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Syntheses of Chiral 1,8-Cineole Metabolites and Determination of Their Enantiomeric Composition in Human Urine After Ingestion of 1,8-Cineole-Containing Capsules

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The chiral metabolites in human urine were investigated after ingestion of a 1,8-cineole (eucalyptol)-containing enterocoated capsule (Soledum). For identification of the various enantiomers the enantiomerically pure (−/+)-α2-hydroxy-1,8-cineole, (−/+)-β2-hydroxy-1,8-cineole, (−/+)-9-hydroxy-1,8-cineole, and (−/+)-2-oxo-1,8-cineole were prepared. To achieve this aim, after acetylation of the synthesized racemic 2- and 9-hydroxy-1,8-cineoles, pig liver esterase- or yeast-mediated hydrolysis provided the (−)-alcohols with their antipodal (+)-acetates with enantiomeric excess of 33–100%. Dess–Martin periodinane oxidation of the alcohol (+)-α2-hydroxy-1,8-cineole, obtained by hydrolysis of the resolved acetate, provided the corresponding (+)-2-oxo-1,8-cineole, meanwhile the oxidation of (−)-α2-hydroxy-1,8-cineole gave (−)-2-oxo-1,8-cineole. Using these standards seven metabolites (+/−)-α2-hydroxy-1,8-cineole, (+/−)-β2-hydroxy-1,8-cineole, (+/−)-α3-hydroxycineole, (+/−)-3-oxo-1,8-cineole, 4-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, and (+/−)-9-hydroxy-1,8-cineole, all liberated from their glucuronides, were identified in urine by GC-MS on a chiral stationary phase after consumption of 100 mg of 1,8-cineole. Metabolite screening using $^{2}H_{3}$-1,8-cineol as the internal standard revealed (+/−)-α2-hydroxy-1,8-cineole as the predominant metabolite followed by (+/−)-9-hydroxy-1,8-cineole. Furthermore, the results showed that one enantiomer is always formed preferentially.

Introduction

The monoterpenoid 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo-[2.2.2]octane), commonly known as eucalyptol, is the main component of eucalyptus essential oil and is abundant in several plant species. Because of its fresh and menthol-like odor it is used for flavoring foods and cosmetics. Furthermore, 1,8-cineole is applied in pharmaceutical preparations to treat cough, bronchitis, asthma, and muscular pain.[1]

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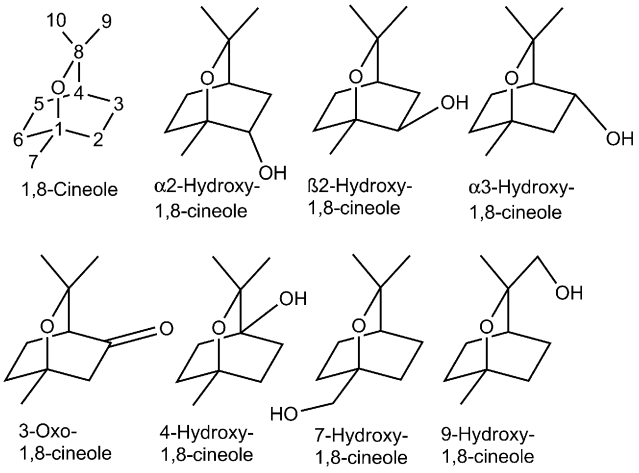
The biotransformation of 1,8-cineole has been studied extensively in brushtail possum (*Trichosurus vulpecula*) and koala. The main metabolites in these species were the hydroxycineolic acids, cineolic acids, and diols with preferred location for the oxidation in position-9 and -7. In minor concentrations also some hydroxycineoles were found: α2-hydroxy-1,8-cineole, β2-hydroxy-1,8-cineole, α3-hydroxy-1,8-cineole, β3-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, 9-hydroxy-1,8-cineole.[2,3,4] After administration of 1,8-cineole to rabbits, which normally do not consume eucalyptus in their regular diet, α/β2-hydroxy-1,8-cineole and α/β3-hydroxy-1,8-cineole were identified. Higher oxidized products were not found.

The metabolism of 1,8-cineole was also investigated in in vitro assays involving human liver microsomes. Mainly the enzymes CYP3A4 and CYP3A5 catalyzed the oxidation to α2- and α3-hydroxy-1,8-cineole.[5]

Only recently the metabolites of 1,8-cineole have also been studied in humans. Horst and Rychlik[6] identified the four metabolites α2-hydroxy-1,8-cineole, α3-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, and 9-hydroxy-1,8-cineole in blood and urine after ingestion of sage tea. The metabolites were quantified after liberation from their glucuronides using stable isotope dilution assays. Also, for the first time, the metabolites of cineole in human milk were examined in another study; thereby, milk samples were analyzed after lactating mothers ingested the non-prescription pharmaceutical Soledum (Klosterfrau Health-



care Group, Cologne, Germany) containing 100 mg of 1,8-cineole. In addition to the previously known human hydroxylated metabolites α2-hydroxy-1,8-cineole, β2-hydroxy-1,8-cineole α3-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, and 9-hydroxy-1,8-cineole, five additional new human metabolites could be identified: 4-hydroxy-1,8-cineole, 2-oxo-1,8-cineole, 3-oxo-1,8-cineole, 2,3-dehydro-1,8-cineole and 2,3-α-epoxy-1,8-cineole.

Nevertheless, in all previous studies until today, no distinction was made between the respective enantiomeric forms of the metabolites. Accordingly, on the basis of the synthesized racemic reference substances, the results were represented as the sum of both enantiomers in each case.

