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Biotransformation of geraniol, nerol and citral by sporulated surface cultures of *Aspergillus niger* and *Penicillium* sp.

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Abstract

The biotransformation of geraniol, nerol and citral by *Aspergillus niger* was studied. A comparison was made between submerged liquid, sporulated surface cultures and spore suspensions. This bioconversion was also carried out with surface cultures of *Penicillium* sp. The main bioconversion products obtained from geraniol and nerol by liquid cultures of *A. niger* were linalool and α-terpineol. Linalool, α-terpineol and limonene were the main products obtained from nerol and citral by sporulated surface cultures, whereas geraniol was converted predominantly to linalool, also resulting in higher yields. Bioconversion of nerol with *Penicillium chrysogenum* yielded mainly α-terpineol and some unidentified compounds. With *P. rugulosum* the major bioconversion product from nerol and citral was linalool. The bioconversion of nerol to α-terpineol and linalool by spore suspensions of *A. niger* was also investigated. Finally the biotransformation with sporulated surface cultures was also monitored by solid phase microextraction (SPME). It was found that SPME is a very fast and efficient screening technique for biotransformation experiments.

Keywords: *Aspergillus niger*; *Penicillium* sp.; Fungal spores; Biotransformation; Bioconversion; Geraniol; Nerol; Citral; Linalool; α-Terpineol; SPME

1. Introduction

In the course of the work related to the bioconversion of monoterpene alcohols and aldehydes by fungi, the biotransformation of geraniol, nerol and citral by *Aspergillus niger* and *Penicillium* sp. was investigated.

The bioconversion of geranyl and neryl acetate by *A. niger* has been described (Madyastha and Krishna Murthy, 1988a,b). The main reaction found was hydrolysis of terpene acetates to the corresponding alcohols, followed by further ω-hydroxylation to the respective 8-hydroxy derivatives. In all these experiments, liquid cultures of *A. niger* were used. The degradation of geraniol by *A. niger* has also been examined by a Japanese group (Goto, 1967). Using a surface culture of the organism, and adding a methanolic solution of the terpene after a good mat had developed, evidence was found to suggest that geraniol was converted to linalool and partially oxidised to citral (Wood, 1969). Recovery of the fungal metabolites from the cultures was done by steam distillation of the media which were covered with mycelia. It appears that no sporulation of the fungi was observed. To date no literature data are available on the bioconversion of geraniol and nerol by fungal spores of *A. niger*. In this paper, the biotransformation of geraniol and nerol by sporulated surface cultures of five *A. niger* strains and three *Penicillium* strains is compared with that of submerged liquid cultures.

2. Results and discussion

2.1. Biotransformation by liquid cultures

In a first experiment, the biotransformation capacity of two *A. niger* strains (marked AN1 and AN3) towards the terpene alcohols nerol and geraniol was tested in liquid medium.

The main bioconversion products obtained from geraniol and nerol by submerged liquid cultures of *A. niger*

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were linalool, α-terpineol, 2,6,6-trimethyl-2-vinyltetrahydropyran (TMVP) and an unidentified compound (see Table 1). In the control flasks, most of the substrate was recovered unchanged, and some traces (< 2%) of 6-methyl-5-hepten-2-one were noticed. The pH of the liquid cultures at the end of the bioconversion period was checked. For this test also liquid cultures of *Penicillium* were grown. The pH of the media of the *A. niger* cultures was 3.5–4, the media of the *Penicillium* cultures had a final pH of 5.5–7. To test the possible chemical conversion of the substrates due to acid conditions, two more control experiments were run, one with the precursor geraniol, one with nerol (each substrate at a concentration of 100 μl in 50 ml solution). Three conditions were compared, namely neutral pH, pH 5 and pH 3.5 and two liquid media were used, water and YMPG–broth. After 1 h, the solutions were extracted for the first time, and after 7 days, the solutions were extracted for a second time. It was found that neither nerol, nor geraniol were converted after a period of 7 days at neutral pH and at pH 5. At pH 3.5 however, linalool was recovered from the water and the YMPG-solution after 7 days (0.54 and 0.51% conversion respectively). As for the test with nerol at pH 3.5, 0.32 and 0.54%, respectively, of linalool was recovered from the water and the YMPG-solution. Formation of α-terpineol from nerol was also noticed at pH 3.5 (0.94 and 1.45% from the water and the YMPG-solution, respectively). It is interesting to note that previous research showed that linalool was not converted to α-terpineol under acid conditions (pH 3.5; Demyttenaere and Willemen, 1998).

