RESEARCH ARTICLE

Quantification of 1,8-cineole and of its metabolites in humans using stable isotope dilution assays

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Received: November 3, 2009
Revised: December 22, 2009
Accepted: January 13, 2010

The metabolism of 1,8-cineole after ingestion of sage tea was studied. After application of the tea, the metabolites 2-hydroxy-1,8-cineole, 3-hydroxy-1,8-cineole, 9-hydroxy-1,8-cineole and, for the first time in humans, 7-hydroxy-1,8-cineole were identified in plasma and urine of one volunteer. For quantitation of these metabolites and the parent compound, stable isotope dilution assays were developed after synthesis of [$^{2}H_3$]-1,8-cineole, [9/10-$^{2}H_3$]-2-hydroxy-1,8-cineole and [^{13}C,$^{2}H_2$]-9-hydroxy-1,8-cineole as internal standards. Using these standards, we quantified 1,8-cineole by solid phase microextraction GC-MS and the hydroxyl-1,8-cineoles by LC-MS/MS after deconjugation in blood and urine of the volunteer. After consumption of 1.02 mg 1,8-cineole (19 μg/kg bw), the hydroxycineoles along with their parent compound were detectable in the blood plasma of the volunteer under study after liberation from their glucuronides with 2-hydroxycineole being the predominant metabolite at a maximum plasma concentration of 86 nmol/L followed by the 9-hydroxy isomer at a maximum plasma concentration of 33 nmol/L. The parent compound 1,8-cineole showed a low maximum plasma concentration of 19 nmol/L. In urine, 2-hydroxycineole also showed highest contents followed by its 9-isomer. Summing up the urinary excretion over 10 h, 2-hydroxycineole, the 9-isomer, the 3-isomer and the 7-isomer accounted for 20.9, 17.2, 10.6 and 3.8% of the cineole dose, respectively.

Keywords:
1,8-cineole / Hydroxy-1,8-cineole / Metabolism / Sage tea / Stable isotope dilution assay

1 Introduction

The monoterpene 1,8-cineole, also known as eucalyptol, is a major component of essential oils from *Eucalyptus polybractea*. Moreover, 1,8-cineole is present in numerous spices, such as rosemary, sage, basil and laurel. It has a characteristic fresh and camphoraceous fragrance and, therefore, is used for flavoring of foods and cosmetics. Besides its flavoring applications, 1,8-cineole is used in pharmaceutical preparations to treat cough, muscular pain, neurosis, rheumatism, asthma and urinary stone [1, 2].

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Abbreviations: HRGC, high resolution GC; **IS,** internal standard; **SIDA,** stable isotope dilution assay; **SPME,** solid phase microextraction

Metabolism of odorants is an actual topic in toxicology and pharmacology, as important herbal compounds such as pulegone from pennyroyal [3], estragole from fennel [4] and coumarin from cinnamon [5] have been shown to undergo bioactivation by metabolizing enzymes. For 1,8-cineole, biotransformation studies have been performed in brushtail possum and rabbits and identified 2α-hydroxy-1,8-cineole, 2β-hydroxy-1,8-cineole, 3α-hydroxy-1,8-cineole, 3β-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, 9-hydroxy-1,8-cineole (Fig. 1) and the respective diols, cineolic acids and hydroxyl cineolic acids as phase I metabolites in urine and blood plasma [6–8]. Regarding toxicity of 1,8-cineole, the oral acute LD_{50} in rats is reported to be 2480 mg/kg bw [9]. Subacute toxicity was shown in rats for dose levels of 600 mg/kg bw and higher. Symptoms were loss of body weight and lesions

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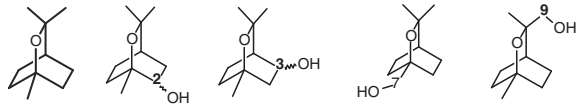

Figure 1. Structures of 1,8-cineole and its monohydroxylated metabolites.

in liver and kidney. There is no evidence for chronic or genotoxic effects of 1,8-cineole [10]. For the hydroxyl metabolites, no toxicological data is available.

In humans, studies on cineole metabolism are rare and up to date, 2-hydroxy-1,8-cineole and 3-hydroxy-1,8-cineole have been identified in urine after a single cineole dose of 100 mg [11]. However, when cineole is administered as herbal tea or as spice, the dose is much lower and dose-dependent metabolism has been shown to be critical for evaluating pharmacology and toxicology. Moreover, interference of other terpenes in metabolism may also occur. Therefore, the aim of the current study was to identify and quantify cineole metabolites in humans after the intake of food-relevant doses.

2 Material and methods

2.1 Chemicals

The following reagents were purchased from the sources given in parentheses: 1,8-cineole, 4-acetyl-1-methylcyclohexene, [$^2H_3$]-methylmagnesium iodide, pyridine, phenyl selenyl chloride, *m*-chloroperbenzoic acid (max. 77%), R(+)-limonene, lead tetraacetate, [^{13}C,$^2H_3$]-methyl iodide, tert-butyllithium in hexane (Aldrich, Steinheim, Germany), *p*-toluenesulfonic acid, mercuric acetate, sodium borohydride, triphenylphosphine (Fluka, Buchs, Switzerland); β-glucuronidase from Helix pomatia (EC 3.2.1.31, Type H-2, *ca.* 100 000 units/mL) (Sigma, Deisenhofen, Germany); toluene, tetrahydrofurane, dichloromethane, ACN, α-terpineol (Merck, Darmstadt, Germany).

3α-Hydroxy-1,8-cineole was a generous gift from Craig J. Wallis/R. M. Carman, Univ. of Queensland, Brisbane, Australia.

[9-$^2H_3$]-1,8-Cineole, 2-hydroxy-1,8-cineole, [9/10-$^2H_3$]-2-hydroxy-1,8-cineole, 9-hydroxy-1,8-cineole, 9-[^{13}C,$^2H_2$]-9-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, and 7- and 9-carboxy-1,8-cineole were synthesized by the following procedures.

2.1.1 Synthesis of [9-$^2H_3$]-α-terpineol (2)

In adaptation of the method described by Bégué *et al.* [12], a solution of 4-acetyl-1-methylcyclohexene (1, limona ketone, 358 mg; 2.6 mmol) in absolute diethyl ether (10 mL) was added dropwise to an ethereal solution (1.0 mol/L) of $^2H_3$-methylmagnesium iodide (10 mL; 10 mmol). The mixture was allowed to stir for 15 min at room temperature and then hydrolyzed with 300 µL ice-cold water. The precipitate was dissolved by adding saturated aqueous NH_4Cl solution. After separating the organic layer the aqueous solution was extracted with dichloromethane (2 × 5 mL). The combined organic extracts were washed with water, dried over Na_2SO_4 and concentrated in a stream of nitrogen. Filtration over Florisil with pentane/diethylether (3/1, v/v) gave pure [9-$^2H_3$]-α-terpineol (2, 300 mg; 1.95 mmol, 75%).

Mass spectrum (EI): m/z (relative intensity): 157 (M; 2), 142 (28), 140 (40), 139 (64), 125 (20), 124 (61), 121 (55), 110 (45), 96 (46), 95 (47), 94 (46), 92 (100), 81 (52), 62 (58), 55 (42), 54 (35), 46 (43), 43 (45), 41 (40), 39 (39).

NMR spectrum: (^1H): 1.2 ppm (s, 1.5 H); 1.21 ppm (s, 1.5 H); 1.28 ppm (m, 2H); 1.52 ppm (m, 1H); 1.68 ppm (s, 3H); 1.95 ppm (m, 5H); 5.4 ppm (m, 1H).

2.1.2 Synthesis of [9-$^2H_3$]-1,8-cineole (4)

Following the description of Bugarčić *et al.* [13] for the unlabeled compound, [9-$^2H_3$]-α-terpineol (2, 50 mg; 0.32 mmol) and pyridine (28 mg, 0.34 mmol) were dissolved in 2 mL anhydrous dichloromethane and phenylselenyl chloride (69 mg; 0.34 mmol) was added at room temperature. The solution was stirred for 1 h and was then successively washed with HCl (1 mol/L, 3 × 2 mL), saturated $NaHCO_3$ (2 × 2 mL) and brine. After drying over Na_2SO_4 and concentration, the residue was purified by chromatography over silica with dichloromethane to give [9-$^2H_3$]-2-phenylselenyl-1,8-cineole (3) as a yellow residue. Reduction of the latter to remove the phenylselenyl group was performed as described by Nicolaou *et al.* [14] using tri-*n*-butyltin hydride (150 µL, 0.5 mmol) and azobisisobutyronitrile (0.02 mol/L in toluene, 300 µL, 6 µmol) in toluene at 110°C for 1 h.

Chromatography over silica with the solvent dichloromethane gave pure [9-$^2H_3$]-1,8-cineole (4, 31 mg, 0.2 mmol, 63%).

High resolution mass spectrum (EI): m/z: 157.1588 ($C_{10}H_{15}D_3O$ requires 157.1546).

Mass spectrum (EI): m/z (relative intensity): 157 (39), 142 (18), 139 (18), 129 (16), 128 (13), 114 (26), 111 (51), 110 (25), 96 (32), 95 (29), 87 (42), 81 (58), 72 (50), 71 (37), 55 (23), 46 (24), 43 (100), 41 (34), 39 (21).

Mass spectrum (CI, methanol): m/z (relative intensity): 140 (100), 81(12), 65(14), 55(8).