However, for determining the enantiomeric ratio of the metabolites the enantiopure references are required. As these references are not commercially available, this involves extensive synthetic and preparative work. Previously, Luzzio and Duveau[7] prepared for the first time (1*R*,3*R*,4*S*)-(−)-3-hydroxy-1,8-cineole by pig liver esterase (PLE)-mediated hydrolysis of the racemic acetate (+/−)-β3-acetyloxy-1,8-cineole in an enantiomeric purity exceeding 99%. After hydrolysis of the antipodal (1*S*,3*S*,4*R*)-(+)-3-acetyloxy-1,8-cineole the two enantiopure β3-hydroxy-1,8-cineoles were oxidized using pyridinium chlorochromate (PCC) to give (1*R*,4*R*)-(+)-3-oxo-1,8-cineole and (1*S*,4*S*)-(−)-3-oxo-1,8-cineole.[8]

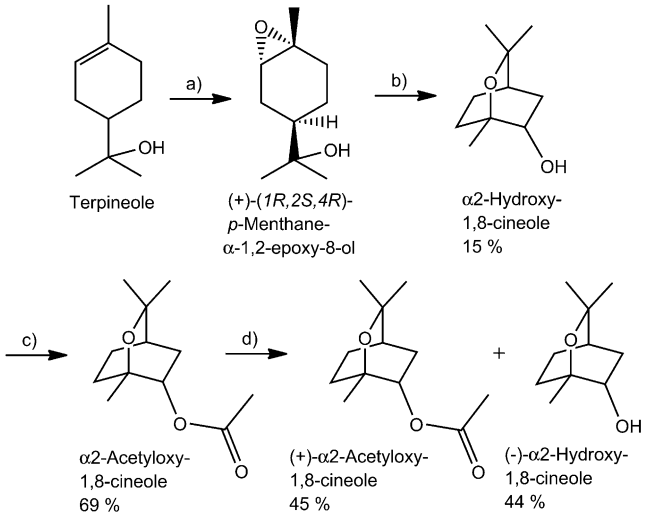
Nevertheless, no other previous study was targeted at determining the natural ratios of cineole metabolites being formed in humans. Accordingly, in the present study, the enantiopure references of α2-hydroxy1,8-cineole, β2-hydroxy1,8-cineole, 2-oxo-cineole, and 9-hydroxy-1,8-cineole were synthesized by referring to or by adapting and optimizing previously described methods.

Using these references, an initial screen was performed to characterize the metabolism profile of 1,8-cineole and the enantiomeric ratios of its metabolites in human urine after ingestion of one Soledum capsule by application of gas chromatography-mass spectrometry on a chiral stationary phase.

Results and Discussion

Synthesis of (−)- and (+)-α2-hydroxy-1,8-cineole

The synthetic sequence commences with epoxidation of the commercially available α-terpineol mediated by *m*-chloroperbenzoic acid as shown in Scheme 1. After a reaction time of 24 hours, upon acid catalysis and ring closure to the [2,2,2]-oxabicyclooctane skeleton, the racemic (−/+)-α2-hydroxy-1,8-cineole was obtained in 15% yield after purification by chromatography on silica gel. Treatment of this alcohol with acetyl chloride in the presence of 4-dimethylaminopyridine (DMAP) in dichloromethane provided the corresponding racemic acetate (−/+)-α2-acetyloxy-1,8-cineole in 69% yield after purification by flash chromatography on silica gel. Inspired by the stereospecific synthesis of (−)- and (+)-β3-hydroxy-1,8-cineole by Luzzio and Duveau[7] we selected a porcine liver esterase (PLE)-mediated desymmetrization of the secondary acetate. After incubation of (−/+)-α2-acetyloxy-1,8-cineole with the commercially available porcine liver esterase (EC 3.1.1.1) for 16 hours at 37 °C afforded a chromatographically separable mixture of (−)-α2-hydroxy-1,8-cineole (43%) and (+)-α2-acetyloxy-1,8-cineole (45%). After separation by flash chromatography the remaining (+)-acetate was saponified with lithium hydroxide at room temperature and provided (+)-α2-hydroxy-1,8-cineole (44%). To determine the enantiomeric purity the products were analyzed on two gas chromatography capillary columns with chiral stationary phases of βDEX-sm and γDEX-sa. The enantiomeric excess of 98% for (−)-α2-hydroxy-1,8-cineole indicated that the PLE is selective for the (−)-ester according to many similar types of substrates.[7] However, the substrate was not fully hydrolyzed showing an enantiomeric excess of just 78% for (+)-α2-hydroxy-1,8-cineole. Nevertheless, the PLE-mediated

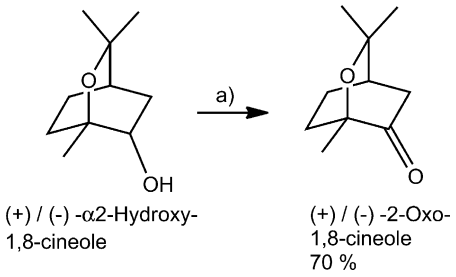


Scheme 1. Synthesis of (−)-α2-hydroxy-1,8-cineole: a) *m*CPBA, CH_2Cl_2, 0 °C, 2 h; b) *p*-toluenesulfonic acid, CH_2Cl_2, RT, 24 h; c) Acetyl chloride, DMAP, CH_2Cl_2, 0–5 °C; d) PLE, pH 7, 12 h, 37 °C.

enantioselective hydrolysis proved to be an effective possibility for the synthesis of enantiopure α2-hydroxy-1,8-cineole.

Synthesis of (−)- and (+)-2-oxo-1,8-cineole

Oxidation of (−)-α2-hydroxy-1,8-cineole using Dess–Martin periodinane cleanly provided the ketone (−)-2-oxo-1,8-cineole with 70% yield (96% *ee*), while the oxidation of (+)-α2-hydroxy-1,8-cineole gave the corresponding (+)-2-oxo-1,8-cineole (77% *ee*) with 71% yield after purification by a short pad of silica gel (Scheme 2). The enantiomeric excess corresponded to