Table 1
Metabolites produced and unchanged substrates recovered from 100 mg of nerol and 100 mg of geraniol by liquid cultures of *Aspergillus niger* (mg)[a]

	Obtained from nerol		Obtained from geraniol	
Metabolites and substrates[b]	AN1	AN3	AN1	AN3
Linalool	4.3	3.9	1.7	2.0
α-Terpineol	5.4	4.5	13.9	14.0
Nerol[b]	0.2	0.2	n.d.	n.d.
TMVP	5.4	5.0	4.3	4.6
Geraniol[b]	n.d.	n.d.	0.1	0.3
Unidentified compound	1.2	0.7	3.8	3.6
Total (mg)	16.5	14.3	23.9	24.5

[a] TMVP = 2,6,6-trimethyl-2-vinyltetrahydropyran; n.d. = not detected; AN1 and AN3 = *A. niger* strains.
[b] Recovery of unchanged substrate.

2.2. Biotransformation with sporulated surface cultures

A biotransformation experiment was run to compare the capability of five *A. niger* strains and three *Penicillium* strains to transform geraniol, nerol and citral as sporulated surface cultures. The five *A. niger* strains were marked AN1, AN2, AN3, AN4 and AN5. The three *Penicillium* species were: *Penicillium chrysogenum* Thom (marked PCT), *P. lividum* Westling (marked PLW) and *P. rugulosum* Thom (marked PRT). The substrates were sprayed onto the sporulated surface cultures as solutions in EtOH absolut (50%). The biotransformation was monitored by dynamic headspace (purge and trap) onto Tenax. Before substrate addition however, a first headspace sample was taken as a control, to test the possible de novo production of volatile products. In the gas chromatography (GC) analyses of these samples, no fungal metabolites were observed. After the last headspace sample, the cultures were extracted by steam distillation–solvent extraction (SDSE; Likens–Nickerson). From geraniol the main products were linalool and 6-methyl-5-hepten-2-one, while the main bioconversion products from nerol were linalool, α-terpineol, limonene, 6-methyl-5-hepten-2-one and TMVP (in order of decreasing contribution). The bioconversion yields of the products from geraniol, nerol and citral are listed in Figs. 1–3.

Since no fungal metabolites were obtained from the control headspace samples, taken before substrate addition, it can be concluded that all products recovered from the cultures were bioconversion products. In general, all strains were able to metabolise the substrates applied and to biotransform them into other terpene compounds. The same bioconversions were repeated. Whilst submerged shaken liquid cultures of *A. niger* gave only poor yields, sporulated surface cultures were able to perform biotransformation reactions. From geraniol, the major bioconversion product was linalool, together with 6-methyl-5-hepten-2-one, limonene and α-terpineol (see Scheme 1). Isomerisation to nerol and further oxidation to geranial and neral was also noticed. Nerol yielded the same products together with *E*-β-ocimene and γ-terpinene (see Scheme 2). As for the *Penicillium* strains, only *P. rugulosum* showed good bioconversion activity.

In a final experiment, the bioconversion of 200 μl (175 mg) nerol and 200 μl (176 mg) geraniol was compared with six cultures: five *A. niger* surface cultures (AN1, AN2, AN3, AN4 and AN5) and one *P. chrysogenum* (PCT) and one control flask (BLA). The biotransformation was monitored by dynamic headspace sampling. At the end of the experiment, the cultures were extracted by SDSE.

In Table 2 the amounts of recovered products from the headspace samples of the cultures treated with nerol are listed.

From Table 2, it is clear that linalool and α-terpineol were the main metabolites, followed by terpinolene and limonene. From *P. chrysogenum* (PCT) a high yield of unidentified compounds was obtained.

