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Journal of Chromatography A, 1027 (2004) 137–146
JOURNAL OF CHROMATOGRAPHY A
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Biotransformation of (*R*)-(+)- and (*S*)-(−)-citronellol by *Aspergillus* sp. and *Penicillium* sp., and the use of solid-phase microextraction for screening

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Abstract

The biotransformation of (*R*)-(+)- and (*S*)-(−)-citronellol by fungi was studied. For screening experiments, solid-phase microextraction (SPME) was used as analytical sampling technique. It was found that sporulated surface cultures of *Aspergillus niger* were able to convert the substrate into *cis*- and *trans*-rose oxides and nerol oxide. The relative contents in the headspace SPME extract of the three bioconversion products *cis*- and *trans*-rose oxide and nerol oxide were up to 54, 21 and 12%, respectively. Rose oxide is found in minor amounts in some essential oils, such as Bulgarian rose oil and geranium oil and contributes to its unique odor. It is one of the most important fragrance materials in perfumery in creating rosy notes. Other bioconversion products were 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, limonene, terpinolene, linalool and α-terpineol. These bioconversion reactions were confirmed by sporulated surface cultures on larger scale and sampling by dynamic headspace sweep and steam distillation solvent extraction. The same conversions were noticed with *A. tubingensis* and *Penicillium roqueforti*. This bioconversion was enantioselective since more of the chiral *cis*- than *trans*-rose oxide was obtained (*cis*/*trans* ratio up to 95/5). Submerged liquid cultures of *P. roqueforti* yielded two unidentified metabolites after conversion of citronellol (yield up to 5%). The stability and acid-catalyzed conversion of citronellol was also investigated. No chemical oxidation or auto-oxidation products were detected in acidified liquid control broths up to pH 3.5. However, when control tests were run with solid media, acid-catalyzed conversion of the substrate to small amounts of *cis*- and *trans*-rose oxides, nerol oxide, linalool and α-terpineol was observed at pH 3.5 and when heat treatment (steam distillation solvent extraction) was applied.

Keywords: *Aspergillus niger*; *Aspergillus tubingensis*; *Penicillium digitatum*; *Penicillium roqueforti*; Solid-phase microextraction; Citronellol; Rose oxides

1. Introduction

In the course of our work related to the bioconversion of monoterpene alcohols by fungi, the biotransformation of citronellol by *Aspergillus niger* and other fungi was investigated. Citronellol (3,7-dimethyl-6-octen-1-ol) has been found in about 70 essential oils and in the oil of *Rosa bourbonia*. The Bulgarian rose oil and the geranium oil from Réunion and Madagascar (Bourbon type) contain more than 50% of (−)-citronellol, whereas East African geranium contains more than 80% of the (+)-isomer [1]. (+)-Citronellol dominates in oils from *Boronia citriodora* (total citronellol content ca. 80 %) and *Eucalyptus citriodora* [2]. The first detailed work on the microbial degradation of the acyclic monoterpene alcohols and aldehydes was reported in the early 1960s [3–6]. Besides the metabolism of citronellol, also the conversion of citronellal, geraniol and geranic acid by the soil bacterium *Pseudomonas citronellolis* was studied. Later, a strain of *A. niger* was isolated from garden soil, which was found to be able to transform geraniol, citronellol and linalool to their respective 8-hydroxy derivatives, a reaction referred to as ω-hydroxylation [7,8]. In their study on the bioconversion of terpene alcohols by *Botrytis cinerea*, Brunerie et al. [9] described the biotransformation of citronellol by this fungus, using grape juice as culture medium. The main conversion product was the ω-hydroxylation product (*E*)-2,6-dimethyl-2-octen-1,8-diol and its reduction product 2,6-dimethyl-1,8-octanediol. When the synthetic medium was used, supplied with some grape juice, other degradation products, such as 6-methyl-5-hepten-2-one and

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doi:10.1016/j.chroma.2003.08.090

citronellic acid were also identified [10]. Another group described the biotransformation of citronellol and other terpene alcohols by *B. cinerea* in model solutions [11]. Again ω-hydroxylation products were noticed as the main metabolites.

More recently, the bioconversion of citronellol by the plant pathogenic fungus, *Glomerella cingulata*, to 3,7-dimethyl-1,6,7-octanetriol was reported [12].

One of the last publications in this area described the biotransformation of citronellol by the basidiomycete *Cystoderma carcharias* in an aerated membrane bioreactor [13]. Again 3,7-dimethyl-1,6,7-octanetriol was obtained as the main product of conversion, together with 3,7-dimethyl-6,7-epoxy-1-octanol as important side-product. Minor compounds were 2,6-dimethyl-2-octene-1,8-diol, 3,7-dimethyl-5-octene-1,7-diol and 3,7-dimethyl-7-octene-1,6-diol. Rose oxide, an important aroma compound, obtained by dehydration and cyclisation of 3,7-dimethyl-5-octene-1,7-diol, was also noticed as minor bioconversion product. Rose oxide was not detectable in the aqueous medium, but small amounts (up to 10 mg per day) were found in the exhaust air of the bioreactor. The microbial formation of rose oxide was here observed for the first time. The authors also carried out control experiments with citronellol in the same medium under equal conditions (pH 2.5–4) to confirm that this conversion was not a chemical or photochemical process. The acid-catalyzed conversion of (*S*)-3,7-dimethyl-5-octene-1,7-diol, a monoterpenoid from petals of *Rosa damascena* Mill., into isomeric rose oxides has been described by another group [14] (see Fig. 1).

Rose oxide (4-methyl-2-(2-methyl-1-propenyl)-tetrahydropyran) is found in minor amounts in some essential oils, such as Bulgarian rose oil and geranium oil and contributes to its unique odor [2]. It is one of the most important fragrance materials in perfumery in creating rosy notes. The olfactory properties of the stereoisomers of rose oxide have recently been reviewed [15]. The biogenesis of rose oxides in plants has been investigated in detail on *Pelargonium* species [16–18].

Rose oxides can be obtained synthetically by photoinduced oxygenation of (−)-citronellol to allylic hydroperoxides and further reduction to the corresponding diols [19]. de Faria et al. [20] reported the electrosynthesis of rose oxide through anodic cyclisation of citronellol with yields up to 70%.

To the best of our knowledge, a straightforward fungal biotransformation from citronellol into high value flavor compounds such as rose oxides has not been published before. The present report highlights this valuable biotransformation for the first time.