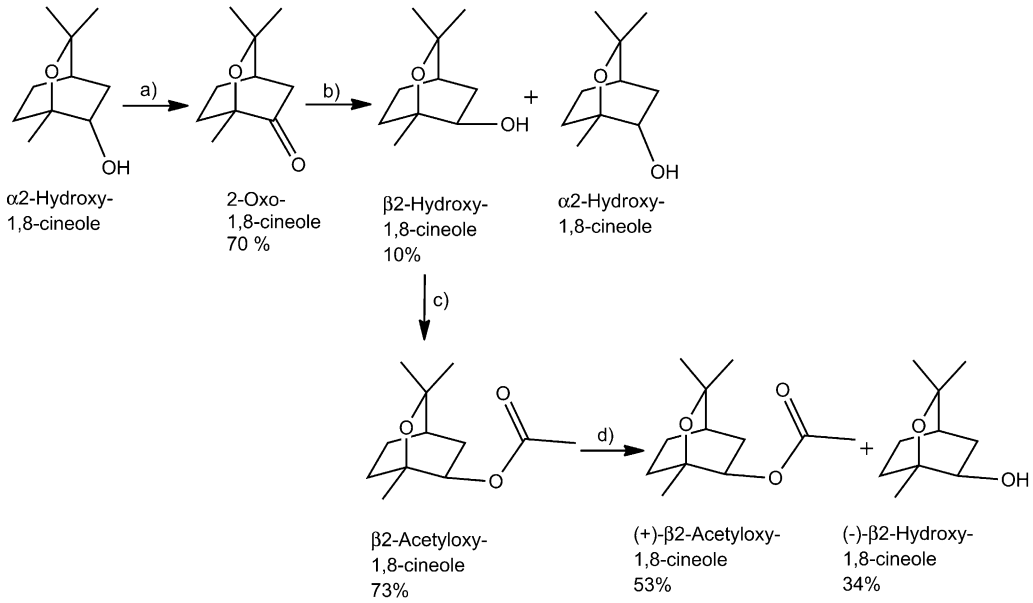
Scheme 2. Synthesis of (+)-2-oxo-1,8-cineole and (−)-2-oxo-1,8-cineole: a) Dess–Martin periodinane, CH_2Cl_2, RT, 24 h.

the enantiomeric purities of the stereospecifically synthesized educts. For comparison, the described pyridinium chlorochromate oxidation was performed with the enantiopure (−)- and (+)-α2-hydroxy-1,8-cineole. Our results showed that similar yields of the desired products were obtained. However, the developed Dess–Martin periodinane oxidation is comparatively easier to perform because it is less laborious. Further, the Dess–Martin periodinane is less toxic than the cancerogenic PCC. In conclusion, an environmentally friendlier oxidation to the enantiopure 2-oxo-1,8-cineole could be developed in addition to the original PCC oxidation.

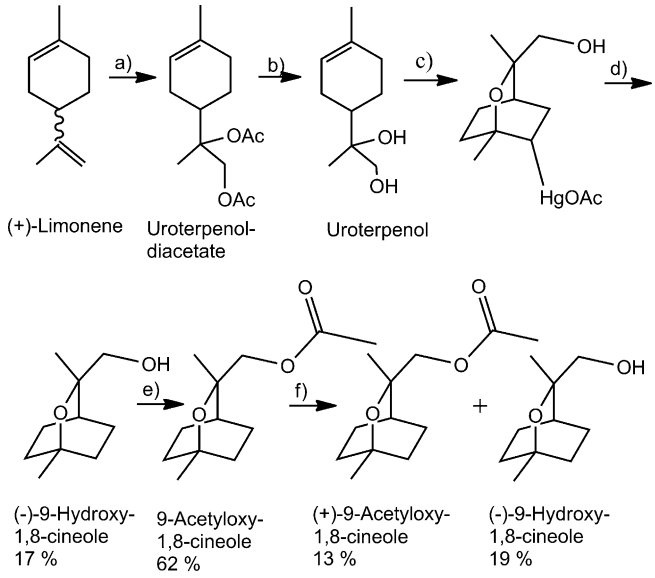
Synthesis of (−)- and (+)-β2-hydroxy-1,8-cineole

As shown in Scheme 3 the synthesis of β2-hydroxycineole starts from its diastereomer α2-hydroxy-1,8-cineole (see above). After Dess–Martin oxidation the received 2-oxo-1,8-cineole (70% yield) was reduced with sodium borohydride to a mixture of α/β2-hydroxy-1,8-cineoles. After separation on an alumina column with an elution gradient from apolar to polar, pure β2-hydroxy-1,8-cineole was obtained with 10% yield. Analogous to the stereospecific synthesis of α2-hydroxy-1,8-cineole described above the alcohol was esterified with acetyl chloride to β2-acetyloxy-1,8-cineole (73%). Incubation with PLE (EC 3.1.1.1) for 18 hours at 37 °C afforded a chromatographically separable mixture of (−)-β2-hydroxy-1,8-cineole (34%) and (+)-β2-acetyloxy-1,8-cineole (53%). After separation by flash chromatography the remaining (+)-acetate was saponified to provide (+)-β2-hydroxy-1,8-cineole (48%).

The determination of the enantiomeric purity by GC-MS on a chiral stationary phase revealed a 100% optical purity for (−)-β2-hydroxy-1,8-cineole corresponding to the expected selectivity of PLE. Despite an incubation time of 18 hours the hydrolysis was not complete, so the resulting enantiomeric excess of (+)-β2-hydroxy-1,8-cineole was only 33%. The poorer results, in comparison to the corresponding diastereomer (+)-α2-hydroxy-1,8-cineole, were presumably due to the *exo*-position of the hydroxyl group and a probably sterically restrained binding in the active center of PLE.



Scheme 3. Synthesis of (−)-β2-hydroxy-1,8-cineole: a) Dess–Martin periodinane, CH_2Cl_2, RT, 24 h; b) 2 M NaOH, 2 h, 25 °C; c) Acetyl chloride, DMAP, CH_2Cl_2, 0–5 °C; d) PLE, pH 7, 18 h, 37 °C.



Scheme 4. Synthesis of (−)-9-hydroxy-1,8-cineole: a) $Pb(Ac)_4$, THF, 4 h, 65 °C; b) KOH (2%), EtOH, 45 min, 60 °C; c) $Hg(Ac)_2$, THF, 24 h, 55 °C; d) $NaBH_4$, NaOH, 16 h, RT; e) Acetyl chloride, DMAP, CH_2Cl_2, 0–5 °C; f) *Saccharomyces cerevisiae*, sucrose, 24 h. THF = tetrahydrofuran.