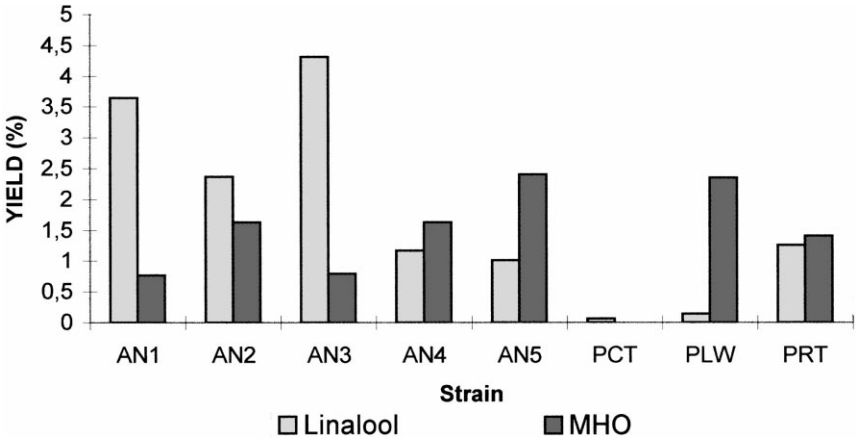


Fig. 1. Yield (%) of the main metabolites produced from geraniol by sporulated surface cultures of *Aspergillus niger* and *Penicillium* sp. MHO = 6-methyl-5-hepten-2-one; AN1, AN2, AN3, AN4, AN5 = *A. niger*; PCT = *P. chrysogenum*; PLW = *P. lividum*; PRT = *P. rugulosum*.

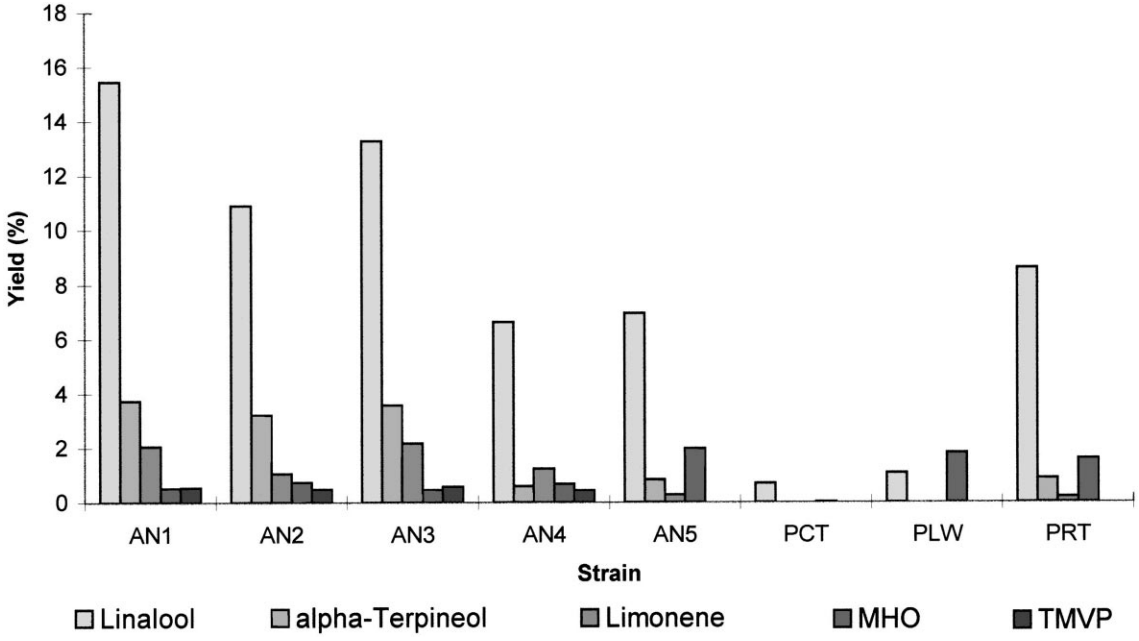


Fig. 2. Yield (%) of the main metabolites produced from nerol by sporulated surface cultures of *Aspergillus niger* and *Penicillium* sp. MHO = 6-methyl-5-hepten-2-one; TMVP = 2,6,6-trimethyl-2-vinyltetrahydropyran; AN1, AN2, AN3, AN4, AN5 = *A. niger*; PCT = *P. chrysogenum*; PLW = *P. lividum*; PRT = *P. rugulosum*.