This study investigates the use of solid-phase microextraction (SPME) as fast monitoring technique to screen fungi for their ability to bioconvert the substrate citronellol into rose oxides. Some common fungi were evaluated in this study, such as *A. niger*, *Aspergillus tubingensis*, *Penicillium digitatum* and *Penicillium roqueforti*.

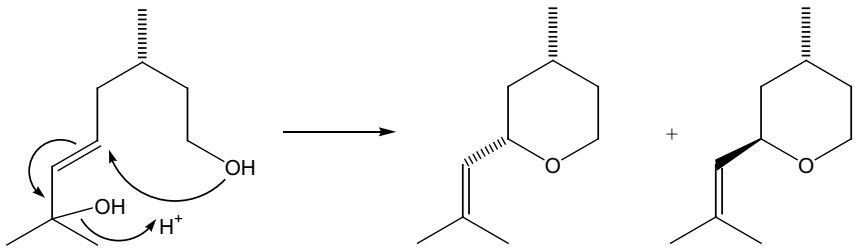
2. Experimental

2.1. Microorganisms and cultivation

For preliminary screening with SPME, more than 60 fungal strains were used as described earlier [21]. Two *A. niger* (ANA and ANT), four *A. tubingensis* (AT), one *A. carbonarius* (AC), two *P. digitatum* (CMC and PDD) and three *P. roqueforti* strains (CCV, CDG and CZW) and one unidentified *Penicillium* sp. (GCC) were used for further study. The fungi were either isolated from spoiled petri dish culture media (*A. niger* strain ANT, *P. roqueforti* strains CCV, CDG and CZW and unidentified *Penicillium* sp. GCC) and from a spoiled mandarin (*P. digitatum* strain CMC), were provided by Wageningen Agricultural University (*A. tubingensis*), or were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) (strain PDD, *P. digitatum* DSM 62840 and strain ANA, *A. niger* DSM 821). The cultures were cultivated and conserved by periodic replications (every 2 weeks) on malt extract agar (MEA: malt extract 2%, bacteriological peptone 0.1%, glucose 2% and agar 2%, pH 5.4).

2.2. Optimisation of SPME methodology

Prior to the preliminary screening experiments, a method was developed to cultivate sporulated surface cultures of the fungi in small vials, and the SPME-parameters were optimized [21,22]. An extensive optimisation was first



(*S*)-3,7-Dimethyl-5-octene-1,7-diol (−)-(2*S*, 4*R*)-*cis*-Rose oxide (−)-(2*R*, 4*R*)-*trans*-Rose oxide

Fig. 1. Acid-catalyzed conversion of (*S*)-3,7-dimethyl-5-octene-1,7-diol into isomeric rose oxides (after [14]).

carried out for the flavor compounds linalool and its bioconversion metabolites linalool oxides during a previous biotransformation study [22]. Five different SPME fibers were then evaluated, namely 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane–divinylbenzene (PDMS–DVB), 50/30 μm divinylbenzene–Carboxen on polydimethylsiloxane (DVB–CAR–PDMS), 65 μm Carbowax–divinylbenzene (CW–DVB) and 85 μm polyacrylate (PA) (all obtained from Supelco), and different extraction times (15, 30 and 60 min) and temperatures (20, 25 and 30 °C) were compared. Extraction of the volatiles (linalool and metabolites) was done by headspace SPME. It was found that the best SPME fiber for extraction of linalool and its metabolites furanoid linalool oxide and α-terpineol was DVB–CAR–PDMS. The optimum adsorption time was 30 min and the best extraction temperature was 25 °C. A desorption temperature of 250 °C and desorption time of 2 min were sufficient to desorb all volatiles into the gas chromatographic inlet.

The SPME method was then further optimized for the current study using a standard solution of (−)-rose oxides (mixtures of isomers) in EtOH at 0.5% (v/v). Agar gels (5 ml) in 22 ml SPME vials were prepared and to each gel 5 μl of the standard solution was sprayed and the vial was closed. After exactly 1 h of equilibration at 25 °C, the vials were sampled by headspace SPME during 7.5, 15, 30 and 60 min at 25 °C (in duplicate). A significant increase in extraction recovery could be observed between 7.5 and 30 min (data not shown), but no further increase in extraction recovery was noticed from 30 to 60 min. Hence, since a maximum recovery was obtained after 30 min (equilibrium), and since the GC–MS run time was about 30 min, an extraction time of exactly 30 min was selected as standard procedure.

2.3. Screening of sporulated surface cultures of fungi by SPME

The fungi were cultivated as small sporulated surface cultures in 40 ml SPME vials (Supelco, Bellefonte, Pa, USA) as described earlier [21]. To each sporulated surface culture, 5 μl of a solution of (*R*)-(+)-citronellol (puriss., 99%, Fluka) in EtOH (10%) was sprayed. The vials were covered with Black Viton Septa and Open Top Phenolic Closures (Supelco) and stored at room temperature until the start of the headspace SPME-extraction. During extraction, the SPME fibers (50/30 μm DVB–CAR–PDMS, Supelco) were exposed to the headspace of the cultures for 30 min at 25 °C, according to the best results obtained after the optimisation studies.

2.4. Bioconversion by liquid cultures

A biotransformation experiment was run with five *Penicillium* strains in duplicate: three *P. roqueforti* (CCV, CDG and CZW), one *P. digitatum* (CMC) and one unidentified *Penicillium* sp. (GCC).

The fungi were cultivated in 250 ml conical flasks, filled with 50 ml liquid medium (YMPG: yeast extract 0.5%, malt extract 1%, bacteriological peptone 0.5%, glucose 1%, pH 6.1). Inoculation was performed with spore suspensions as described earlier [21]. The test substrate citronellol was added as a 5% solution (v/v) in absolute EtOH. At different time intervals, 5 ml samples were taken and extracted with 2 × 2 ml Et_2O. After addition of 1 ml of a standard solution of 0.1% (v/v) *n*-decane in Et_2O, the samples were directly analyzed by GC–MS. Experiments were also run with control flasks, containing sterile culture broth, acidified to pH 3.5 with HOAc, but not inoculated, and to which the substrate was added. The culture flasks were stirred at 150 rpm, at 24 °C.