Synthesis of (−)- and (+)-9-hydroxy-1,8-cineole

The racemic 9-hydroxy-1,8-cineole (Scheme 4) was obtained by dihydroxylation of limonene to uroterpenol and subsequent ring closure with mercuric acetate and sodium borohydride as described by Horst and Rychlik[6] After acetylation 9-acetyloxy-1,8-cineole (62%) was incubated with PLE. Enantiomeric analysis showed that PLE did not discriminate the racemate of the primary acetate, which was completely hydrolyzed to the corresponding racemic alcohol 9-hydroxy-1,8-cineole. The same observation was already described by Loandos et al.[8] Thus, we have confirmed that PLE does not show selectivity for primary cineolyl acetates. This outcome corresponds with the described syntheses, which showed high stereospecifity of PLE only for primary *meso*-diols and secondary or tertiary alcohols.[9]

On the other hand, under the same conditions the synthesized racemic 9-acetyloxy-1,8-cineole was not a substrate for the recombinant γ-isoenzyme of LPE expressed in *E. coli* so that no conversion occurred. Thus, after purification, the unchanged acetate was obtained.

Finally the incubation of the primary ester 9-acetyloxy-1,8-cineole with yeast (*Saccharomyces cerevisiae*) and sucrose provided (−)-9-hydroxy-1,8-cineole in 13% yield and (+)-9-acetyloxy-1,8-cineole in 19% yield after separation by flash chromatography. After saponification of the remaining acetate with lithium hydroxide, enantiomeric analysis by GC-MS on a chiral stationary phase was performed. The results showed that the hydrolysis of the racemic primary acetate by the esterases present in *S. cerevisiae*, is not totally stereospecific. (−)-9-Hydroxy-1,8-cineole was obtained with an enantiomeric excess of 40%, whereas the enantiomeric excess of its enantiomer (+)-9-hydroxy-1,8-cineole was 73%. In conclusion, the stereospecific hydrolysis of the primary acetate 9-acetyloxy-1,8-cineole was only possible with the esterases present in yeast yielding moderate results. To achieve an improved enantioselective hydrolysis the reaction conditions have to be optimized, or further esterases have to be tested with regard to their ability of enantiospecific conversion of 9-acetyloxy-1,8-cineole.

Determination of the enantiomeric composition of chiral 1,8-cineole-metabolites in human urine

A further goal of the present study was to investigate the enantiomeric composition of chiral 1,8-cineole metabolites in human urine after ingestion of 1,8-cineole using the synthesized enantiopure references. After ingestion of an enterocoated Soledum capsule containing 100 mg of 1,8-cineole, samples were collected at regular time intervals (2 h). Accordingly, characterization of each metabolite or enantiomer, respectively, was accomplished based on the confirmation of the structural identities of the compounds in the human urine samples in comparison to the respective mass spectral and chromatographic data (retention indices, compare with Table 1) of the reference substances.

Overall, the metabolites (+/−)-α2-hydroxy-1,8-cineole, (+/−)-β2-hydroxy-1,8-cineole, (+/−)-α3-hydroxy-1,8-cineole, (+/−)-3-oxo-1,8-cineole, 4-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, and (+/−)-9-hydroxy-1,8-cineole along with traces of their parent compound were detectable in urine of four volunteers (two men and two women) after liberation from their glucuronides. Not every metabolite could be found in every sample at each time point after ingestion, but in the samples with the highest excretion rate each metabolite could be iden-

Table 1. Retention indices (RI).

Substance	CAS number	RI [FFAP]	RI [TG-SQC]	RI [β-DEX-sm][a]	RI [γDEX-sa][a]
(+/−)-α2-hydroxy-1,8-cineole	18679-48-6	1890	1534	1389/1389	1488/1492
(+/−)-α2-acetyloxy-1,8-cineole	–	1714	1752	1375/1390	1484/1500
(+/−)-β2-hydroxy-1,8-cineole	92999-78-5	1707	n.d.	1328/1332	1452/1459
(+/−)-β2-acetyloxy-1,8-cineole	57709-95-2	1774	n.d.	1408/1428	1520/1528
(+/−)-2-oxo-1,8-cineole	70222-88-7	1675	1628	1307/1340	1507/1512
(+/−)-α3-hydroxy-1,8-cineole	98920-24-2	n.d.	n.d.	1423/1425	1515/1521
(+/−)-3-oxo-1,8-cineole	–	n.d.	n.d.	1290/1312	1429/1453
4-hydroxy-1,8-cineole	–	n.d.	n.d.	1351	1457
7-hydroxy-1,8-cineole	–	n.d.	n.d.	1390	1473
(+/−)-9-hydroxy-1,8-cineole	–	1860	1673	1401/1417	1504/1506
(+/−)-9-acetyloxy-1,8-cineole	–	1816	1811	1456/1456	1554/1558
(+/−)-2,3-dehydro-1,8-cineole	92760-25-3	n.d.	n.d.	1052/1061	1091/1091
(+/−)-2,3-epoxy-1,8-cineole	–	n.d.	n.d.	1258/1269	1339/1343

[a] First (+) enantiomer, second value (−) enantiomer. n.d. = not determined.

tified for each volunteer. All seven metabolites found in human urine have been previously reported in humans: 9-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, α2-hydroxy-1,8-cineole, β2-hydroxy-1,8-cineole, and α3-hydroxy-1,8-cineole were already known as animal metabolites since long.[4] 7-Hydroxy-1,8-cineole was first described by Horst and Rychlik as human metabolite.[6] Recently 4-hydroxy-1,8-cineole and 3-oxo-1,8-cineole were identified in human milk after ingestion of a Soledum capsule by Kirsch et al.[10] In this study the authors proved that the Soledum capsules themselves contained traces of all metabolites, but the amounts were much lower than the concentrations observed in human milk samples. Thus, it can be excluded that the metabolites in milk, as well as in the present case in human urine, cannot be derived from the capsule content but have to be built presumably inside the body.

Metabolism of 1,8-cineole in humans has been studied extensively in human liver microsomes. According to present knowledge, the cytochrome P450 enzymes CYP3 A4 and CYP3 A5 are mainly responsible for the first oxidative steps in 1,8-cineole metabolism leading to 2- and 3-hydroxy-1,8-cineole.[5, 11]

Furthermore, a semiquantitative assessment was carried out in relation to the concentration of the internal standard (labeled 1,8-cineole), still, without consideration of the presumably different responses. Thus, these ratios have to be treated with caution, but they can be used to compare the different samples to each other and to discuss inter- and intra-individual differences. In addition, considering the different extent of concentration of the urine samples, the metabolite concentration was expressed as a ratio relative to μmol of creatinine.