NMR spectrum: (^1H): 1.07 ppm (s, 3 H); 1.26 ppm (s, 3 H); 1.43 ppm (m, 1H); 1.45–1.60 ppm (m, 4H); 1.68 ppm (m, 2H); 2.04 ppm (m, 2H).

2.1.3 Synthesis of unlabeled 2-hydroxy-1,8-cineol

α-Terpineol (170 mg, 1.1 mmol) was treated with *m*-chloroperbenzoic acid (260 mg, max. 77%, ~1.5 mmol) as described by Kopperman *et al.* [15]. The obtained epoxides were

stirred with *p*-toluenesulfonic acid (50 mg) in dichloromethane for 24 h according to Carman and Fletcher [16]. Clean up was performed with column chromatography on silica and hexane/ether as described by Miyazawa and Hashimoto [17].

Mass spectrum (EI): m/z (relative intensity): 170(10), 137(10), 126(22), 111(24), 109(13), 108(100), 93(55), 83(14), 71(30), 69(21), 57(17), 55(22), 43(63), 41(28), 39(30).

Mass spectrum (CI, methanol): m/z (relative intensity): 171(3), 153(100), 135(63), 109(7), 95(15), 55(6).

Mass spectrum (ESI^+, MS/MS energy of collision 10 V): 171(13), 153(62), 135(100), 109(12), 107(20), 97(9), 95(12), 93(11).

NMR spectrum: (^1H in CD_3OD): 1.06 ppm (s, 3H); 1.2 ppm (s, 3H); 1.28 ppm (s, 3H); 1.35 ppm (m, 1H); 1.47–1.63 ppm (m, 3H); 1.85–2.07 ppm (m, 2H); 2.52 ppm (m, 1H; 3.63 ppm (m, 1H).

NMR spectrum: (^{13}C): 22.19 ppm, 24.07 ppm, 24.93 ppm, 28.63 ppm, 29.04 ppm, 34.27 ppm, 34.60 ppm, 34.60 ppm, 71.13 ppm, 72.52 ppm, 73.45 ppm.

2.1.4 Synthesis of [9/10-$^2H_3$]-2-hydroxy-1,8-cineole (6)

[9-$^2H_3$]-α-terpineol (2, 107 mg, 0.62 mmol) was dissolved in dry dichloromethane (2.5 mL) and added dropwise to a suspension of *m*-chloroperbenzoic acid (max. 77%, 160 mg, 0.7 mmol) in 2.5 mL dichloromethane at 0°C. The mixture was stirred under argon at 0°C for 2 h and then filtrated. *m*-Chlorobenzoic acid was removed by successively washing with aqueous $NaHSO_3$ (5%), $NaHCO_3$ (5%) and water. GC-MS revealed [9-$^2H_3$]-1,2-epoxy-*p*-menthane-8-ol (5) as the main product with traces of [9/10-$^2H_3$]-2-hydroxycineole (6) and [9/10-$^2H_3$]-2,8-epoxy-*p*-menthane-1-ol. The solution was diluted with dichloromethane to 15 mL and *p*-toluene sulfonic acid (30 mg, 0.17 mmol) was added. After stirring at room temperature for 24 h, the solution was washed with 10% aqueous $NaHCO_3$. After separation of the latter solution by column chromatography over silica and ether/hexane (1/2 v/v) as the mobile phase and evaporation of the solvent of the fractions, needles of [9/10-$^2H_3$]-2-hydroxy-1,8-cineole (6, 47 mg, 0.27 mmol, 43%) were obtained.

Mass spectrum (EI): m/z (relative intensity): 173(41), 155(6), 140(6), 129(56), 114(60), 111(98), 100(19), 97(34), 96(38), 95(38), 94(33), 93(40), 86(40), 83(35), 79(23), 73(32), 71(100), 69(33), 67(30), 62(38), 58(39), 55(33), 53(29), 46(33), 43(70), 41(33), 39(32).

Mass spectrum (CI, methanol): m/z (relative intensity): 174(4), 156(100), 138(59), 112(6), 95(11),

Mass spectrum (ESI^+, MS/MS energy of collision 10 V): 174(13), 156(70), 138(100), 137(10), 112(10), 110(14), 98(5), 97(7), 95(11), 93(7).

NMR spectrum: (^1H in CD_3OD): 1.06 ppm (s, 3 H); 1.2 ppm (s, 1.5 H); 1.28 ppm (s, 1.5 H); 1.35 ppm (m, 1 H); 1.47–1.63 ppm (m, 3 H); 1.85–2.07 ppm (m, 2 H); 2.52 ppm (m, 1 H; 3.63 ppm (m, 1H).

2.1.5 Synthesis of unlabeled 9-hydroxy-1,8-cineole

In short, 4-acetyl-1-methylcyclohexene (limonaketone, 1) was converted in a Wittig reaction with methyl triphenylphosphonium iodide into limonene. Reaction of the latter with lead acetate and subsequent hydrolysis of the acetates gave the respective diol uroterpenol, which yielded 9-hydroxy-1,8-cineole upon reaction with mercuric acetate and sodium borohydride.

R-(+)-Limonene (540 mg, 4 mmol) was oxidized as described by Dean *et al.* [18] using lead tetraacetate (moistened with ~15% acetic acid, 10.3 g, ~20 mmol) in toluene (10 mL) at 65°C for 4 h. The mixture was filtered and after washing with an excess of water, drying over Na_2SO_4 and evaporation of the organic phase, a yellow oil was obtained (980 mg), which was hydrolyzed for 45 min at 60°C with KOH (2%) in ethanol (20 mL). The mixture was allowed to cool down, then diluted with water (140 mL) and thoroughly extracted with dichloromethane. Drying and evaporation of the solvent gave uroterpenol as a yellow oil (540 mg). The latter was dissolved in toluene (20 mL), extracted with water and the combined aqueous extracts were re-extracted with dichloromethane. The dichloromethane phases were combined, dried over Na_2SO_4 and concentrated to give raw uroterpenol as a colorless residue (230 mg, 1.35 mmol, 34%).

Mass spectrum (EI) of uroterpenol: m/z (relative intensity): 152(25), 139(30), 121(88), 105(12), 95(54), 94(71), 93(40), 81(28), 79(50), 77(17), 75(38), 71(38), 67(36), 57(36), 55(25), 53(15), 43(100), 41(22), 39(13).

Following a procedure reported by Flynn and Southwell [19], raw uroterpenol (230 mg, 1.35 mmol) was oxymercurated with mercuric acetate (435 mg, 1.4 mmol) in dry tetrahydrofuran (10 mL) at 55°C for 24 h. Then, aqueous NaOH (12%, 5 mL) was added and the mixture was treated with a solution of sodium borohydride (0.5 mol/L) in 12% aqueous NaOH (5 mL, 2.5 mmol) and stirred for 16 h at room temperature. Then, NaCl was added to saturate the aqueous layer, which was then separated and extracted with diethyl ether. The combined organic phases were dried over Na_2SO_4 and concentrated. Chromatography over silica with the solvent chloroform gave 9-hydroxy-1,8-cineole (52 mg, 0.31 mmol, 23%) as a white solid.

Mass spectrum (EI) of 9-hydroxycineol: m/z (relative intensity): 155(1), 139(70), 121(5), 109(3), 96(5), 95(33), 93(10), 81(15), 71(16), 67(10), 55(10), 43(100).

Mass spectrum (CI, butanol): m/z (relative intensity): 171(10), 153(86), 135(100), 107(8).

Mass spectrum (ESI^+, MS/MS energy of collision 10 V): 171(15), 153(55), 135(100), 107(57), 95(11), 93(21).

2.1.6 Synthesis of [9-^{13}C,$^2H_2$]-9-hydroxy-1,8-cineole (9)

The reaction sequence shown in Fig. 5 was performed to generate labeled 9-hydroxy-1,8-cineole (9). In short, the

[^{13}C,$^{2}H_3$]-label was introduced in a Wittig reaction according to Engel [20] and Zeller and Rychlik [21] *via* [^{13}C,$^{2}H_3$]-methyl triphenylphosphonium iodide into 4-acetyl-1-methylcyclohexene (limonaketone, 1) to give [9-^{13}C,$^{2}H_2$]-limonene (7). The latter was then reacted to the respective diol [9-^{13}C,$^{2}H_2$]-uroterpenol (8), which gave [9-^{13}C,$^{2}H_2$]-9-hydroxy-1,8-cineole upon reaction with mercuric acetate and sodium borohydride.

2.1.6.1 Preparation of [^{13}C,$^{2}H_3$]-methyl-triphenylphosphine

Following the instructions of Becker [22], triphenylphosphine (1.9 g, 7.1 mmol) was treated with a chilled solution of [^{13}C,$^{2}H_3$]-methyl iodide (1.0 g, 7 mmol) in 10 mL absolute toluene and then heated to 130°C for 20 h in a sealed tube. The precipitate was isolated by filtration and washed with hot toluene to obtain [^{13}C,$^{2}H_3$]-methyl triphenylphosphonium iodide (2.84 g, 7 mmol, 100%) as a bright yellow solid.