2.5. Biotransformation of (±)-citronellol by sporulated surface cultures

In the first experiment with sporulated surface cultures, two strains of *A. niger* (ANA, ANT), four strains of *A. tubingensis* (AT) and two strains of *P. roqueforti* (CCV and CZW) were compared. Conical flasks of 250 ml were used, filled with 50 ml MEA medium and inoculated with 0.5 ml of freshly prepared spore suspensions, containing 3.5×10^8 spores/ml (*Aspergillus*) or 1.8×10^8 spores/ml (*Penicillium*). After inoculation, the cultures were incubated at 30 °C for 24 h and at room temperature during the rest of the experiment. Substrate addition was carried out by spraying 100, 250 and 250 μl of a solution of (±)-citronellol in EtOH (10%) onto the sporulated surface cultures after 8, 13 and 19 days, respectively. The biotransformation was monitored by dynamic headspace sampling as described previously [23] and by steam distillation solvent extraction (SDSE) using a Likens–Nickerson device [24] (see the following paragraph).

For the second experiment with sporulated surface cultures, a spore suspension of *P. digitatum* (CMC) containing 4.2×10^8 spores/ml (as counted with Kova® slides) was used to inoculate the medium. Four different culture media were used, namely malt extract agar (MEA), potato dextrose agar (PDA: potato extract 0.4%, glucose 2%, agar 2%), sabouraud dextrose agar (SAB: mycological peptone 1%, glucose 4%, agar 2%) and Czapex Dox medium (CZD), all obtained from Oxoid. Two times 250 μl of a solution of (±)-citronellol in EtOH (10%) was added to the sporulated surface cultures, after 11 and 14 days, respectively. The biotransformation was monitored as described above.

2.6. Dynamic headspace sampling and steam distillation solvent extraction

For dynamic headspace sampling the conical culture flasks were fitted with a glass inlet and outlet for aeration. To the outlet of the flasks, a Tenax adsorption tube was attached (length: 22 cm, i.d.: 13 mm, o.d.: 14 mm), containing 100 mg of Tenax TA (Alltech, Lokeren, Belgium). The system was continuously aerated from an air cylinder at

30 ml min^{-1}. The volatiles were adsorbed on the Tenax trap during 8–24 h. After the sampling time, the tubes were disconnected from the culture flasks and each Tenax tube was eluted with 3 × 2.5 ml of Et_2O. To the headspace extracts 1 ml of a standard solution of 0.1% (v/v) *n*-decane in Et_2O was added as internal standard and the samples were directly analyzed by GC and GC–MS, respectively for quantification and identification of the bioconversion products. At the end of the bioconversion experiment, half of the sporulated surface culture was cut in small pieces (1 cm^2) and put in a round bottomed 250 ml-flask for steam distillation using a Likens–Nickerson device [24]. Distilled water was added to adjust the volume to 100 ml. The extraction was carried out with 12 ml of CH_2Cl_2 by simultaneously boiling both the aqueous phase and the solvent during 1.5 h. After addition of 1 ml of the internal standard solution [0.1% (v/v) *n*-decane in Et_2O] to the dichloromethane extract, the sample was directly analyzed by GC and GC–MS.

2.7. Biotransformation of enantiomerically pure (+)- and (−)-citronellol by sporulated surface cultures

The bioconversion of enantiomerically pure (+)- and (−)-citronellol by sporulated surface cultures was investigated using six different strains of *A. niger*, four of *A. tubingensis* and three strains of *P. roqueforti*. SPME was applied as the monitoring technique to evaluate the biotransformation capacity of the fungi.

2.8. Control tests and stability tests

To test the chemical stability of citronellol in submerged liquid broths, three conical flasks of 250 ml were filled with 50 ml YMPG medium. The pH of two broths was adjusted with HOAc to pH 5 and pH 3.5, respectively. The pH of the third broth was not adjusted, and was kept at pH 6.3. To every flask 0.5 ml of a 10% (v/v) solution of citronellol in EtOH was added. Samples were taken and extracted after 15 min and after 7 days.

The possible acid-catalyzed chemical conversion of citronellol in solid media was monitored by headspace SPME. Eight SPME vials of 40 ml each (Supelco) were used, two of which were filled with 10 ml MEA-medium and six of which were filled with pure agar gel. The pH of the MEA medium (pH 6) was not adjusted; two agar gels were adjusted to pH 5, two were adjusted to pH 3.5 and two were not adjusted (pH 7.3). To every vial 10 μl of a 10% (v/v) solution of citronellol in EtOH was added. The non-acidified media were sampled by headspace SPME (during 30 min at room temperature with a 50/30 μm DVB–CAR–PDMS) immediately and after 3 days, whereas the acidified media were only sampled after 3 days, as described recently [21].

The chemical conversion of citronellol in solid media was also monitored by dynamic headspace and SDSE. Six conical flasks of 500 ml were used, three of which were filled with 100 ml MEA-medium and three were filled with 100 ml agar gel (2%). For each medium, three pH values were applied: normal non-adjusted pH (pH 6 and 7.3 for MEA and agar gel, respectively), adjusted to 5 and adjusted to 3.5. To every gel, 1 ml of a 10% (v/v) solution of citronellol in EtOH was added. After 24 h, a dynamic headspace sample was taken during 24 h. Again 1 ml of this 10% (v/v) solution of citronellol in EtOH was added. After 48 h a second dynamic headspace sample was taken during 8 h, followed by SDSE of the complete gels.

2.9. Chemical compounds

The substrates used for the biotransformation experiments were racemic (±)-citronellol (97%, Aldrich, Belgium), (*R*)-(+)-citronellol (99%, Fluka, Belgium) and (*S*)-(−)-citronellol (99%, Fluka, Belgium). As reference compounds, (+)-rose oxide (99%, mixture of *cis* and *trans*, Fluka, Belgium) and (−)-rose oxide (99%, mixture of *cis* and *trans*, Fluka, Belgium) were used.

2.10. Analysis of the samples with GC and GC–MS

GC and GC–MS analyses of the liquid samples were performed as described earlier [21]. Quantification of the substrate citronellol and the bioconversion products *cis*- and *trans*-rose oxides was done by comparison of their peak area with that of the internal standard *n*-decane, taking into account the relative response factors to correct for the difference in response between *n*-decane and the terpenoids. The response factor of citronellol was 1.05 and that of the rose oxides 0.67.