The first sample was collected no earlier than 30 minutes after the absorption of 1,8-cineole. The successful transfer of 1,8-cineole into the blood could be detected by the eucalyptol-like odor of the exhaled breath.[12] As already observed in previous investigations of our group, the absorption of the capsule content varied from subject to subject between 0.5 and 1.5 hours. These variations are presumably influenced by differences in the underlying digestion processes spanning different time periods after capsule ingestion.

Figure 1 shows the average relative concentrations of each metabolite in the three samples. It can be clearly seen that the first samples (0.5 h–1 h after absorption) contained the lowest concentrations of all metabolites. (+/−)-β2-Hydroxy-1,8-cineole, (+/−)-3-oxo-1,8-cineole, and 4-hydroxy-1,8-cineole were not detectable or only traces of them. Generally these substances were the metabolites with the lowest concentrations. The highly concentrated metabolites showed their maximum in the second sample. It is interesting to note that this is divergent to the metabolites with low concentrations as these substances showed their maximum in the last samples, indicating differences in the underlying biotransformation or their pharmacokinetic processes.

Generally, decrease of the detected metabolites in the series of urine samples after reaching their maximum was rather slow as even more than 8 hours after absorption the concentrations of some metabolites were remarkably high. Similar observations were made by Beauchamp et al.[12] who detected 1,8-cineole in the exhaled breath of volunteer panelists even 25 hours after consumption of one Soledum capsule.

Also, as can be seen from the error bars in Figure 1, metabolism profiles of the three samples were significantly different from subject to subject and the ratio of metabolites between each individual varied drastically. This variation could be explained by the different metabolic activities of each person. Higher concentrations in one sample could be due to especially intensive metabolic processes of the respective donor. Furthermore, it has to be considered that the amount of 1,8-cineole, which was absorbed, was apparently different for each person. In addition, the different metabolism profiles could be caused by different times of sampling under the assumption that the time–concentration curves of the metabolites do not run in parallel. Therefore, the timing of the sampling can significantly influence the concentrations of the metabolites. This phenomenon has also been described previously by several other authors.[12, 13] Studies thereby showed that the expressed amounts of metabolic enzymes may differ by a factor of up to 50 between each individual, being related with the respective differences in biotransformatory conversion rates.[14] Accordingly, the inter-individual differences of the elimination rate of 1,8-cineole and its metabolites are most likely high, as can be deduced both from our studies on the elimination of 1,8-cineole via breath as well as the urine studies.[15]

Summation of the excretion rates of all metabolites in urine during the time interval up to 8 hours shows that the predominant metabolite in all samples was (+/−)-α2-hydroxy-1,8-cineole followed by (+/−)-9-hydroxy-1,8-cineole, (+/−)-3-hydroxy-1,8-cineole, and 7-hydroxy-1,8-cineole. The same result were obtained by Horst and Rychlik after consumption of sage tea (1.02 mg of 1,8-cineole).[6] However, the authors did not detect the less abundant metabolites (+/−)-β2-hydroxy-1,8-cineole, 3-oxo-1,8-cineole, and 4-hydroxy-1,8-cineole. It is likely that the concentrations of these compounds were below the limit of detection as the ingested dose of 1,8-cineole was 100-fold lower than in the present study. Moreover, higher concentrations of xenobiotica can significantly change the metabolism owing to enzyme induction or saturation, so that other metabolism pathways might be involved than in case of low doses.

In previous studies, the metabolites of 1,8-cineole in urine and plasma were found to be mainly composed of their corresponding glucuronides and were virtually not detected in their free form. Accordingly, in our study, the metabolite profile of one urine sample was compared once with and once without glucuronidase treatment. Thereby, we could confirm that only traces of free metabolites were detectable. Therefore, the major part of the functionalized metabolites (phase I reaction) were conjugated to glucuronic acid by phase II reaction.

Table 2 shows the overall enantiomeric ratios of the chiral metabolites (results of all time points are integrated into combined data). The temporally resolved results are additionally provided in Table S1 in the Supporting Information. It can be clearly seen that each enantiomeric pair displayed one preferred enantiomer, that means either the (+)- or the (−)-form predominated.