2.1.6.2 Synthesis of [9-^{13}C,$^{2}H_2$]-limonene

A solution of *tert*-butyllithium in hexane (2.4 mL, 1.6 mol/L) was added dropwise to a suspension of [$^{2}H_3$]-methyl triphenylphosphonium iodide (1.5 g, 3.7 mmol) in 80 mL absolute diethyl ether. The mixture was stirred under an atmosphere of nitrogen until a clear, orange solution evolved. Then, 4-acetyl-1-methylcyclohexene (1, 230 mg, 1.7 mmol) in 20 mL absolute diethyl ether was added slowly. A white precipitate appeared and the solution turned to bright yellow. Stirring under N_2 was continued for 1 h. Subsequently, the reaction mixture was washed with aqueous KH_2PO_4 (0.5%) and then the organic phase was dried over Na_2SO_4. GC-MS revealed [9-^{13}C,$^{2}H_2$]-limonene (7) as the main reaction product.

Mass spectrum (EI) of [9-^{13}C,$^{2}H_2$]-limonene: m/z (relative intensity): 139(25), 124(30), 110(25), 96(32), 93(83), 79(28), 71(89), 68(100), 67(52), 53(20), 41(23).

Mass spectrum (CI, methanol) of [9-^{13}C,$^{2}H_2$]-limonene: m/z (relative intensity): 140(100), 139(14), 138(10), 137(2), 110(6), 95(6).

Spectra of unlabeled limonene for comparison:

Mass spectrum (EI) of limonene: m/z (relative intensity): 136(30), 121(30), 107(28), 94(47), 93(82), 91(28), 81(39), 79(32), 68(41), 67(100), 53(28), 41(25).

Mass spectrum (CI, methanol) of limonene: m/z (relative intensity): 137(70), 136(11), 135(10), 134(2), 107(5), 95(15).

2.1.6.3 Synthesis of [9-^{13}C,$^{2}H_2$]-9-hydroxy-1,8-cineole

Raw [9-^{13}C,$^{2}H_2$]-limonene (7) was used without further clean-up and treated with lead tetraacetate (1.5 g, ~3 mmol) with subsequent hydrolysis using ethanolic KOH (2%, 1.5 mL) as described for the unlabeled compound. The obtained labeled uroterpenol (60 mg, 0.35 mmol) was then oxymercurated with mercury(II) acetate (130 mg, 0.4 mmol) in dry THF (5 mL) and treated with a solution of sodium borohydride (0.5 mol/L) in 12% aqueous NaOH (5 mL, 2.5 mmol) to yield [9-^{13}C,$^{2}H_2$]-9-hydroxy-1,8-cineole (9).

Mass spectrum (EI) of [9-^{13}C,$^{2}H_2$]-uroterpenol: m/z (relative intensity): 155(42), 139(46), 121(93), 105(6), 95(65), 94(58), 93(43), 81(27), 79(42), 78(36), 71(32), 67(39), 60(31), 55(22), 53(15), 43(100), 41(18), 39(13).

Mass spectrum (EI) of [9-^{13}C,$^{2}H_2$]-9-hydroxy-1,8-cineole: m/z (relative intensity): 158(1), 139(89), 121(5), 112(3) 97(10), 96(7), 95(34), 93(8), 81(15), 71(16), 67(13), 55(10), 43(100).

Mass spectrum (CI, methanol) [9-^{13}C,$^{2}H_2$]-9-hydroxy-1,8-cineole: *m/z* (relative intensity): 174(8), 156(78), 138(100).

Mass spectrum (ESI^+, MS/MS energy of collision 10 V): 174(17), 156(64), 138(100), 137(10), 110(38), 109(13), 96(7), 95(18), 93(9).

2.1.7 Synthesis of 7-hydroxy-1,8-cineole

In the first stage, δ-terpineol was synthesized according to Bull and Carman [23]. Therefore, (−)-β-pinene (300 mg, 2.2 mmol) was treated with *N*-bromosuccinimide (450 mg, 2.5 mmol) in acetone/water (10 mL, 4:1; v/v) at 0°C. After stirring for 30 min, the solution was diluted with water (10 mL) and extracted with diethyl ether (3 × 10 mL). The organic phase was dried over Na_2SO_4 and the solvent evaporated to obtain a yellow oil. The latter was dissolved in diethyl ether (8 mL) and acetic acid (2 mL) as well as zinc dust (200 mg) were added. The mixture was stirred at 0°C for 30 min and then washed with aqueous $NaHCO_3$ (saturated, 3 ×). After drying over Na_2SO_4, the organic phase was concentrated and the raw product was purified by column chromatography over silica with pentane/ether (5:1; v/v) as the mobile phase. The obtained δ-terpineol (180 mg, 1.2 mmol; 55%) was contaminated with about 7% of α-terpineol, which could not be removed by recrystallization as stated in the original reference [23].

Mass spectrum (EI): m/z (relative intensity): 154 (M; 2), 139 (M-Me; 5), 136 (M-H_2O, 20), 121 (9), 96 (15), 93 (55), 81 (55), 67 (20), 59 (100), 43 (23).

Subsequent synthetic steps to yield 7-hydroxy-1,8-cineole were described by Bull *et al.* [24]. δ-Terpineol (180 mg, 1.2 mmol) was dissolved in dry dichloromethane (10 mL), stirred and cooled to 0°C before *m*-chloroperbenzoic acid (340 mg, max. 77%, ~1.5 mmol) was added in small portions. After a further 15 min of stirring, the solution was washed with aqueous NaOH (10%), which formed a white precipitate. The latter was removed together with the aqueous phase. The organic phase was dried over Na_2SO_4 and concentrated (~5 mL). Then, *p*-toluenesulfonic acid (5 mg) was added and the mixture was stirred at 0°C for 15 min. The organic phase was washed with aqueous $NaHCO_3$ (saturated, 3 ×), dried over Na_2SO_4 and concentrated. Column chromatography over silica with pentane/

ether (1:1 v/v) as the solvent gave 7-hydroxy-1,8-cineole (yield 28%, 58 mg, 0.34 mmol).

Mass spectrum (EI): m/z (relative intensity): 170 (M; 12), 155 (M-Me; 45), 139 (M-CH_2OH; 15), 137 (18); 112 (16), 111 (95), 94 (23), 93 (60), 79 (43), 69 (100), 67 (23), 59 (44), 55 (40), 43 (66), 41 (38).

Mass spectrum (CI, methanol): m/z (relative intensity): 171 (10), 153 (90), 135 (100).

Mass spectrum (ESI^+, MS/MS energy of collision 10 V): 171(13), 153(42), 135(100), 125(8), 107(29), 93(17), 79(5), 69(6).

NMR spectrum: (^1H): 1.26 ppm (s, 6H); 1.41 ppm (m, 2H); 1.48 ppm (m, 1H); 1.56 ppm (m, 3H); 1.81 ppm (m, 2H); 2.06 ppm (m, 2H); 3.34 ppm (s, 2H).

2.1.8 Synthesis of 2α,4-dihydroxy-1,8-cineole

Following a route described by Carman and Rayner [25], a stirred, ice-cold solution of terpinolene (259 mg, 1.9 mmol) in dry dichloromethane was treated dropwise with a suspension of *m*-chloroperbenzoic acid (max. 77%, 440 mg, ≥2 mmol) in dichloromethane. Stirring was continiued for 1 h until the reaction mixture was washed with saturated $NaHCO_3$ and water. The organic phase was dried over $NaSO_4$ and the solvent was removed. The residue was dissolved in 6% sulfuric acid (10 mL) and stirred in an ice bath for 2 h. Then the solution was adjusted to pH 7 with aqueous $NaHCO_3$ and extracted with diethyl ether (2 ×). The organic extract was washed with water and dried. GC-MS revealed *p*-menth-1-ene-4,8-diol as main product.

Mass spectrum (EI) of p-menth-1-ene-4,8-diol: m/z (relative intensity): 170(2), 155(5), 152(37), 137(35), 119(26), 110(100), 97(33), 95(38) 93(92), 91(41), 84(36), 81(41), 79(39), 77(37), 67(37), 59(46), 55(42), 53(30), 43(38), 41(34), 39(29).

The solvent was removed and the residue (239 mg) was dissolved in dichloromethane (10 mL) and again treated with *m*-chloroperbenzoic acid (max. 77%, 245 mg, 1.1 mmol) and suspended in dichloromethane at 0°C. After stirring for 1 h, the solution was washed with aqueous NaOH (10%), dried over Na_2SO_4 and the solvent evaporated. The residue was heated to 210°C for 1.5 h in a capped sovirel tube. Column chromatography (silica, pentane/diethyl ether 2/3) gave 2α,4-dihydroxycineole (6.5 mg).

Mass spectrum (EI) of 2α,4-dihydroxy-1,8-cineol: m/z (relative intensity): 186 (8), 142(93), 124(19), 110(24), 109(45), 99(39), 84(40), 71(80), 69(23), 59(35), 58(42), 55(23), 43(100), 41(25).

Mass spectrum (ESI^+, MS/MS energy of collision 10V): 187(100), 172(12), 169(10), 157(35), 151(31), 123(100), 121(15), 107(26).

2.1.9 Synthesis of 7- and 9-cineolic acid

In analogy to Bull *et al.* [24], the respective hydroxycineole (3 mg, 18 mmol) was dissolved in dichloromethane (300 μL), pyridinium chlorochromate (10 mg, 46 mmol) was added and the mixture was stirred for 15 h at room temperature. The solution was diluted with 300 μL diethyl ether and filtered over Florisil. In the filtrate the respective cineolaldehyde was detected using high resolution GC (HRGC)-MS.

Mass spectrum (EI) of 9-Cineolal: m/z (relative intensity): 139 (M-HCOOH; 95), 95 (50), 71 (40), 43 (100).