For the analysis of the SPME extracts, a HP 6890 GC Plus coupled with a HP 5973 MSD (mass selective detector, quadrupole type), equipped with a CIS-4 PTV (programmed temperature vaporisation) injector (Gerstel), and a HP5-MS capillary column (30 m × 0.25 mm i.d.; coating thickness 0.25 μm) was used. Working conditions were: injector 250 °C, transfer line to MSD 250 °C, oven temperature: start 40 °C, held 2 min; programmed from 40 to 150 °C at 5 °C min^{-1}, from 150 to 170 °C at 10 °C min^{-1} and from 170 to 250 °C at 30 °C min^{-1}, held 2 min; carrier gas (He) 1.2 ml min^{-1}; split 1/10; electron impact (EI) ionization: 70 eV; acquisition parameters: scanned *m*/*z*: 40–200 (5–15 min), 40–300 (15–20 min), 40–400 (>20 min).

Substances were identified by comparison of their mass spectra and retention indexes (Kováts indexes) with those of reference substances (where possible) and by comparison with the US National Institute of Standards and Technology (NIST) mass spectral library (Version 1.6d, 1998).

Chiral GC analyses were performed with a HP 6890 GC Plus, equipped with a split/splitless injector and a flame ionization detection (FID) system, and a Cydex-B chiral column (SGE: 30 m × 0.25 mm i.d.; coating thickness 0.25 μm). Working conditions were: injector 250 °C, detector 280 °C (make-up gas He 10 ml min^{-1}), oven temperature: start

83 °C, held 45 min at 83 °C, programmed from 83 to 100 °C at 1 °C min^{-1}, held 2 min at 100 °C; carrier gas (He), column head pressure 30 psi (1 psi = 6894.76 Pa); flow rate 1.1 ml min^{-1} (average velocity 26 cm s^{-1}); split 1/50.

2.11. *cis*- and *trans*-Rose oxide, nerol oxide

cis-Rose oxide. Kováts retention index: 1106. EIMS 70 eV. *m/z*: (rel. int.): 154 [M^+] (9), 139 (100), 69 (80), 41 (40), 55 (36), 83 (28), 42 (13), 67 (13), 85 (13), 43 (11), 84 (11). *trans*-Rose oxide. Kováts retention index: 1125. EI–MS 70 eV. *m/z* (rel. int.): 154 [M^+] (6), 69 (100), 139 (77), 41 (45), 55 (36), 83 (29), 43 (17), 84 (14), 42 (12), 67 (11), 56 (9). Nerol oxide. Kováts retention index: 1156. EI–MS 70 eV. *m/z* (rel. int.): 152 [M^+] (7), 68 (100), 67 (81), 83 (81), 85 (32), 41 (21), 53 (19), 69 (18), 55 (15), 96 (12), 81 (9).

2.12. Two unidentified metabolites obtained from *P. roqueforti* and *Penicillium sp.*

Two metabolites were recovered from the cultures of *P. roqueforti* and another *Penicillium* sp. They could not be identified, despite extensive mass spectral investigation.

Metabolite 1. Kováts retention index: 1335. EI–MS 70 eV. *m/z* (rel. int.): 59 (100), 55 (45), 41 (40), 81 (38), 71 (37), 43 (36), 85 (31), 69 (28), 57 (27), 68 (24). Metabolite 2. Kováts retention index: 1344. EI–MS 70 eV. *m/z* (rel. int.): 59 (100), 55 (42), 41 (40), 81 (39), 43 (38), 71 (37), 85 (32), 69 (27), 57 (27), 68 (24). It is very likely that those metabolites are the *cis*- and *trans*-isomers of one compound.

3. Results and discussion

3.1. Stability and acid catalyzed conversion of citronellol

3.1.1. Chemical stability of citronellol in submerged liquid broths

To check the chemical stability of citronellol in submerged liquid broths, flasks were filled with YMPG medium the pH of which was adjusted to pH 5 and 3.5 and shaken (see Section 2).

After 1 week of shaking, no chemical oxidation or auto-oxidation products were detected in the culture broths, apart from impurities that were already present in the substrate, e.g. rose oxides (<0.1%). At least, 60% of the initial added citronellol was evaporated after this period. The pH values of the broths did not change after 1 week.

3.1.2. Monitoring of chemical conversion of citronellol in solid media by SPME

The possible acid-catalyzed chemical conversion of citronellol in solid media was monitored by headspace SPME (see Section 2).

Table 1
Relative composition (%) of headspace SPME extracts of the control agar gels at different pH, 3 days after addition of citronellol

Substrate/product	Normal pH (7.3)	pH 5	pH 3.5
Citronellol	100.0	95.7	88.1
cis-Rose oxide	0.0	3.1	7.9
trans-Rose oxide	0.0	1.2	3.1
Nerol oxide	0.0	0.0	0.9

The results with the composition of the headspace SPME extracts of the agar gels after 3 days are displayed in Table 1.

In some vials, traces of rose oxides were detected in the first headspace SPME extract, suggesting that the substrate, citronellol, contained small traces of rose oxides as artifacts. At pH 3.5 and 5, small traces (<0.2%) of α-terpineol were noticed. It can be concluded that at pH 5 and especially at pH 3.5 citronellol can be chemically converted to *cis*- and *trans*-rose oxide after 3 days. However, in the culture media and the agar gels at normal pH, no significant chemical conversion of citronellol was observed.

3.1.3. Monitoring of chemical conversion of citronellol in solid media by dynamic headspace and SDSE

Although SPME is a very sensitive method for headspace extraction of solid media, it suffers from the drawback that it only gives qualitative data because quantitative data are very difficult to obtain. Therefore, the chemical conversion of citronellol in solid media at different pH values was monitored by dynamic headspace and SDSE.

The results with the yields of recovered citronellol and formed chemical conversion products from the headspace and SDSE extracts are depicted in Table 2.

From the results displayed in Table 2, it can be concluded that citronellol was stable at pH ≥5 when no thermal treatment was applied to the substrate. Only small traces (<0.1%) of rose oxides and nerol oxide were recovered from the headspace samples of the solid media at normal pH or pH 5. At acid pH (3.5) or when heat was applied to the gels during SDSE, acid-catalyzed conversion of the substrate to significant amounts of *cis*- and *trans*-rose oxides, nerol oxide, linalool and α-terpineol was observed. Linalool is probably formed through oxidation of citronellol to geraniol or nerol during heating of the acid media and is further converted to α-terpineol by cyclisation [25]. The recuperation of the substrate during SDSE was quite high (up to 87% from the agar gel at pH 7.3), but decreased with decreasing pH. The pH did not drop significantly after SDSE.