In Fig. 4, the amounts of recovered products from geraniol are displayed; from this graph it is clear that linalool was the main metabolite recovered from all cultures. The highest yield was obtained by the *A. niger* strain 'AN5', which also produced most geranyl acetate of all strains.

To test the possible acidic conversion of geraniol and nerol due to acid conditions and during heating (solvent extraction) a control experiment was run. For each substrate (geraniol and nerol) six control flasks with different medium at different pH were used. The conversion of the substrate was monitored by dynamic headspace and by SDSE (see Experimental). The results of these control tests are displayed in Table 3 for geraniol and Table 4 for nerol.

From the data displayed in Table 3, it can be concluded that no geraniol conversion was noticed under acidic conditions at room temperature (during headspace sampling). However, during steam distillation, a significant conversion of geraniol to linalool was noticed at pH 3.5 (6.6% conversion in MEA-medium, 11.8% conversion in AGAR-medium). Some conversion to α-terpineol was also noticed under these conditions. In all pH conditions, some isomerisation of geraniol to nerol was observed, but most of the substrate (38–59%) was recovered unchanged from the headspaces and dis-

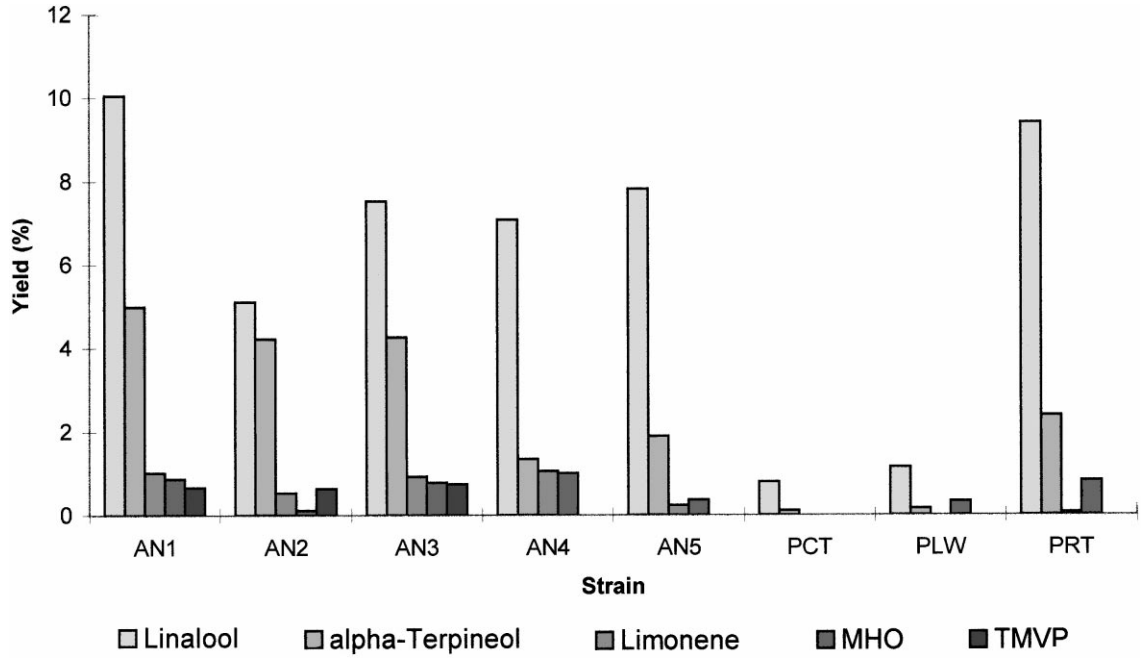
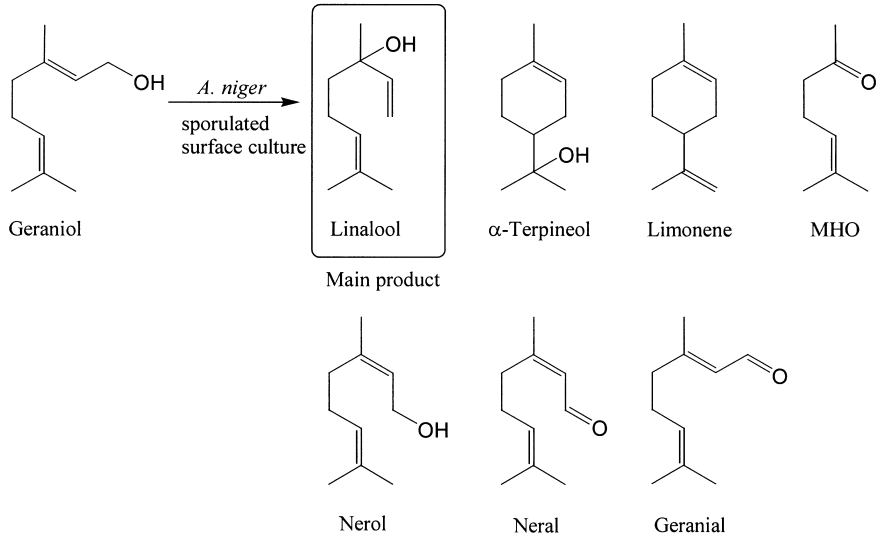