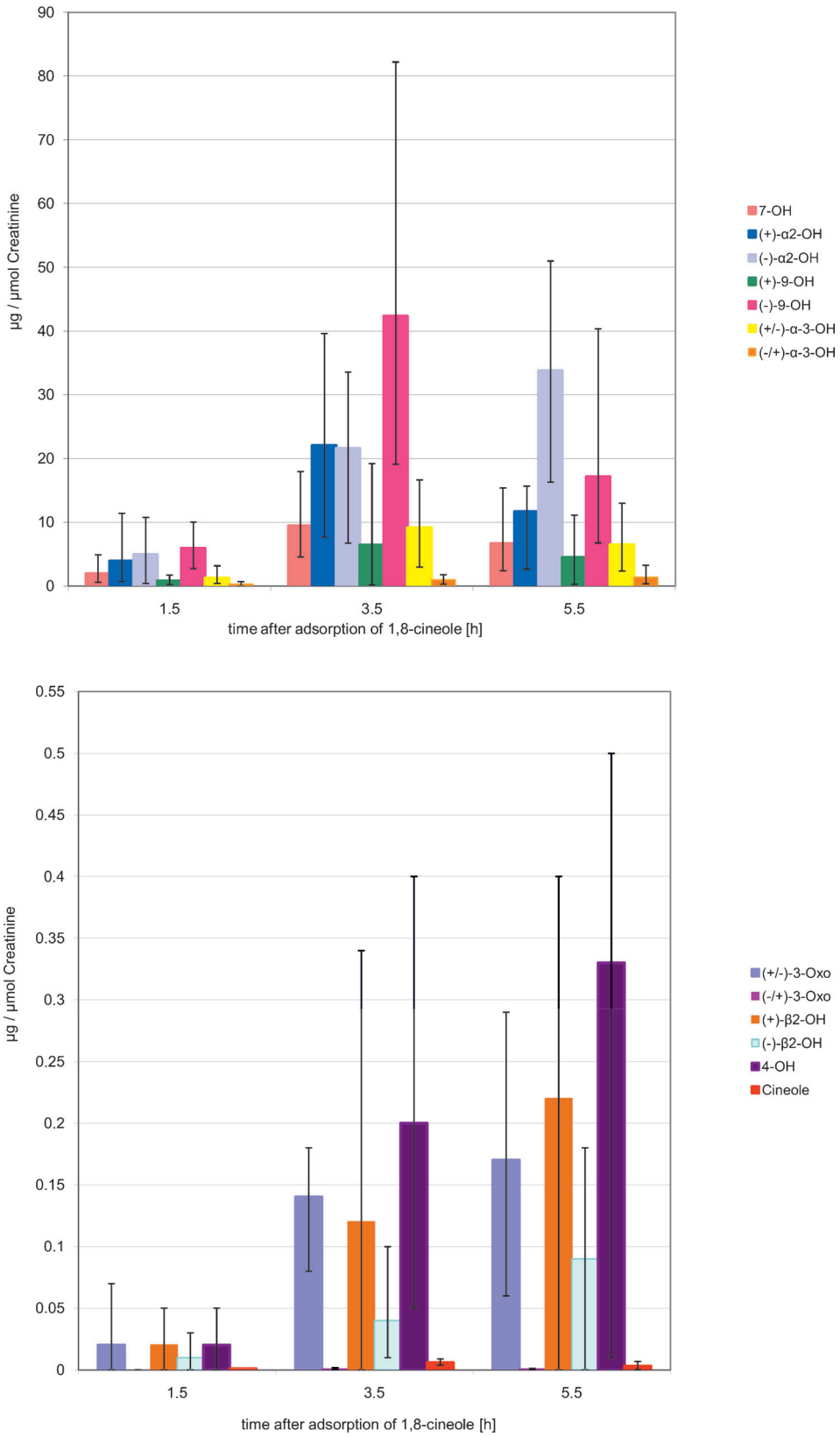


Figure 1. Average profile of a) high and b) low concentrated metabolites of 1,8-cineole in human urine after ingestion of a Soledum capsule. Error bars show the range of values measured for the individual volunteers.

Similar results were reported by Slessor et al., who determined the stereo and enantioselectivity of $CYP450_{cin}$ and (1*R*)-2β-hydroxy-1,8-cineole-dehydrogenase from *Citrobacter braakii* after incubation with 1,8-cineole.[16] The authors described (1*R*)-2β-hydroxy-1,8-cineole to be the predominantly formed enantiomer with cytochrome $P450_{cin}$. Furthermore, only (1*R*)-2β-hydroxy-1,8-cineole was oxidized to (1*R*)-2-keto-1,8-cineole by (1*R*)-2β-hydroxy-1,8-cineole-dehydrogenase, whereas the remaining three isomers of 2-hydroxy-1,8-cineole were not converted.

Overall, the question arises if in any case the same CYP monooxigenase produces both enantiomers or if different enzymes are involved in catalyzing these reactions. The latter might be more likely in view of results described by Garcia et al. for the diasteromers α/β2-hydroxy-1,8-cineole and α/β3-hydroxy-1,8-cineole.[17] These authors reported that biotransformation of 1,8-cineole by a strain of *Aspergillus terreus* isolated from Eucalyptus provided four oxygenated compounds. The inhibition of CYP450 using ketoconazole or metronidazole showed that only the *endo* position α2-hydroxy-1,8-cineole and α3-hydroxy-1,8-cineole were inhibited, meanwhile the *exo* position was not affected by the inhibitors. Consequently, these authors concluded that the β-isomers could be built by another oxidase or could be generated from the corresponding α-hydroxy-1,8-cineole.

In our study a shift of the ratios of the respective enantiomers with time was observed, as well as major variations from person to person (metabolite concentrations of each volunteer, compare Table S3–S6). Thus, different reaction pathways may be responsible for the formation of the respective enantiomers. Accordingly, further studies are needed to address the mechanistic aspects of the bioconversion of 1,8-cineole.

Table 2. Percentages of the enantiomeric ratios of 1,8-cineole metabolites in human urine.

1,8-Cineole derivatives	Range [μg μmol^{-1} x creatinine]	Ø Total content [μg μmol^{-1} x creatinine]	Enantiomeric ratio [%] in urine[a]
(+/−)-3-oxo	0.21–0.36	0.32	99.5
(−/+)-3-oxo	0.0005–0.003	0.0016	0.5
(+)-β2-OH	0.2–0.79	0.36	72
(−)-β2-OH	0.06–0.25	0.14	28
(+)-α2-OH	15.36–66.72	37.58	38
(−)-α2-OH	34.44–95.41	60.30	62
(+)-9-OH	1.24–30.87	11.68	15
(−)-9-OH	38.49–91.83	65.32	85
(+/−)-α3-OH	6.68–24.13	16.84	86
(−/+)-α3-OH	0.72–4.17	2.66	14

[a] [100 % = amount of both enantiomers].

Conclusion

Pig liver esterase (PLE) can be efficiently employed for the enantioselective hydrolysis of secondary racemic cineolyl acetates. Using this enzyme (−)-α2-hydroxy-1,8-cineole and (−)-β2-hydroxy-1,8-cineole could be stereospecifically synthesized with high enantiomeric excess and chemical yield. For the primary 9-acetyloxy-1,8-cineol PLE showed no selectivity, as this substance was completely hydrolyzed to the corresponding racemic 9-hydroxy-1,8-cineole. In contrast to this, for the recombinant γ-isoenzyme of PLE the primary acetate was shown not to be a substrate. Only after incubation with *Saccharomyces cerevisiae* an enantioselective hydrolysis was observed with moderate enantiomeric excess and chemical yield. Further, an environmentally friendlier oxidation to the enantiopure (−/+)-2-oxo-1,8-cineole with Dess–Martin-periodinane was developed in addition to the PCC oxidation used in former studies.

Another objective of this study was to investigate the enantiomeric composition of the chiral metabolites in human urine after consumption of 100 mg of 1,8-cineole using the newly synthesized enantiopure reference compounds. Seven metabolites could be clearly identified after liberation from their glucuronides. The individual metabolite profiles varied widely from person to person. Enantiomeric analyses showed that one enantiomer always predominated for each metabolite.