Mass spectrum (EI) of 7-Cineolal: m/z (relative intensity): 168 (M; 3), 153 (100), 135 (10), 111 (15), 110(21), 93 (38), 83 (25), 81 (25), 79 (18), 69 (54), 67 (12), 59 (18), 55 (23), 43 (52), 41 (28), 39 (11).

For further oxidation to the respective acids, the solvent was removed and the residue dissolved in ethanol (200 μL). Subsequently, silver nitrate (4 mg, 23 mmol) was added followed by slow addition of aqueous KOH-solution (10%, 50 μL). Stirring was continued for 40 min and then the solution was diluted with water (300 μL), filtered to remove a black precipitate and washed with diethyl ether. The aqueous phase was then acidified with HCl (0.1 mol/L) to enable extraction of the acids with diethyl ether. The ethereal phase was dried over Na_2SO_4 and evaporated carefully to obtain 7-carboxycineole (yield 70%, 2.3 mg, 12.5 μmol) and 9-carboxycineole (yield 64%, 2.1 mg, 11.5 μmol), respectively.

2.1.9.1 7-Cineolic acid

Mass spectrum (EI): m/z (relative intensity): 169 (M-Me; 100), 151 (M-Me-H_2O; 15), 126 (25), 123 (30), 111 (20), 108 (18), 81 (40), 79 (30), 69 (53), 67 (18), 59 (23), 55 (33), 45 (34), 43 (53), 41 (30).

Mass spectrum (CI, methanol): m/z (relative intensity): 185 (M+1; 100), 167 (10), 149 (5), 139 (3), 121 (5).

Mass spectrum (ESI^+, MS/MS energy of collision 10V): 185 (M+1; 100), 167 (M+1-H_20; 80), 149 (12), 139 (M+1-HCOOH; 78), 125 (10), 121 (139- H_20; 90), 111 (16), 93 (8), 83 (13).

2.1.9.2 9-Cineolic acid

Mass spectrum (EI): m/z (relative intensity): 139 (M-HCOOH; 95), 95 (40), 71 (22), 43 (100).

Mass spectrum (CI, methanol): m/z (relative intensity): 185 (M+1; 100), 167 (10), 149 (5), 139 (3), 121 (5).

Mass spectrum (ESI^+, MS/MS energy of collision 10 V): 185 (M+1; 100), 167 (M+1-H_20; 80), 149 (12), 139 (M+1-HCOOH; 78), 125 (10), 121 (139- H_20; 90), 111 (16), 93 (8), 83 (13).

2.2 Design of the human study

The protocol of the study was approved by the Ethics Committee of the Faculty of Medicine of the Technische

Universität München (1996/07). For wash out, the volunteer (female, 26 years old, body mass index 19.2) used toothpaste devoid from terpenes according to Engel [20] and avoided spices, herbs and fruits and other foods and cosmetics containing 1,8-cineole during 3 days prior to the study. Dried sage (6.4 g) was weighed into a tea filter and was brewed with 600 mL of boiling water. After letting it steep for 15 min in a capped bottle, the filter was removed.

Blank samples from urine and blood were collected as controls before consumption of the sage tea. On an empty stomach, the volunteer drank the tea (404 g≡1017 μg 1,8-cineole) within 10 min and urine was collected at 2, 5, 7, 10, 17, 21, 28, 32, 35, 44, 50, 53, 60 and 69 h after consumption. Quantity of each sample was determined by weighing and NaN_3 (0.1%) was added for conservation. Samples were split into aliquots and stored at –70°C until analysis. Additionally, venous blood samples were taken after 0.75, 1.7, 3.25, 6.75 and 24 h using sterile 9-mL EDTA tubes (VACUETTE, Greiner Bio One). Plasma and red blood cells were separated by centrifugation (4°C, 3000 rpm, 15 min) and stored in aliquots at −70°C before analysis.

2.3 Stable isotope dilution assay (SIDA) of 1,8-cineole in tea

Aliquots of the tea infusion were weight in capped tubes, cooled to room temperature and the internal standard (IS) [9-$^2H_3$]-1,8-cineole (22.5 μg, 143 mmol) was added as ethereal solution. After stirring for 1 h, the tea was extracted with dichloromethane. The extract was dried over Na_2SO_4 and analyzed by HRGC-MS in the selected ejection chemical ionization mode. The concentration of 1,8-cineole was 2.52 ± 0.11 mg/kg, quantified by relative area counts of analyte (A) and IS in their mass traces $m/z = 137$ and $m/z = 140$, respectively, using the linear equation $y = 0.9926x + 0.0906$ ($y = \text{area}_{(IS)}/\text{area}_{(A)}$; $x = n_{(IS)}/n_{(A)}$), which was determined by analyzing definite mixtures of analyte and IS.

2.4 Analysis of hydroxycineoles in urine and plasma

2.4.1 Plasma sample preparation

To thawed plasma (1 g), acetic buffer (0.1 mol/L, pH 5, 1 mL), an aqueous solution containing the labeled compounds [9/10-$^2H_3$]-2-hydroxy-1,8-cineole and [9-^{13}C,$^2H_2$]-9-hydroxy-1,8-cineole (20–240 ng each) and β-glucuronidase (5000 units/mL sample; 1 unit liberates 1.0 μg phenolphthalein from its glucuronide *per* hour at pH 5.0 at 37°C) were added. Samples were stirred at 37°C for 15 h and then heated (100°C, 10 min) to precipitate the proteins. After centrifugation (16 000 rpm, 4°C, 15 min), the supernatant was subjected to SPE.

2.4.2 Urine sample preparation

Aqueous solutions containing the labeled compounds [9/10-$^{12}H_3$]-2-hydroxy-1,8-cineole and [9-^{13}C,$^2H_2$]-9-hydroxy-1,8-cineole (20–240 ng each), and β-glucuronidase (5000 units/mL sample; 1 unit liberates 1.0 μg phenolphthalein from its glucuronide *per* hour at pH 5.0 at 37°C) were added to thawed urine (1 g). Samples were stirred at 37°C for 15 h and after centrifugation (16 000 rpm, 4°C, 15 min), the supernatant was subjected to SPE.

2.4.3 SPE

The clear extract was loaded on SPE tubes (ENVI-18, 100 mg, Supelco; prepared with 2×1 mL methanol and 2×1 mL water) and was allowed to slowly pass through by suction (app. 1 drop/min). The protein residue was re-extracted with water/methanol (95:5, v/v, 1 mL), and the extract was also loaded on the columns. The tubes were rinsed with water/methanol (95:5, v/v, 2×1 mL) and then drawn to dryness carefully. Analytes were slowly eluted with water/ACN (50:50, v/v, 500 μL). The extracts were diluted with water (500 μL) and analyzed by LC-MS/MS.

2.4.4 LC-MS/MS

LC was performed using a Surveyor Plus HPLC system coupled to a TSQ Quantum Discovery mass spectrometer (both Thermo Finnigan, Dreieich, Germany). The stationary phase was a Polar RP-column (150×2 mm, 4 μm, 80 A, Phenomenex), which was equipped with a C18 guard column. For gradient elution, formic acid (0.1%, solvent A) and ACN (solvent B) were used at a flow rate of 0.2 mL/min. The column was equilibrated for 15 min with 20% B. After injection (full loop mode, 10 μL), solvent B was increased to 100% within 10 min and then kept for further 6 min, before it was brought back to 20% B within 1 min. In the elution range between 4 and 11 min, the column effluent was directed into the mass spectrometer. The ion source was operated in the ESI^+ mode, with a spray needle voltage of 3.5 kV and sheath and auxiliary gas at 35 and 5 arbitrary units, respectively. The source CID was adjusted to 12 V. Argon was used as collision gas at a pressure of 1.0 arbitrary units. For method development, the four OH-cineoles ($[M+1]^+$: $m/z = 171$) were analyzed with full scan in the product mode and gave $m/z = 153$ ($M+1-H_2O$) and 135 ($M+1-2H_2O$) as the most intense ions. Maximum areas were obtained with collision energies at 10 and 11 V, respectively. The labeled compounds ($[M+1]^+$: $m/z = 174$) gave the corresponding ions $m/z = 156$ and 138, for which the same collision energy was used. For MS/MS of hydroxycineoles, the mass transitions (m/z precursor ion/m/z product ion) 171/135 and 171/153 for the unlabeled and 174/138 and 174/156 for [9/10-$^2H_3$]-2-hydroxy-1,8-cineole

and [9-^{13}C,$^{2}H_2$]-9-hydroxy-1,8-cineole, respectively, were chosen. The voltages applied to the precursor ion to obtain the product ions m/z 135 or 138 and m/z 153 or 156 were 10 and 11 V, respectively. The peak width was adjusted to 0.7 full width at half-maximum, the scan time for each transition was 0.2 s and the scan width was $\pm$ 0.7 amu.

2.4.5 Calibration and calculation

For the secondary alcohols 2- and 3-hydroxy-1,8-cineole, labeled [9/10-$^{2}H_3$]-2-hydroxy-1,8-cineole served as the IS; for the primary alcohols 7- and 9-hydroxy-1,8-cineole, [9-^{13}C,$^{2}H_2$]-9-hydroxy-1,8-cineole was used. Unlabeled and labeled compounds were added to a blank sample (urine and plasma, respectively) in five different ratios ranging from 0.3 to 7. The samples were worked up as described above and analyzed by LC-MS/MS. From the relation of area ratios to molar ratios calibration curves were constructed, and the corresponding linear equation was used to calculate the concentration of the analytes in samples by considering the area ratios, added amounts of labeled standard and the sample weights.