Generally, it can be concluded that citronellol can be chemically converted to *cis*- and *trans*-rose oxides, nerol oxide, linalool and α-terpineol due to a combination of acid catalysis (pH 3.5) and heat treatment (SDSE) (see Fig. 2). This chemical conversion did not take place in the liquid control broths, even at pH 3.5.

Table 2
Recovered citronellol (%) and amounts of chemical conversion products (%) from control media (MEA vs. agar gel) and influence of pH of the medium and method of sampling (headspace vs. steam distillation solvent extraction)

Substrate and metabolites	MEA medium						Agar gel					
	Normal pH (6)		pH 5		pH 3.5		Normal pH (7.3)		pH 5		pH 3.5	
	HS	SDSE	HS	SDSE	HS	SDSE	HS	SDSE	HS	SDSE	HS	SDSE
Recovered citronellol	3.3	84.5	3.8	68.0	3.1	34.6	2.9	87.3	2.0	82.5	0.5	73.3
cis-Rose oxide	tr.	0.6	0.1	0.6	0.3	0.2	tr.	0.4	tr.	0.4	tr.	0.4
trans-Rose oxide	0.0	0.3	tr.	0.3	0.1	tr.	0.0	0.2	tr.	0.2	tr.	0.1
Neroloxide	0.0	0.1	tr.	0.1	tr.	tr.	0.0	0.1	0.0	0.1	0.0	0.1
Linalool	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.3
α-Terpineol	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.4

HS: headspace; SDSE: steam distillation solvent extraction; tr.: traces (<0.1%); MEA: malt extract agar.

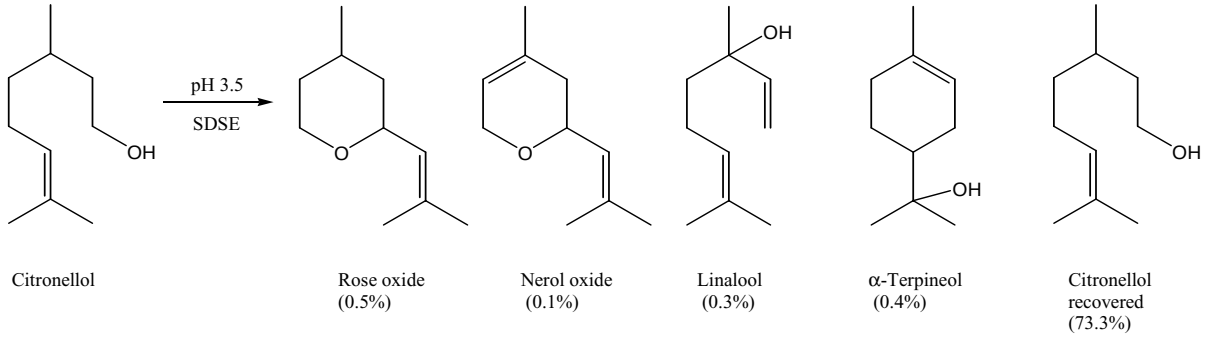


Fig. 2. Partial acid-catalysed conversion of citronellol in solid media at pH 3.5 during heat treatment (steam distillation solvent extraction: SDSE).

3.2. Biotransformation of citronellol

3.2.1. Screening of sporulated surface cultures of fungi by SPME

More than 60 fungal strains, grown as sporulated surface cultures, were screened for their ability to bioconvert the substrate (*R*)-(+)-citronellol, using SPME as the monitoring technique [21] (see Section 2).

From the GC–MS analyses of the SPME extracts of the sporulated surface cultures, it could be concluded that some *Aspergillus* sp. and *P. roqueforti* cultures were able to convert the substrate mainly to *cis*- and *trans*-rose oxide.

Table 3
Relative contribution (%) of bioconversion products obtained from citronellol, other fungal metabolites and non-converted citronellol in the headspace SPME extract of sporulated surface cultures

No.	Compound	*Aspergillus* sp.			*Penicillium roqueforti*			GCC
		AC	ANA	AT	CCV	CZW	CDG	
1	α-Pinene	2.93	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	6-Methyl-5-hepten-2-one	0.13	6.20	3.17	5.68	2.79	0.73	9.03
3	6-Methyl-5-hepten-2-ol	n.d.	0.25	0.17	1.13	0.41	0.45	0.88
4	Limonene	6.56	0.12	10.60	0.05	0.08	0.09	0.03
5	Terpinolene	3.52	0.08	4.91	n.d.	0.04	0.07	0.02
6	Linalool	12.11	0.41	3.91	1.01	0.74	1.03	0.20
7	*cis*-Rose oxide	49.14	32.08	39.83	45.78	44.24	53.70	42.31
8	*trans*-Rose oxide	4.68	10.82	2.48	18.46	18.62	20.98	16.62
9	Nerol oxide	12.01	5.12	10.65	5.06	7.58	5.49	3.56
10	α-Terpineol	0.69	0.22	1.45	0.13	0.10	0.14	n.d.
11	Citronellol	6.32	39.85	16.29	10.26	9.36	7.58	5.66
12	β-Elemene	n.d.	0.04	n.d.	2.48	3.11	1.99	3.85
13	(+)-Aristolochene	n.d.	1.17	0.63	8.87	11.30	6.69	15.21
14	Valencene	n.d.	3.28	4.80	0.69	1.48	0.32	2.36
15	SPME-artifact	1.91	0.35	1.11	0.41	0.14	0.73	0.27

AC: *Aspergillus carbonarius*; ANA: *A. niger*; AT: *A. tubingensis*; CCV, CDG and CZW: *Penicillium roqueforti*; GCC: unidentified *Penicillium* sp.; n.d. = not detected.