On the whole, in future studies these results can be compared with the enantiomeric composition of 1,8-cineole metabolites in blood and human milk for better understanding of 1,8-cineole metabolism. Such investigations are currently under way.

Experimental Section

Chemicals

The following reagents were purchased from the sources given in parentheses: potassium hydroxide, silica gel 60 (0.040–0.063 mm), silica gel (0.063–0.200 mm), sodium acetate, sodium carbonate 99 %, sodium hydrogen carbonate, sodium hydroxide, pentane, toluene (Merck, Darmstadt, Germany); 4-dimethylamino pyridine, α-terpineole, β-glucuronidase from *Helix pomatia* (EC 3.2.1.31, Type HP-2, 100 000 units mL^{-1}), aluminium oxide (neutral), lead tetraacetate, Dess–Martin periodinane, acetyl chloride 98 %, lithium hydroxide, *m*-chloroperbenzoic acid 77 %, sodium azide, sodium borohydride > 96 %, phosphate buffer (pH 7), *p*-toluenesulfonic acid, yeast (*Saccharomyces cerivisiae*), pig liver esterase (EC 3.1.1.1, ≥ 150 units mg^{-1} protein), γ-isoenzyme of porcine liver esterase expressed in *E. coli* (≥ 1.2 units mg^{-1}; Sigma Aldrich, Steinheim, Germany); dichloromethane, acetic acid 99.5 %, diethyl ether 99.5 %, hexane 99.5 %, (*R*)-limonene, sodium sulfate 98.5 %, sodium thiosulfate 99 %, tetrahydrofuran 99.9 % (Th. Geyer, Renningen, Germany), and $^{2}H_{3}$-1,8-cineol (aromaLAB AG, Munich, Germany). 2,3-Dehydro-1,8-cineole, 2,3-epoxy-1,8-cineole, α3-hydroxycineole, 3-oxo-1,8-cineole, 4-hydroxy-1,8-cineole and 7-hydroxy-1,8-cineole were a generous gift from Frauke Kirsch (Friedrich-Alexander-University of Erlangen-Nuremberg, Germany).

Syntheses

Detailed experimental procedures are given in the Supporting Information. Some of the experimental procedures were adapted from literature methods.[18,19,20]

Methods

Column chromatography

Column chromatography was carried out using silica gel 60 (0.063–0.200 mm) or neutral aluminium oxide (water-cooled glass column; 500 mm × 20 mm i.d.). Flash column chromatography was performed with silica gel 60 (0.040–0.063 mm; water-cooled glass column; 400 mm × 20 mm i.d.) and nitrogen pressure of 0.2 bar.

Gas chromatography–mass spectrometry

GC-MS analysis was performed with a Finnigan Trace DSQ Single Quadrupole GC/MS (Thermo Fisher Scientific) with GERSTEL CIS 4C injection system and GERSTEL MPS 2 autosampler (GERSTEL GmbH & Co. KG, Duisburg) using the following capillary columns: DB-FFAP (30 m × 0.32 mm, film thickness 0.25 μm; J&W Scientific, Fisons Instruments, Mainz-Kastel, Germany) and GOLD TG-SQC (30 m × 0.25 mm I.D. film thickness 0.25 μm, Thermo Scientific, Schwerte, Germany). For chiral analysis were used the two capillary columns: γDEX-sa and βDEX-sm (30 m × 0.32 mm i.d., film thickness 0.25 μm, Restek Chromatography Products, Kriftel, Germany).

For all analyses the following temperature program was used: The samples were applied by the cold-on-column injection technique at 40 °C. After 2 min, the temperature of the oven was raised at 4 °C min^{-1} to 170 °C, then raised at 40 °C min^{-1} to 230 °C and held for 5 minutes. The flow rate of the helium carrier gas was 2.5 mL min^{-1}.

EI-mass spectra were generated in full scan mode (*m/z* range 40 to 250) at 70 eV ionization energy.

Nuclear magnetic resonance spectroscopy

^{1}H NMR and ^{13}C spectra were recorded on a Bruker AMX400 (Bruker, Karlsruhe, Germany) at 297 K in $CDCl_3$ with TMS as internal standard ($\delta = 0$ ppm). Optical rotations were measured on a polarimeter P 3000 A. KRÜSS Optronic GmbH (Germany).

Study design

The study design was in concordance with the requirements of the declaration of Helsinki, and was conducted after consultation of the local ethical committee of the University of Erlangen-Nuremberg for approval of the study.

Donors

Donors were volunteers (non-smokers, Germans of Caucasian ethnicity, 2 females and 2 males, age range 23–33, mean age 28, body-mass-index: 21.7–22.6) exhibiting no known illnesses at the time of examination, and consuming their freely chosen meals without any specified dietary protocol.

Prior to sample collection and analysis written consent was obtained from all participants providing urine after a full explanation of the purpose and nature of the study. Withdrawing from the study was possible at any time.

Samples

Urine was collected in sterile 500 mL amber glass bottles. The urine samples were either processed and analyzed directly after donation as described below or frozen at −20 °C until analysis. Frozen samples were thawed at room temperature and then analyzed according to the same procedure described below.

One blank sample from urine was collected as control before ingestion of one Soledum capsule (100 mg 1,8-cineole). After perceiving a eucalyptus-like odor in the breath the urine was collected three times during the workday (up to total duration of 8 h).

Solvent Extraction and Solvent Assisted Flavor Evaporation of Urine Volatiles

Solvent assisted flavor evaporation (SAFE)[21] was applied for the fast and careful isolation of the urinary volatiles. The urine was either subjected to the SAFE directly for isolation of the volatile fraction, or immediately subjected to the hydrolysis procedure with β-glucuronidase prior to SAFE as described below, and subsequently subjected to volatile isolation via SAFE.