2.4.6 Precision

Precision was checked by quintuplicate determination of the hydroxycineole content of a urine sample on two different days within 1 wk. For plasma, a quadruplicate analysis of a sample was performed.

2.4.7 Stability

Stability of the labeled compounds during work up was verified by preparing a stock solution of labeled and unlabeled compounds. Aliquots were diluted with acetic buffer (0.1 mol/L, pH 5, 1:1 v/v) and either stored at 37°C (15 and 24 h) or heated to 100°C (10 and 60 min) before LC-MS/MS. The stock solution was also analyzed directly and the resulting area ratio of analyte to standard was compared with those of the stored or heated solutions.

2.4.8 Determination of detection and quantification limits

Human blood plasma and urine, which were both devoid of the analytes under study, were used for determination of the LOD and the LOQ. The following amounts of analytes were added to the respective matrices: 2, 4, 10 and 20 μg/kg plasma for all hydroxycineoles; 1, 2, 5 and 10 μg/kg urine for 2- and 9-hydroxy-1,8-cineole, and 5, 10, 25, and 50 μg/kg urine for 2- and 9-hydroxy-1,8-cineole. Each sample was analyzed in triplicate by SIDA as described before. However, upon enzymatic hydrolysis, blank samples free from all analytes could not be obtained, thus indicating that traces cannot be eliminated by any washout protocol. To still use an authentic matrix and not a simple surrogate, we chose authentic urine and plasma, but without enzymatic hydrolysis. LOD and LOQ were determined according to the method of Vogelgesang and Hädrich [26]. LOD is the addition value referring to the 95% confidence limit of the calibration line at the zero addition level. LOQ is the addition level which lowers the 95% confidence limit to meet the upper 95% confidence limit of the addition level at the LOD.

2.4.9 Recoveries of analytes during work up

A plasma sample containing 12.9 μg/kg 2-hydroxy-1,8-cineole, 15.3 μg/kg 9- hydroxy-1,8-cineole; 10.1 μg/kg 3- hydroxy-1,8-cineole and 8.45 μg/kg 7- hydroxy-1,8-cineole was analyzed with four different approaches (duplicate each), which were varied in the point of adding the IS. The ISs were either added in the very beginning of sample work up (a), after the incubation time for glucuronide hydrolysis (bI), after the protein precipitation (bII) and after the SPE directly before LC-MS/MS analysis (bIII). The results were compared by setting approach A to 100% recovery.

2.5 Analysis of cineolic acids in urine

For LC-MS/MS analysis of cineolic acids, urine samples were prepared as described for hydroxycineoles, but prior to SPE the samples and the washing solutions were adjusted to pH 1 (HCl, 1 mol/L). Similar LC conditions were used as for the hydroxycineoles, except that the gradient elution started at 15% B for 5 min, was then raised to 100% within 15 min and kept for 2 min. Column effluent from 11 to 19 min was directed into the MS, while the most intense product ions $m/z = 121$ (CE = 14 V) and $m/z = 139$ (CE = 9 V) from parent M+1 ($m/z = 185$) were scanned.

2.6 Analysis of 1,8-cineole in urine and plasma by Solid phase microextraction (SPME)-GC-MS

2.6.1 Urine sample preparation

Thawed urine samples (10 g) were weighed into 20 mL headspace vials (VWR, Darmstadt, Germany) and the IS ([9-$^{2}H_3$]-1,8-cineol) was added in an aqueous solution ($c = 700$ ng/mL, 20 μL). Urine samples were saturated with NaCl and sealed with a septum crimp cap before being equilibrated at 55°C for at least 2 h prior to analysis. During equilibration, samples were shaken occasionally.

2.6.2 Plasma sample preparation

One gram of thawed plasma was diluted with an aqueous saturated NaCl solution (9 mL) and the IS ([$^2H_3$]-1,8-cineol) was added in an aqueous solution ($c = 700$ ng/mL, 20 μL) before the vials were sealed. Subsequently, the vials were equilibrated at 55°C by occasionally shaking for at least 2 h prior to analysis.

2.6.3 SPME-GC-MS

Analysis was performed using a Trace GC Ultra (Thermo-Quest, Dreieich, Germany), equipped with a Combi Pal autosampler and an ion trap mass spectrometer Saturn 2100 T (Varian, Darmstadt, Germany) with methanol as the chemical ionization gas. A DB 5 column (30 m × 0,32 mm, 25 μm, J&W Scientific) was used with constant flow (helium, 1.2 mL/min). For solid phase microextraction, a carboxen/polydimethylsiloxan fibre (StableFlex, d_f 85 μm; needle size 24 ga, Supelco) was used. Before each extraction, the fibre was conditioned in a needle heater (270°C, 15 min). The sample tray was heated to 55°C. The fibre was exposed to sample headspace for 13 min and then desorbed at the hot injector (250°C, splitless, 5 min). The liberated analytes were cryo-focussed in a cold trap 915 cooled with liquid nitrogen to 150°C. At the end of the desorption time, the trap was heated (15°C/min, 250°C) and the GC run started with an oven temperature of 40°C (1 min). The temperature was raised to 86°C (3°C/min) and then to 240°C (40°C/min, 3 min).

For quantification, the respective mass traces given in parentheses were used: 1,8-cineole (m/z 137) and [9-$^2H_3$]-1,8-cineole (m/z 140).

2.6.4 Calibration and calculation

For all described hydroxycineoles, the respective unlabeled and labeled compound were added to a saturated NaCl solution (10 mL) in four different ratios ranging from 0.5 to 5, and analyzed as described above. From the relation of area ratios to molar ratios calibration curves were constructed, and the corresponding linear equation was used to calculate the concentration of the analytes in samples by considering the area ratios, added amounts of labeled standard and the sample weights.

2.7 Further validation studies

Precision was checked by quintuplicate determination of the 1,8-cineole content of an urine sample on two different days within 1 wk.

Stability of the labeled compounds during analysis was verified by repeating the calibration analysis with the solutions being stored at 55°C for 24 h prior to analysis.

Detection and quantification limits were determined according to Vogelgesang and Hädrich [26]. 1,8-Cineole was added to saturated NaCl solution in four different amounts ranging from 133 to 1330 ng/L, triplicate each, and analyzed as described above.

2.8 NMR Spectroscopy

^{1}H-NMR and ^{13}C-spectra were recorded on a Bruker AMX 400 (Bruker, Karlsruhe, Germany) at 297 K in $CDCl_3$ (unless stated otherwise) with TMS as IS ($\delta = 0$ ppm).

2.9 HRGC/high resolution MS

HRGC was performed by means of a type 5300 gas chromatograph (Carlo Erba, Hofheim, Germany) using capillary DB-5 (30 m × 0.32 mm fused-silica capillary, film thickness of the stationary phase $d_f = 0.25$ μm; Fisons Instruments, Mainz, Germany). The samples were applied by the cold on-column technique at 40°C. One minute after injecting the sample, the temperature of the oven was raised to 250°C by a rate of 10°C/min. The flow rate of the carrier gas helium was 2 mL/min.

High resolution mass spectra were recorded by means of an MAT 95 S (Finnigan MAT, Bremen, Germany) coupled to capillary DB-5 at a resolution of 5000 using perfluorocerosene for calibration.

2.10 HRGC-MS

A Varian 3000GC, equipped with a DB-FFAP column (30 m × 0.32 mm, 25 μm film; J&W Scientific), a Combi PAL autosampler and a Saturn 2000 mass spectrometer was used. Helium served as the carrier gas and methanol for selected ejection chemical ionization. Samples (2 μL) were injected on column at 40°C. After 2 min, the temperature was first raised to 70°C (5°C/min) and finally to 250°C (40°C/min, 5 min).

3 Result and discussion

3.1 Qualitative analysis of 1,8-cineole metabolites in humans

Despite its frequent pharmacologic and flavoring use, knowledge on *in vivo* metabolism of 1,8-cineole, particularly in humans, is scarce. In the early 1990s, first studies in brushtail possums revealed monohydroxylated derivatives as the main metabolites [27], *i.e.* the mainly the primary alcohols 9-hydroxyl-1,8-cineole (Fig. 1) along

with 7-hydroxy-1,8-cineole and the secondary alcohols 2-hydroxy-1,8-cineole (both α- and β-isomers) and 3-hydroxyl-1,8-cineole (both α- and β-isomers). Besides these, the dihydroxylated derivatives, cineolic acids and hydroxycineolic acids have been identified as cineole metabolites [6, 7].

Due to eucalyptus leaves being their main diet, possums and koalas are adopted to high intake of 1,8-cineole. However, for other species, particularly for humans, the spectrum of metabolites might be completely different. Indeed, up to now, only 2- and 3-hydroxy-1,8-cineole have been detected in mammals, and additionally 9-hydroxy-1,8-cineole in insects and human liver preparations as well as 7-hydroxy-1,8-cineole in rats treated with phenobarbital [28]. Likewise, human *in vivo* studies revealed only 2- and 3-hydroxy-1,8-cineole in urine after pharmacological doses of a cold medication [8]. In all studies up-to-date, urine had to be subjected to glucuronidase treatment as the metabolites mainly were found as glucuronides and not in their free form. As we applied a low dose of 1.02 mg 1,8-cineole *via* herbal tea, we expected only to detect the two secondary alcohols 2- and 3-hydroxy-1,8-cineoles in the urine of the volunteer. For a sensitive and unequivocal confirmation by LC-MS/MS or GC-MS, the access to the pure reference compounds is inevitable. Therefore, we synthesized 2-hydroxy-1,8-cineole by epoxidation of α-terpineol and subsequent ring closure upon acid catalysis. The 3-isomer was generously provided by Dr. Carman, Australia. After tuning our mass spectrometers for these substances, we indeed detected both metabolites in the urine after liberation from the respective glucuronides.