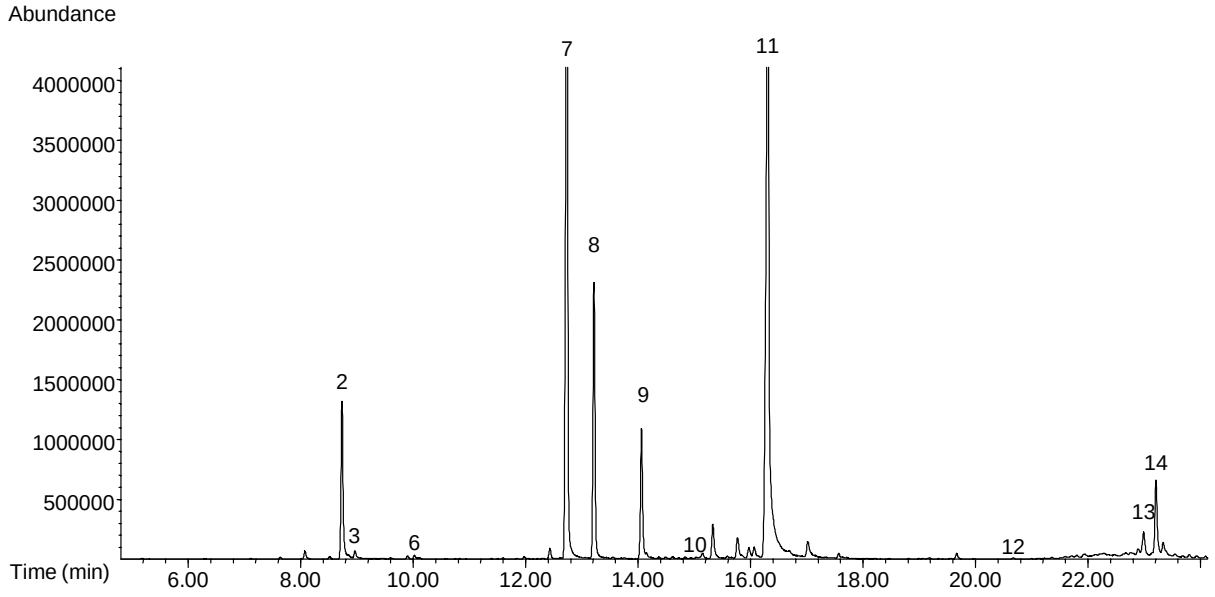


Fig. 3. Chromatogram of the headspace SPME extract of *Aspergillus niger* culture after biotransformation of citronellol. Peak numbers refer to the compounds listed in Table 3.

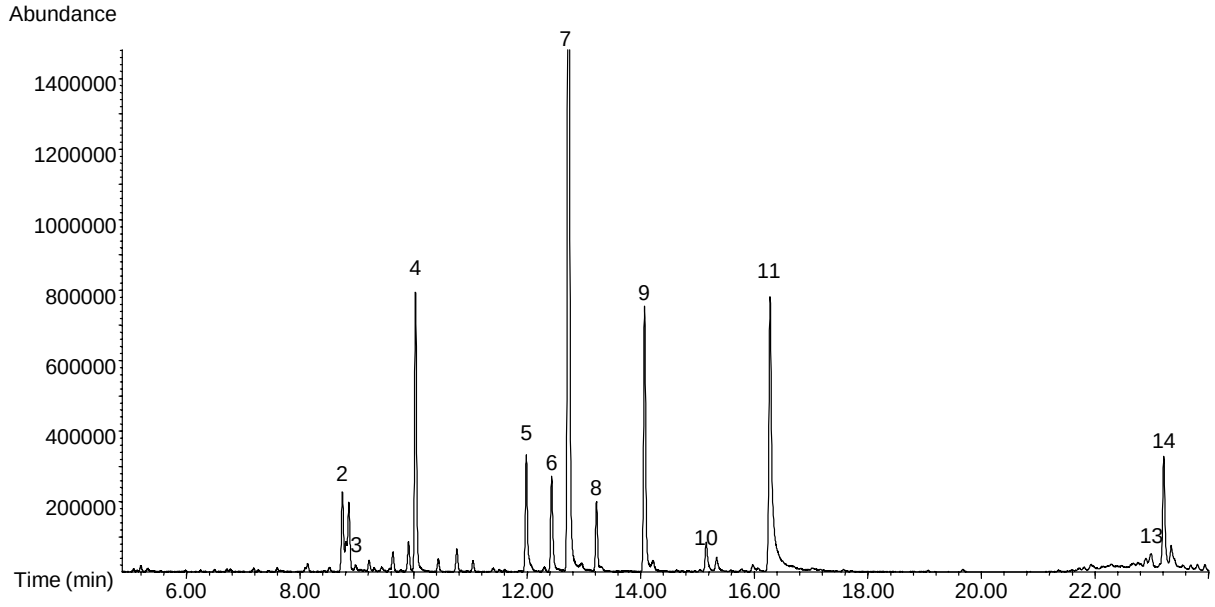


Fig. 4. Chromatogram of the headspace SPME extract of *Aspergillus tubingensis* culture after biotransformation of citronellol. Peak numbers refer to the compounds listed in Table 3.

Minor metabolites were nerol oxide, linalool, limonene and 6-methyl-5-hepten-2-one (see Table 3). In Figs. 3–5, the chromatograms of the headspace SPME extracts of sporulated surface cultures of *A. niger*, *A. tubingensis* and *P. roqueforti* are depicted. Peak numbers refer to the compounds listed in Table 3. *A. niger*, *A. tubingensis*, an unidentified *Penicillium* sp. (strain GCC), *P. roqueforti* and *P. digitatum* were selected for further bioconversion studies.

3.2.2. Biotransformation of citronellol by submerged liquid cultures of Penicillium sp.

Five different *Penicillium* sp. were cultivated (see Section 2) in duplicate. Again two control flasks (pH 3.5) were run parallel with the cultures. From the *P. roqueforti* cultures, marked CCV, CDG and CZW and the unidentified *Penicillium* sp., marked GCC, two unidentified metabolites were recovered (for spectrometric details see Section 2), whereas no conversion products were obtained