Fig. 3. Yield (%) of the main metabolites produced from citral by sporulated surface cultures of *Aspergillus niger* and *Penicillium* sp. MHO = 6-methyl-5-hepten-2-one; TMVP = 2,6,6-trimethyl-2-vinyltetrahydropyran; AN1, AN2, AN3, AN4, AN5 = *A. niger*; PCT = *P. chrysogenum*; PLW = *P. lividum*; PRT = *P. rugulosum*.



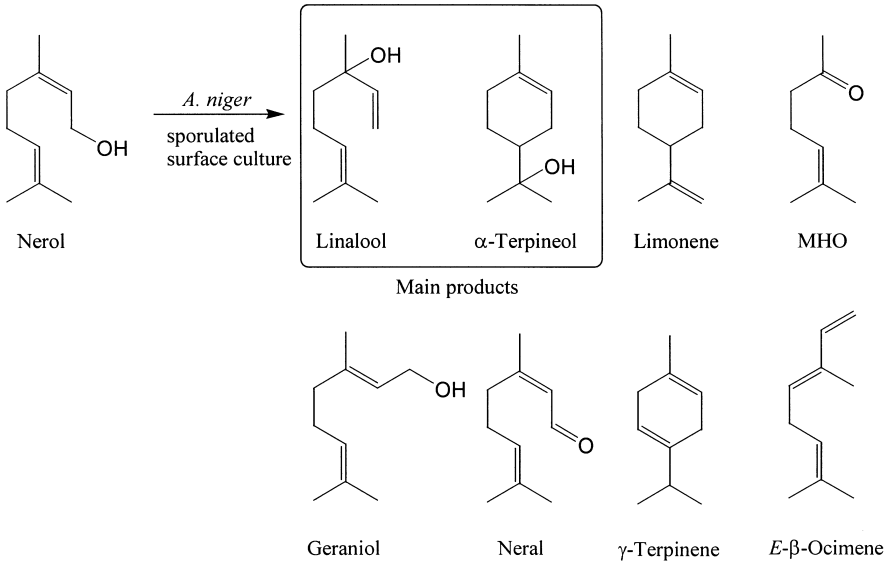
Scheme 1. Bioconversion of geraniol by *Aspergillus niger* as sporulated surface cultures in conical flasks (headspace samples); MHO = 6-methyl-5-hepten-2-one.

tillates. Since the total recovery of bioconversion products from geraniol is displayed in Fig. 4, this conversion of geraniol during heat treatment (steam distillation) is also taken into account (control flask 'BLA' in Fig. 4).