Identification of the hydroxycineoles was rather straightforward as LC-MS/MS of urine gave unequivocal signals in the highly specific MS/MS mode at the identical retention time of the reference compounds.

However, the mass traces in LC-MS/MS revealed two additional compounds with the same molecular ion at *m/z* 171 and the common MS/MS signals at *m/z* 153 and *m/z* 135 of hydroxy-1,8-cineoles. Our assumption was that these additional peaks may be assigned to the primary alcohols 7- and 9-hydroxy-1,8-cineole, which required confirmation by synthesis and analysis of the respective reference compounds. Therefore, on the one hand, 7-hydroxy-1,8-cineole was prepared by bromination of β-pinene and subsequent elimination of hydrobromide to give δ-terpineol, which was then epoxydated using *meta*-chloroperbenzoic acid followed by ring closure upon catalysis with *para*-toluenesulfonic acid. On the other hand, 9-hydroxy-1,8-cineole was obtained by dihydroxylation of limonene to uroterpenol and subsequent ring closure.

With access to these reference compounds, we were able to confirm the identity of 7-hydroxy-1,8-cineole and 9-hydroxyl-1,8-cineole by LC-MS and GC/MS in human urine after application of 1,8-cineole *via* herbal tea.

Due to the unprecedented identification of all four hydroxycineoles in humans *in vivo*, we also tested for dihydroxycineoles and the cineloic acids. In analogy to the hydroxycineoles, we first synthesized 2α,4-dihydroxy-1,8-cineole, 7-cineolic acid and 9-cinelic acid as reference compounds. However, we were neither able to detect any dihydroxymetabolite nor cineolic acids in human urine samples.

3.2 Syntheses of isotope labeled 1,8-cineole and its metabolites

From the detection of the four hydroxy-1,8-cineoles in human urine, the question arose whether these metabolites are also detectable in blood plasma and in which relative amounts they and the parent substance 1,8-cineole are excreted. For their sensitive detection and due to frequent interferences in clinical matrices, the development of SIDAs was inevitable. This kind of assay is based on the addition of stable isotopically labeled analogues of the analytes to the sample prior to extraction. Because of their structural similarity to the analytes, isotopologues show best accordance of chemical and physical properties. Therefore, losses during extraction, clean-up or detection are best compensated for.

For analysis of a series of structurally different isomers such as the hydroxy-1,8-cineoles, the most accurate way is a multi SIDA by application of a stable isotopologue for each analyte. For 1,8-cineole, the [$^2H_3$]-isotopologue was easily accessible by introducing a [$^2H_3$]-label *via* Grignard reaction starting from limonaketone and ring closure of the intermediate [9-$^2H_3$]-α-terpineol (2) by phenylselenation (Fig. 2).

In accordance with the incorporation of the three deuterium labels, the EI mass spectrum of labeled 1,8-cineole revealed a signal at *m/z* 157 for the molecule ion compared to *m/z* 154 from the unlabeled compound. As expected, elimination of a methyl group from labeled 1,8-cineole gave two signals at *m/z* = 139 ($M^{+\bullet} - CD_3$) and *m/z* = 142 ($M^{+\bullet} - CH_3$), respectively, with similar intensity. High resolution MS even revealed two different signals at *m/z* = 139 for the loss of water and the [$^2H_3$]-methyl group, respectively. Elimination of C_2H_5 and C_3H_7 gave signals at *m/z* = 128 ($C_8H_{10}D_3O^{+\bullet}$) and 114 ($C_7H_8D_3O^{+\bullet}$), which are not present in the mass spectrum of the unlabeled compound, but correspond to the respective signals at *m/z* = 125 and 111. Also the signals *m/z* = 87 ($C_5H_5D_3O^{+\bullet}$), *m/z* = 72 ($C_5H_6D_3^{+\bullet}$ or $C_{4H_2}D_3O^{+\bullet}$) and *m/z* = 46 ($C_3H_4D_3^{+\bullet}$) reveal corresponding signals at *m/z* = 84, 69 and 43, respectively, in the spectrum of the unlabeled cineole and indicate the presence of the three deuterium labels.

However, for the four hydroxy-1,8-cineoles, only two of them, *i.e.* the 2- and the 9-isomer, were reasonably accessible as labeled analogues. As the other two, the 3- and the 7-isomer, would have required intense synthetic work, we decided to synthesize at first the two former ones and to test

the labeled 2-hydroxy-1,8-cineole as standard for both secondary hydroxyls, *i.e.* the 2-hydroxy and 3-hydroxy isomer, and the labeled 9-hydroxyl-1,8-cineole as standard for the primary hydroxyls, the 7-hydroxy and 9-hydroxy isomer. In that way, we synthesized [9/10-$^2H_3$]-2-hydroxy-1,8-cineole starting from the before mentioned [9-$^2H_3$]-α-terpineol (2) following the route for unlabeled 2-hydroxy-1,8-cineole (Fig. 3).

The identity of the labeled compound was unequivocally confirmed by its MS/MS spectrum of the protonated molecule ion at *m/z* 174 showing two signals at *m/z* 156 and *m/z* 138, which is in accordance with the introduction of the three deuterium labels (Fig. 4).

9-[^{13}C,$^2H_2$]-9-Hydroxy-1,8-cineole was prepared by introducing a [^{13}C,$^2H_2$]-label *via* Wittig reaction into [^{13}C,$^2H_2$]-limonene and treating the latter in an analogous way to the unlabeled compound (Fig. 5).

Analogously to labeled 2-hydroxycineole, the [^{13}C,$^2H_2$]-label was clearly visible in the MS/MS spectrum showing two product ions at *m/z* 156 and *m/z* 138 (Fig. 6).

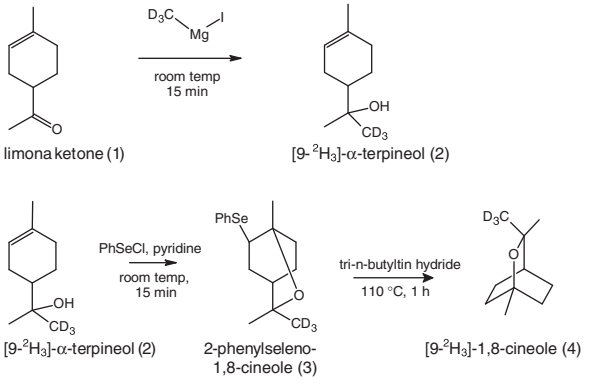

Figure 2. Synthetic pathway leading to [9-$^2H_3$]-1,8-cineole (4).

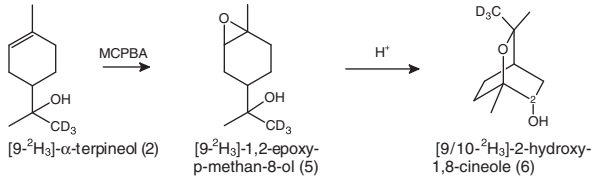

Figure 3. Synthetic pathway leading to [9/10-$^2H_3$]-2-hydroxy-1,8-cineole (6).

3.3 Development of SIDAs for 1,8-cineole by SPME-GC-MS

For the detection of 1,8-cineole in plasma and urine, we found GC-MS more sensitive compared to LC-MS. In preliminary experiments for isolating the odorant, we tested solvent-assisted flavor evaporation [29] prior to GC-MS. This cleanup worked well for urine samples, but for plasma the sensitivity was insufficient as sample volume for blood is restricted. Therefore, we applied SPME for isolation, which resulted in increased sensitivity and enabled us also to quantify 1,8-cineole in plasma. As SPME is known to be affected by matrix effects, we used [$^2H_3$]-1,8-cineole as the IS to compensate for any kind of interferences.

For calibration, a set of analyte/standard mixtures was analyzed to convert area ratios A_A/A_S of analyte and IS to

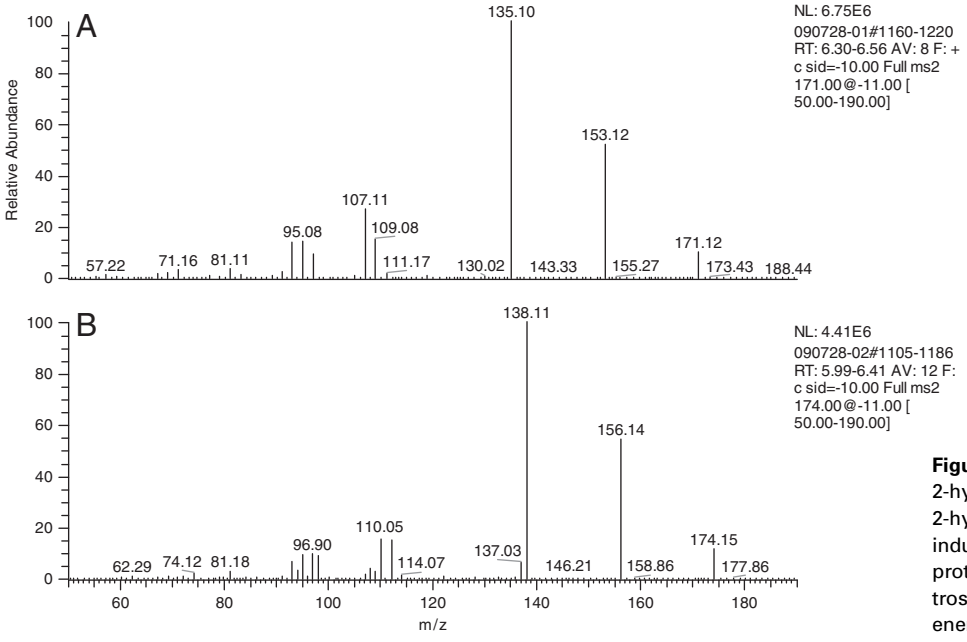

Figure 4. LC-MS/MS spectrum of (A) 2-hydroxy-1,8-cineole and (B) [9/10-$^2H_3$]-2-hydroxy-1,8-cineole after collision-induced dissociation (CID) of the protonated molecules in positive electrospray ionization mode at a collision energy of 11 V.

molar ratios n_A/n_S. Thus, we obtained as response equation $A_{St}/A_A = 1.0772\ n_{St}/n_A + 0.0458$.