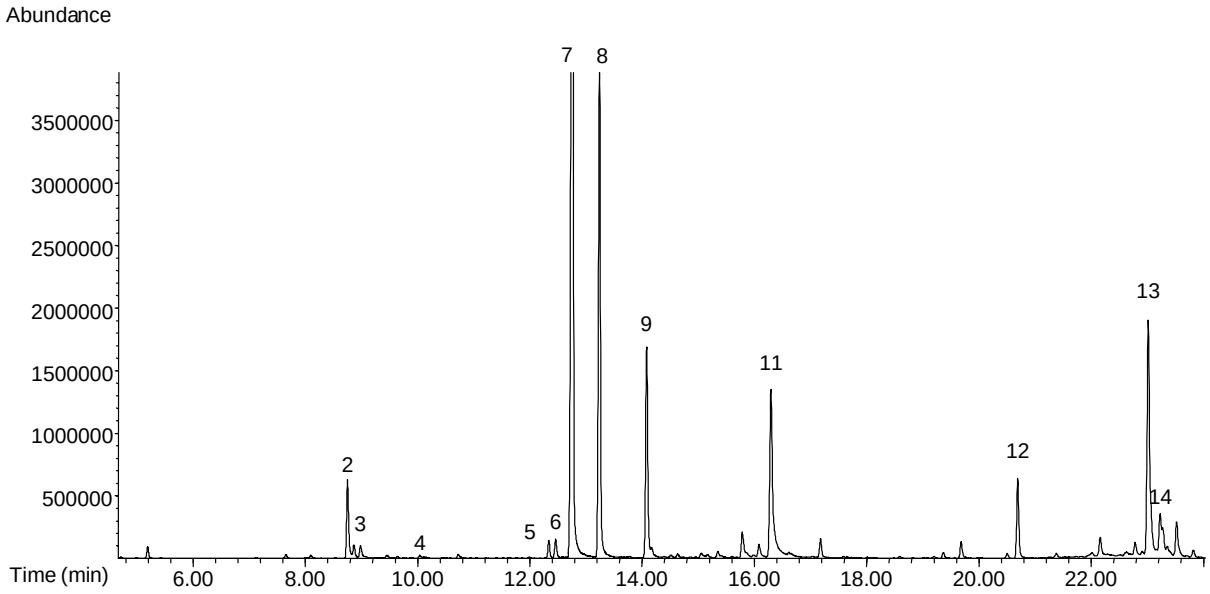


Fig. 5. Chromatogram of the headspace SPME extract of *Penicillium roqueforti* culture (strain CZW) after biotransformation of citronellol. Peak numbers refer to the compounds listed in Table 3.

from the *P. digitatum* cultures, marked CMC, and the control flasks. The results with the yields of the metabolites obtained and substrate recovered are depicted in Table 4.

From these results, it is also clear that the substrate recovery is highest from the *P. digitatum* cultures ($50 \pm 4.7\%$) and the control flasks ($40.1 \pm 0.0\%$).

3.2.3. Biotransformation of (±)-citronellol by sporulated surface cultures of Aspergillus sp. and Penicillium sp.

In the first experiment, the bioconversion of citronellol by sporulated surface cultures of six different strains of *Aspergillus* sp., two *A. niger* and four strains of *A. tubingensis* and two strains of *P. roqueforti* was studied (see Section 2). The yields were rather low (1–2% *cis*-rose oxide, 0.5–1% *trans*-rose oxide and nerol oxide, 0.5–1% α-terpineol). The data confirmed the results obtained by SPME. From these data and the results of the control experiments (Table 2), it can be concluded that the formation of rose oxides is probably due to a combination of bioconversion and acid catalyzed chemical conversion. The formation of linalool and α-terpineol is believed to be due to acid catalysis exclusively, since the final pH of the culture broths of the *Aspergillus* strains after SDSE extraction varied between pH 2 and 3, whereas the final pH of the *P. roqueforti* cultures was 4–4.5 after extraction. Neither linalool nor α-terpineol was recovered from the *P. roqueforti* cultures.

In the second experiment, the bioconversion of citronellol by one strain of *P. digitatum* was investigated, using four different culture broths (see Section 2). The results with the recovery of non-converted substrate and yields of metabolites are given in Table 5 (average of

Table 5
Total amount of recovered citronellol (%) and yields of bioconversion products (%) obtained after dynamic headspace and SDSE of a *Penicillium digitatum* culture grown in various culture media

Substrate and metabolites	Culture medium			
	MEA	PDA	SAB	CZD
Recovered citronellol	15.6	19.5	20.1	18.7
cis-Rose oxide	0.9	1.0	0.8	0.8
trans-Rose oxide	0.3	0.3	0.3	0.3
Neroloxide	0.1	0.1	0.0	0.1
pH after SDSE	3.9	5.2	6.6	4.6

MEA: malt extract agar; PDA: potato dextrose agar; SAB: sabouraud dextrose agar, CZD: czapek dox agar.

Table 4
Recovered citronellol and yields of bioconversion products (%) by five *Penicillium* strains (in duplicate) and two control cultures

Substrate and metabolites	CCV		CDG		CZW		GCC		CMC		Control	
	1	2	1	2	1	2	1	2	1	2	1	2
Recovered citronellol	11.8	15.3	10.9	9.5	15.1	12.4	24.6	20.9	46.7	53.3	40.1	40.1
Unknown 1	1.4	2.6	1.2	2.5	2.1	1.9	2.7	2.6	0.0	0.0	0.0	0.0
Unknown 2	3.0	4.7	2.2	3.8	3.7	2.9	4.3	5.1	0.0	0.0	0.0	0.0

CCV, CDG and CZW: *P. roqueforti*; CMC: *P. digitatum*; GCC: unidentified *Penicillium* sp.