Isolation of the volatile fraction of human urine with enzymatic hydrolysis (β-glucuronidase assays)

Acetic acid-sodium acetate buffer (10 mL) adjusted to pH = 5 were added to freshly collected urine (10 mL). Then 0.1 mL of a sodium azide solution at a concentration of 200 μg mL^{-1}, and 0.2 mL of the β-glucuronidase-solution were added. $^2H_3$-1,8-Cineol (2.25 μg) was used as internal standard. The mixture was stirred for 15 hours at 37 °C. Afterwards, 10 mL of purified dichloromethane were added and the mixture was stirred for 30 minutes. After distillation of the mixture additional aliquots of 5 mL of dichloromethane were administered and distillation was re-performed thrice to achieve complete transfer of the respective odor compounds. The obtained aqueous distillate phase was additionally extracted thrice with 20 mL of dichloromethane. Then all combined dichloromethane phases were dried over anhydrous Na_2SO_4, and finally concentrated to a total volume of 100–200 μL at 50 °C by means of Vigreux-distillation and micro-distillation.

The samples were analyzed on the DSQ system using the two chiral capillaries βDEX-sm and γDEX-sa.

Isolation of the volatile fraction of human urine without enzymatic hydrolysis

To estimate the concentrations of free metabolites, one probe were subjected directly to SAFE distillation without glucuronidase treatment.

Therefore, 2.25 μg $^2H_3$-1,8-cineole and 30 mL dichloromethane were added to 50 mL urine and stirred 30 minutes at room temperature. Subsequently, the untreated sample was worked up by SAFE distillation analogous to the outline described above for the glucuronidase treated samples.

The sample was analyzed on the DSQ system using the two chiral capillaries βDEX-sm and γDEX-sa.

Photometric creatinine determination in urine (Jaffé reaction)

Creatinine concentration in urine is a marker for concentration of urine. It was used as reference level of the excretion of the analytes. Photometrical creatinine determination was performed using the Jaffé reaction.[22] After dilution of the urine (1 + 49, v + v) with distilled water, 100 μL of the diluted urine was mixed with 500 μL sodium hydroxide (300 mmol L^{-1}) and 500 μL picric acid (8.7 mmol L^{-1}). The orange-red complex formed was measured spectrophotometrically at 492 nm in relation to an internal standard (177 μmol L^{-1} creatinine).

Semiquantification of the metabolites in urine

Triply deuterated $^2H_3$-1,8-cineole was used as internal standard and the concentration of each metabolite was calculated as percentage of $^2H_3$-1,8-cineole added to the samples prior to sample preparation. Therefore, the peak area of the individual metabolite was divided trough the peak area of labeled 1,8-cineole and multiplied with the absolute concentration of $^2H_3$-1,8-cineole (2.25 μg). After normalizing to the sample amount the concentration was expressed in relation to the molar creatinine concentration in urine.

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[1] F. A. Santos, V. S. Rao, *Phytother. Res.* **2000**, *14*, 240–244.
[2] R. M. Carman, W. T. Robinson, C. J. Wallis, *Aust. J. Chem.* **2005**, *58*, 785–791.
[3] R. Boyle, S. McLean, N. W. Davies, *Xenobiotica* **2000**, *30*, 915–932.
[4] R. M. Carman, K. D. Klika, *Aust. J. Chem.* **1992**, *45*, 651–657.
[5] M. Duisken, F. Sandner, B. Blomeke, J. Hollender, *Biochim. Biophys. Acta-Gen. Subj.* **2005**, *1722*, 304–311.
[6] K. Horst, M. Rychlik, *Mol. Nutr. Food Res.* **2010**, *54*, 1515–1529.
[7] F. A. Luzzio, D. Y. Duveau, *Tetrahedron: Asymmetry* **2002**, *13*, 1173–1180.

[8] M. d. H. Loandos, M. B. Villecco, E. Burgueno-Tapia, P. Joseph-Nathan, C. A. N. Catalan, *Nat. Prod. Commun.* **2009**, *4*, 1537 – 1545.
[9] U. T. Bornscheuer, R. J. Kazlauskas, *Hydrolases In Organic Synthesis: Regio- And Stereoselective Biotransformations, Vol. 2*, Wiley-VCH, Weinheim, **2006**.
[10] F. H. Kirsch, K. Beauchamp, J. Rychlik, M. Buettner, *Wartburg Symp. Flavor Chem. Biol.* **2010**.
[11] M. Miyazawa, M. Shindo, *Nat. Prod. Lett.* **2001**, *15*, 49 – 53.
[12] J. Beauchamp, F. Kirsch, A. Buettner, *J. Breath Res.* **2010**, *4*, 026006.
[13] M. Miyazawa, H. Kameoka, K. Morinaga, K. Negoro, N. Mura, *J. Agric. Food Chem*. **1989**, *37*, 222 – 226.
[14] K. Aktories, W. Forth, U. Förstermann, *Allgemeine und spezielle Pharmakologie und Toxikologie: für Studenten der Medizin, Veterinärmedizin, Pharmazie, Chemie und Biologie sowie für Ärzte, Tierärzte und Apotheker, mit 303 Tabellen, Vol. 9*, Elsevier, Urban & Fischer, Amsterdam, New York, **2005**.
[15] F. Kirsch, J. Beauchamp, A. Buettner, *Clin. Nutr.* **2012**, *31*, 682.
[16] K. E. Slessor, J. E. Stok, S. M. Cavaignac, D. B. Hawkes, Y. Ghasemi, J. J. De Voss, *Bioorg. Chem.* **2010**, *38*, 81 – 86.
[17] C. García, P. Rodríguez, E. Días, H. Heinzen, P. Menéndez, *J. Mol. Catal. B* **2009**, *59*, 173 – 176.
[18] H. L. Kopperman, R. C. Hallcher, Sr. , A. Riehl, R. M. Carlson, R. Caple, *Tetrahedron* **1976**, *32*, 1621 – 1626.
[19] R. M. Carman, M. T. Fletcher, *Aust. J. Chem.* **1984**, *37*, 1117 – 1122.
[20] I. C. Macrae, V. Alberts, R. M. Carman, I. M. Shaw, *Aust. J. Chem.* **1979**, *32*, 917 – 922.
[21] W. Engel, W. Bahr, P. Schieberle, *Eur. Food Res. Technol.* **1999**, *209*, 237 – 241.
[22] H. H. Taussky, *Clin. Chim. Acta* **1956**, *1*, 210 – 224.

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