3.4 Development of SIDA for hydroxy-1,8-cineoles by LC-MS/MS

During LC-MS/MS, the unlabeled hydroxycineoles revealed the same MS/MS transition from the protonated molecular ion at m/z 171 and the most intense product ions at m/z 153 and m/z 135 corresponding to a sequential loss of two molecules of water. In contrast to this, labeled 2-hydroxycineole and labeled 9-hydroxycineole gave the transitions of m/z 174 to m/z 156 and to m/z 138, which enabled unequivocal differentiation from the unlabeled compounds.

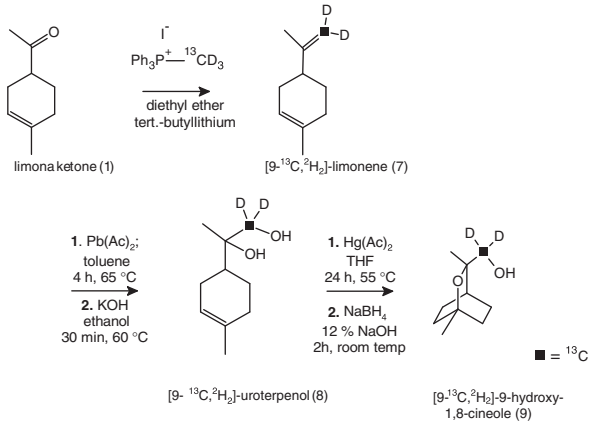

Figure 5. Synthetic pathway leading to [9-^{13}C,^{2}H$_2$]-9-hydroxy-1,8-cineole (9).

For quantitation of all cineoles, chromatographic separation of all isomers is inevitable as they do not differ in their MS/MS spectra. The separation was achieved by a formic acid/ACN gradient on a polar endcapped reversed phase (RP) as shown in Fig. 7, which presents an example of the urinary LC-MS/MS chromatogram showing all four hydroxycineoles and the respective two isotopically labeled isotopologues.

To convert area ratios A_{St}/A_A of analytes and the respective standards to molar ratios n_{St}/n_A, we analyzed different mixtures of the four hydroxycineoles with their respective ISs in blank urine and blank plasma and constructed response curves for the respective matrices. For 2-hydroxy-1,8-cineole and 3-hydroxy-1,8-cineole, relative to [9/10-^{2}H$_3$]-2-hydroxy-1,8-cineole as IS, we obtained the equations $A_{St}/A_A = 0.7964\ n_{St}/n_A\ 0.0026$ and $A_{St}/A_A = 1.6274\ n_{St}/n_A + 0.0096$, respectively, in urine and $A_{St}/A_A = 0.8628\ n_{St}/n_A + 0.0483$ and $A_{St}/A_A = 1.0345\ n_{St}/n_A\ 0.1341$, respectively, in plasma.

For 7-hydroxy-1,8-cineole and 9-hydroxy-1,8-cineole, relative to [^{13}C,^{2}H$_2$]-9-hydroxy-1,8-cineole as IS, we obtained the equations $A_{St}/A_A = 1.202\ n_{St}/n_A + 0.1406$ and $A_{St}/A_A = 0.8962\ n_{St}/n_A\ 0.0117$, respectively, for urine and $A_{St}/A_A = 0.8943\ n_{St}/n_A + 0.2021$ and $A_{St}/A_A = 0.8334\ n_{St}/n_A\ 0.0023$, respectively, for plasma. Expectedly, the equations for urine and plasma were quite identical for 2-hydroxy-1,8-cineole and 9-hydroxy-1,8-cineole, whereas they differed for urine and plasma in the case of 3-hydroxy-1,8-cineole and 7-hydroxy-1,8-cineole. This effect for the latter metabolites obviously was due to their structural differences to the ISs [9/10-^{2}H$_3$]-2-hydroxy-1,8-cineole and [^{13}C,^{2}H$_2$]-9-hydroxy-1,8-cineole.

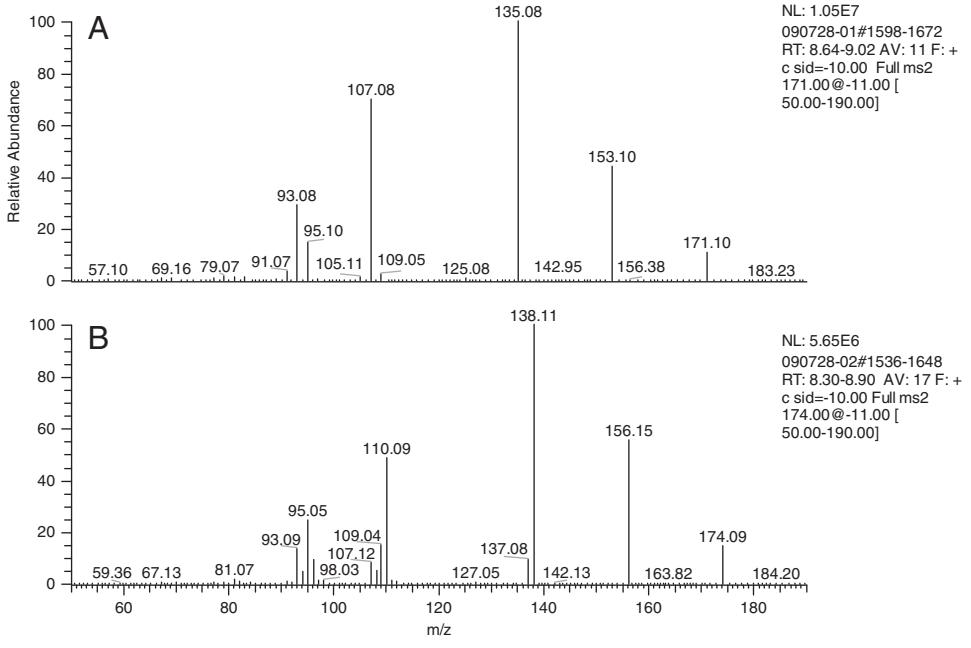

Figure 6. LC-MS/MS spectrum of A. 9-hydroxy-1,8-cineole and B. [9-^{13}C,^{2}H$_2$]-9-hydroxy-1,8-cineole after collision-induced dissociation (CID) of the protonated molecules in positive electrospray ionization mode at a collision energy of 11 V.

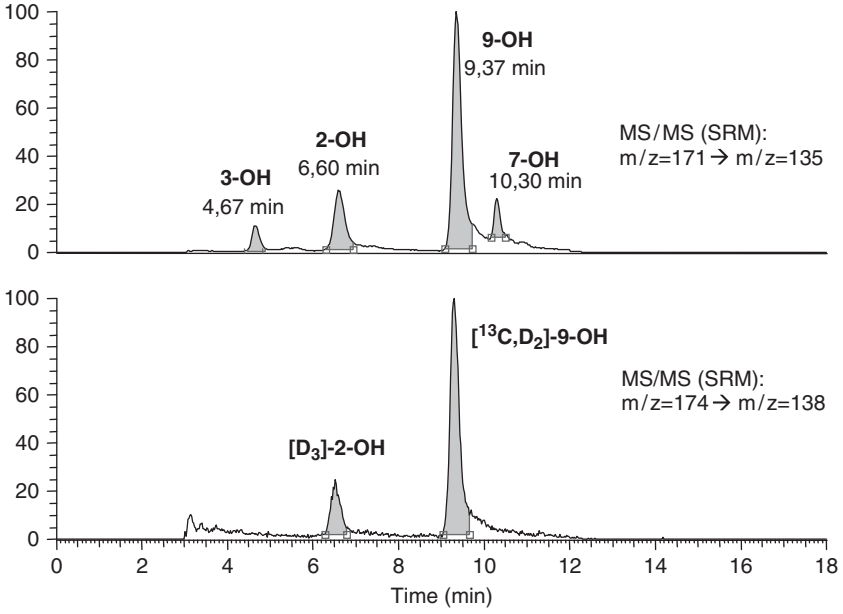

Figure 7. LC-MS/MS chromatogram of an urine sample containing 2-hydroxy-1,8-cineole, 3-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, and 9-hydroxy-1,8-cineole. [9/10-$^{2}H_3$]-2-Hydroxy-1,8-cineole and [9-^{13}C,$^{2}H_2$]-9-hydroxy-1,8-cineole were used as the internal standards. Measurements were performed in selected reaction monitoring (SRM).

