>
<td>0.3 to 1097.5</td>
</tr>
<tr>
<td>LMN-8,9-OH</td>
<td>97</td>
<td>1080.7</td>
<td>&lt;0.1 to 18427.7</td>
</tr>
<tr>
<td>CRN-10-OH</td>
<td>0</td>
<td>&lt;0.1</td>
<td></td>
</tr>
</tbody>
</table>

* Estimated LOD derived from data gained from (1S,2S)-LMN-1,2-OH.

and LMN in 89–100% of the samples (Table 3). The urinary concentrations ranged from the LODs up to more than 10.000 µg.L⁻¹. Particularly, the major metabolite of LMN, LMN-8,9-OH, exhibited a wide range of up to five orders of magnitude. Comparable variability was found for PA and (1S,2S)-LMN-1,2-OH, as well as for the potential α-PN metabolite MYR. Due to the low LOQs, even minor metabolites could be quantified with median concentrations ranging from 2.0 µg.L⁻¹ for POH to 10.3 µg.L⁻¹ for cVER. These results indicate the necessity of a procedure that provides both high sensitivity and a broad operating range to detect the lowest and also the highest background exposure of the population. In contrast to the frequent and distinct quantities of (1S,2S)-LMN-1,2-OH (with diaxial hydroxyl groups; Fig. 1), the (1R,2R)-stereoisomer of LMN-1,2-OH (with diequatorial hydroxyl groups; Fig. 1) could not be detected in any of the samples. This indicates that hydrolysis of only one diastereomer of the oxirane precursor cis-LMN-1,2-epoxide (cis-1-methyl-4-[(1-methylhexyl)-7-oxacyclo[4.1.0]heptane] is efficient in humans in vivo. The alternative explanations, for instance, that only one, namely cis-LMN-1,2-epoxide, is formed upon enzymatic epoxidation of limonene or that hydrolysis of both cis- and trans-epoxides affords the same diaxial diol, are also possible, though seem to be less probable [29]. For a native (1R,2R)-LMN-1,2-OH reference substance, which was not detected in urine, we predicted the corresponding mass transitions and isobars from the GC–PCI-MS/MS behaviour of (1R,2R)-LMN-1,2-OH. The detection of (1S,2S)-LMN-1,2-diol in humans is consistent with the results of studies on the incubation of LMN-1,2-epoxide with fungi and bacteria, which exhibited stereoselective epoxide hydrolysis to (1S,2S)-LMN-1,2-OH as well [35,36].

The expected metabolite of CRN, CRN-10-OH, was not detected in any of the samples. This may be due to the absence of CRN exposure in the investigated individuals. Alternatively, CRN-10-OH may not represent the most appropriate biomonitoring parameter of CRN. To date, this metabolite has only been identified in vitro using human liver microsomes [12,15]. Future studies need to clarify this issue. However, the general applicability of the procedure may provide for the determination of other oxidised CRN metabolites.

4. Conclusion

The present method is the first procedure that enables the simultaneous trace level analysis of the 10 monoterpoid metabolites cVER, iVER, MYR, cCAR, tCAR, CRN-10-OH, POH, PA, LMN-1,2-OH, and LMN-8,9-OH in human urine. The use of SSLSE in combination with optimised two-step trimethylsilylation enables fast extraction, enrichment and derivatisation of the analytes without severe losses, independently from the constitutional isomerism and stereoisomerism. The sample preparation procedure is universally adaptable, even if the spectrum of analytes will include further oxidised monoterpene metabolites. High selectivity and sensitivity is gained by application of tandem mass spectrometry in combination with positive chemical ionisation. Accordingly, the reliability data underlines high reproducibility, accuracy, and precision of the present method. Compared to other published analytical procedures, this method enables the analysis of a broad spectrum of possible monoterpene metabolites with competitive or improved analytical sensitivity. Thus, it is the first monoterpene biomonitoring procedure that is applicable and recommendable for the determination of LMN and α-PN, as well as evaluation of CRN metabolite background exposures in population studies.

Conflict of interest

The authors declare no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anacli.2013.07.046.

References

[34] ACD/Labs, 1994–2013.