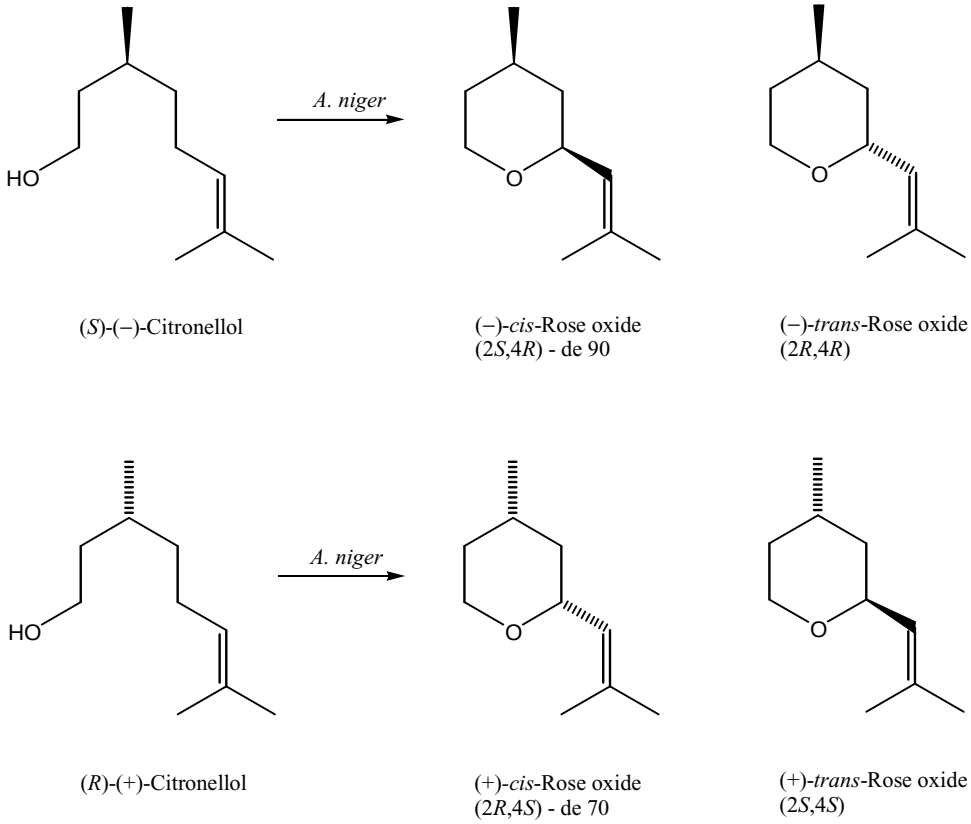


Fig. 6. Enantioselective bioconversion of (*R*)-(+)- and (*S*)-(−)-citronellol to *cis*- and *trans*-rose oxide by *Aspergillus niger*.

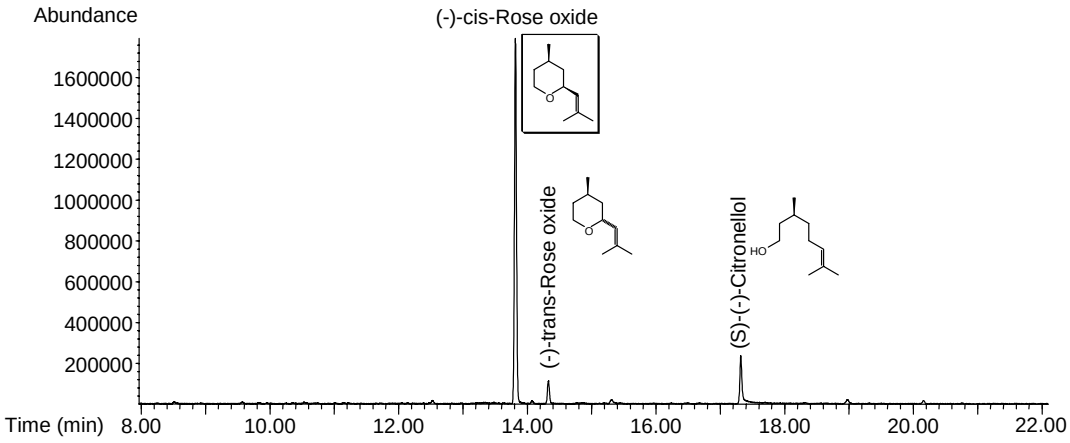


Fig. 7. Chromatogram of an SPME extract of a sporulated surface culture of *Aspergillus niger*, strain ANA, treated with enantiomerically pure (*S*)-(−)-citronellol, yielding (−)-*cis*-rose oxide with a diastereomeric excess (d.e.) of 0.91.

two cultures). It can be noticed that the medium composition has a strong influence on the final pH of the culture broths. However, there was no important influence of the final pH on the yields of conversion products. It is assumed that the products are formed by a combination of biotransformation and acid catalyzed conversion from citronellol.

3.2.4. Biotransformation of enantiomerically pure (+)- and (−)-citronellol by sporulated surface cultures of Aspergillus sp. and Penicillium sp.

The bioconversion of enantiomerically pure (*R*)-(+)- and (*S*)-(−)-citronellol by six different *Aspergillus* strains (two *A. niger* and four *A. tubingensis*) and three *P. roqueforti* strains was compared to check the stereospecificity of the

conversion. This was done using SPME as the monitoring technique. It was found that (*S*)-(−)-citronellol was converted by *Aspergillus* specifically to (−)-*cis*-rose oxide with a *cis*/*trans* ratio up to 95/5 (Fig. 6). A typical chromatogram of an SPME extract of a sporulated surface culture of *A. niger*, treated with enantiomerically pure (*S*)-(−)-citronellol, is depicted in Fig. 7.

For *P. roqueforti* the *cis*/*trans* ratio was 70/30. (*R*)-(+)-citronellol was also converted more or less specifically to *cis*-rose oxide (the (+)-isomer in that case) with a *cis*/*trans* ratio of 85/15 and 75/25 for *Aspergillus* and *P. roqueforti*, respectively (Fig. 6). This specific formation of *cis*-rose oxide relative to *trans*-rose oxide can be due to enzymatic fungal conversion, but can also be explained because the *cis*-forms of rose oxide are thermodynamically more stable than the *trans*-forms [26]. The enantioselective bioconversion of (−)-citronellol to (−)-*cis*-rose oxide is very promising from a commercial point of view, since the (−)-*cis*-isomer is the most powerful of the four, exhibiting a bloomy green geranium type odor [15].

4. Conclusion

It can be concluded that the combination of acid-catalyzed heat conversion and fungal bioconversion of citronellol yields rose oxides as the main products. Although the yields obtained after fungal bioconversion of citronellol are not very high, the yield of rose oxides can be increased by further method optimisation and study of the different culture conditions involved. This method offers a gentle and promising way of producing the natural sought after rose oxide flavor compound. Especially the bioconversion of (*S*)-(−)-citronellol to (−)-*cis*-rose oxide is very interesting since (−)-*cis*-rose oxide has a much lower odor threshold value than the other stereoisomers and contributes substantially to the unique bloomy green top notes of geranium and rose oils [15]. SPME was shown to be a very efficient and convenient technique for further screening and method optimisation. It is fast, solvent free and enables to screen sporulated fungal cultures on solid media for their capacity to bioconvert the substrate in a non-destructive way.

Acknowledgements

This work was supported by a grant form the European Community (FAIR-CT98-3559). The authors are indebted to the Fund for Scientific Research, Flanders (Belgium) (F.W.O., Vlaanderen) for a post-doctoral fellowship.

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