In previous reports [6, 8, 30], hydroxycineoles were liberated in higher amounts when applying enzymatic incubation with β-glucuronidase to urine of animals or human. As hydroxycineoles also might be conjugated with sulfuric acid, a mixture of β-glucuronidase and sulfatase was also tested. The amount of enzymatic solution was chosen as previously reported by Zeller *et al.* [4]. However, incubations either with combined β-glucuronidase and sulfatase or with pure β-glucuronidase did not differ in liberated hydroxycineoles. Therefore, glucuronidation is the major phase II reaction.

Deconjugation was applied for both the plasma and urine samples followed by SPE clean-up.

For monitoring the hydroxycineoles in plasma and urine, we developed clean-up procedures for both matrices, which were based on SPE on RP 18 sorbents and from which the analytes were eluted by ACN/water. Mixtures in plasma, proteins were precipitated by heating after addition of the IS and deconjugation. For urine, the procedure was simpler since the heating step could be omitted.

As cleanup included a glucuronidase treatment at 37°C and protein precipitation at 100°C, the stability of the analyte/standard ratio during these treatments has to be tested. Therefore, we incubated various mixtures of analytes and standards at pH 5 at 37°C for 15 and 24 h as well as at 100°C for 10 min and for 1 h. We compared analyte/standard ratios before and after the treatments. For all analyte/standard combinations, no significant differences ($p > 0.05$) were found except for 7- hydroxy-1,8-cineole at 37°C, which showed lower stability than the respective standard [9/10-$^{2}H_3$]-2-hydroxy-1,8-cineole. However, this discrimination was compensated for by response experiments, in which the calibration mixtures were treated the same.

3.4.1 Validation

3.4.1.1 Precision

Repeatability (intra-assay precision) and reproducibility (inter-assay precision) was evaluated by analyzing urine and plasma samples from the human study twice in multiplicate during 3 wk. The coefficients of variation for intra-assays ($n = 5$) ranged from 2.1% for 2-hydroxycineole to 19.4% for 7-hydroxycineole and for inter-assay precision from 2.9% for 2-hydroxycineole to 15.4% for 7-hydroxycineole. These results are well in line with the structural properties of the respective IS, which is identical for 2-hydroxycineole and only similar for 7-hydroxycineole.

3.4.1.2 Recovery

Control plasma and urine were spiked (each in triplicate) with unlabeled cineole and hydroxycineoles above their respective LODs. Recovery was determined (a) for the whole SIDAs and (b) after different steps of the sample cleanup. For (a), SIDAs were performed as detailed before and recoveries in urine were calculated to be 100.8, 99.4, 106.9, 100.5 and 103.1% for 2-hydroxy-1,8-cineole, 3-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, 9-hydroxy-1,8-cineole and 1,8-cineole, respectively. In plasma, the recoveries for (a) were 90.9, 96.1, 90.0, 100.5 and 103.1% for 2-hydroxy-1,8-cineole, 3-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, 9-hydroxy-1,8-cineole and 1,8-cineole, respectively. The addition levels in the recovery studies were approximately twice to threefold the LOQ. For the evaluation of possible sources of losses during the whole assay, sample workup was started and the labeled standards were added at different steps: (bI) after glucuronidase treatment at 37°C for 15 h, (bII) after protein

precipitation and centrifugation and (bIII) after SPE cleanup. The differences to 100% uncover losses of the analytes in all steps, which are performed before the standards were added. The results are presented in Table 1 and indicate that losses before protein precipitation (experiment bI) are less than 12%, before SPE (experiment bII) are less than 20% and that the main losses occur during SPE (experiment bIII).

3.4.1.3 LOD and LOQ

To determine the LOD and LOQ, we applied the calibration procedure proposed by Vogelgesang and Hädrich [26]. The respective matrix devoid of the analytes was spiked with increasing amounts of analytes and ISs prior to analyzes. LODs in urine for 2-hydroxy-1,8-cineole, 3-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole and 9-hydroxy-1,8-cineole were 1.4, 3.3, 1.2 and 0.9 μg/kg and LOQs were 4.2, 15.6, 3.7 and 2.8 μg/kg, respectively. In plasma, LODs were 2.8, 1.1, 2.0 and 1.6 μg/kg and LOQs were 8.1, 5.0, 6.0 and 4.9 μg/kg, respectively. For 1,8-cineole, the LOD (0.7 μg/kg plasma, 0.07 μg/kg urine) and LOQ (2.1 μg/kg plasma, 0.2 μg/kg urine) were decisively lower because of the more sensitive detection by SPME-GC-MS.

3.5 Application of SIDAs in a pilot human study

One goal of the present study was to investigate 1,8-cineole metabolism of ingestion-correlated amounts [3] occurring after consumption of spices or herbal teas. Of all 1,8-cineole containing spices including laurel, rosemary, peppermint, basil and sage, we chose the latter as it was likely to provide the highest dose among all of these foods. To enable a realistic dietary intake of 1,8-cineole, we prepared a sage tea containing 2.5 mg 1,8-cineole *per* kg, of which the volunteer drank 400 g.

Our first control samples prior to consumption of the sage tea, however, revealed a significant background of cineole metabolites, which presumably originate from the ubiquitous occurrence of this terpene in cosmetics and foods. Therefore, our volunteer was forced to avoid all foods and cosmetics containing 1,8-cineole, which required abstinence to all spices and commercial tooth pastes and detergents. The latter products, therefore, had to be prepared in our laboratory for this single use.

Table 1. Absolute recoveries of 1,8-cineole metabolites after different steps of the analytical procedure: (bI) after glucuronidase treatment at 37°C for 15 h; (bII) after protein precipitation and centrifugation; and (bIII) after SPE cleanup

Recovery	After step		
	bI (%)	bII (%)	bIII (%)
2-Hydroxy-1,8-cineole	97.8	89.8	84.9
3-Hydroxy-1,8-cineole	100.4	97.1	94.4
7-Hydroxy-1,8-cineole	88.0	79.1	74.4
9-Hydroxy-1,8-cineole	98.2	87.6	76.6

The concentration determined by stable isotope dilution assays was set to 100%.

After consumption of 1.02 mg 1,8-cineole (19 μg/kg bw), the metabolites 2-hydroxy-1,8-cineole, 3-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, and 9-hydroxy-1,8-cineole along with their parent compound were detectable in the blood plasma of the female volunteer under study after liberation from their glucuronides. All compounds peaked in plasma after 0.75 h with 2-hydroxycineole being the predominant metabolite at a plasma concentration of 86 nmol/L followed by the 9-hydroxy isomer at a plasma concentration of 33 nmol/L. The 7- and the 3-isomer were detectable, but their plasma concentrations were below their LOQ. The parent compound 1,8-cineole showed a low plasma concentration of 19 nmol/L, thus indicating that its metabolism occurs very fast and effective. Assuming the volunteer's plasma volume of 2.4 L [31], the sum of all metabolites and the parent compound appearing in plasma was quite low being less than 7% at t_{max}. In plasma samples drawn later than 0.75 min after application, neither 1,8-cineole nor its metabolites were above their LOQ.

In contrast to blood, hydroxy-1,8-cineoles were higher abundant in urine showing highest contents during the first 2 h. In accordance with the plasma levels, 2-hydroxycineole showed highest contents in urine followed by its 9-isomer. However, in contrast to the plasma contents, 3-hydroxycineole was more abundant in urine than the 7-isomer. Summing up the urinary excretion over 10 h, 52.5% of the 1,8-cineole dose was identified as metabolites, of which 2-hydroxycineole, the 9- isomer, the 3-isomer and the 7-isomer accounted for 20.9%, 17.2%, 10.6% and 3.8%, respectively. After 10 h, only traces of metabolites could be detected.

In agreement with previous reports [11, 30], we could confirm the predominant formation of 2-hydroxy-1,8-cineol as the main metabolite of 1,8-cineole in humans. Moreover, the formation of the 3- and 9-isomer was also confirmed [30]. The 7-isomer, which up to date only has been identified in brushtail possums and koala [24, 30], was for the first time identified in human urine and plasma. Further metabolites such as dihydroxycineols or cineolic acids were not detected and are obviously formed only in animals consuming high amounts of 1,8-cineole such as brushtail possums or koala.

4 Concluding remarks

In the present *in vivo* study, the human metabolism of 1,8-cineole is reported for the first time after consumption of amounts endogenously occurring in foods. The metabolism was found to occur very fast within the first hour after consumption and gave rise to four hydroxycineols, of which the 7-isomer was identified for the first time in humans.

Regarding the sum of metabolites, the fate of 47.5% of the dose is still unknown. Parts of this missing amount may include non-absorbed fractions of 1,8-cineole in the gastrointestinal tract. Moreover, further routes of metabolism could include excretion *via* bile into feces or *via* exhalation of the parent compound or as carbon dioxide.

Up to date, the antiasthmatic and antiallergic effect of 1,8-cineole mainly has been assigned to the parent compound, but as metabolism occurs fast, it may also be attributed to the high amounts of metabolites formed. However, up to date these were not subjected to bioactivity assays [32], and, therefore, this question remains open.

Moreover, the present study was intended to develop analytical methods, which were then applied to only one human. As metabolism can vary decisively among individuals, a higher number of volunteers is necessary for obtaining representative results. These studies are currently under way.

The provision of 3α-hydroxy-1,8-cineole from Craig J. Wallis/ R. M. Carman, University of Queensland, Brisbane, Australia, is greatly acknowledged.

The authors have declared no conflict of interest.

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