From the data displayed in Table 4, again it can be concluded that no formation of linalool and α-terpineol from the substrate (nerol) was observed during headspace sampling (no heat treatment). However, during steam distillation (heat treatment) a significant conversion of nerol to linalool and α-terpineol was noticed at pH 3.5. Since in Table 2 only the recovered bioconversion products from the headspace samples of the fungal cultures are displayed, there is no product formation due to heat treatment.

2.3. Biotransformation of nerol with spore suspensions of *A. niger*

The biotransformation of nerol was tested with spore suspensions of *A. niger*. This experiment was conducted



Scheme 2. Bioconversion of nerol by *Aspergillus niger* as sporulated surface cultures in conical flasks (headspace samples); MHO = 6-methyl-5-hepten-2-one.

Table 2
Total amounts of recovered products from the headspace samples of cultures of *Aspergillus niger* and *Penicillium* sp. after bioconversion of 200 μl nerol (in μl)[a]

	AN1	AN2	AN3	AN4	AN5	PCT	BLA
MHO	0.45	0.86	0.47	0.15	0.99	0.08	0.03
Limonene	2.16	1.05	2.01	0.21	0.75	0.07	0.00
E-β-ocimene	0.90	0.34	0.38	0.03	0.14	0.01	0.00
Terpinolene	4.33	3.14	3.34	0.22	1.37	0.34	0.01
Linalool	7.09	7.64	6.52	0.77	8.57	0.90	0.02
Unknown compounds	3.07	2.76	2.04	0.59	1.34	22.29	0.05
α-Terpineol	11.99	12.51	8.73	5.87	8.17	6.17	0.00
Nerol	15.21	29.78	17.61	11.02	22.92	22.45	35.06
Neral	0.76	1.03	0.39	0.15	0.52	0.87	1.52
Geraniol	10.29	0.75	0.43	10.24	0.38	0.29	0.34
Geranial	0.09	0.07	0.06	0.04	0.26	0.05	0.46
Neryl acetate	0.10	0.11	0.08	0.03	0.09	0.09	0.23
Geranyl acetate	0.02	0.02	0.01	0.02	0.02	0.00	0.01

[a] MHO = 6-methyl-5-hepten-2-one; AN1, AN2, AN3, AN4, AN5 = *A. niger*; PCT = *P. chrysogenum*; BLA = Blanco.

to test the biotransformation capacity of spores of *A. niger* (strains AN1 and AN3) in spore suspensions, in the absence of medium. In this experiment, nerol was added to the spore suspensions as bioconversion substrate. In a total period of 2 weeks, 100 μl nerol was added to both suspensions and the bioconversion was monitored by taking 5-ml liquid samples, which were extracted with diethyl ether.

In the GC-analyses of the samples, it was observed that both strains of *A. niger* produced mainly α-terpineol. The total yields of the bioconversion products are displayed in Table 5. The total volume of compounds in the medium was calculated as the sum of all samples, plus the expected amount present in the remaining suspension, which was calculated from the last sample. The total amount of recovered unreacted nerol was only 5.5% from AN1 and 8.2% from AN3. This means that the substrate was either converted or metabolised, while part of it could also be lost due to evaporation. It can be concluded that spores of *A. niger* are able to convert nerol to α-terpineol and linalool. The amount of linalool recovered in the spore suspension was much lower than its recovery from the headspace samples of the sporulated surface cultures in the previous experiment. It is assumed that linalool is the intermediate in the conversion of nerol to α-terpineol: indeed, when linalool was given to a spore suspension of *A. niger*, it was also converted to α-terpineol (Demyttenaere and Willemen, 1998). The conversion of linalool to α-terpineol has also been found in citrus (Attaway and Buslig, 1968).

2.4. Use of SPME for biotransformation experiments

The biotransformation of geraniol and nerol by sporulated surface cultures of *A. niger* was monitored using another method, namely solid phase microextraction (SPME), based on the absorption of volatiles on a fibre coated with stationary phase. Two fibres were used: polydimethylsiloxane (100 μm) for the extraction of volatiles and polyacrylate (85 μm) for the extraction of polar semivolatiles.

The fungi were grown as small sporulated surface cultures in 40-ml SPME-vials. The precursors were added as solutions in EtOH. The cultures were sampled by headspace SPME at different time intervals during 30 min.

It was shown that SPME is a very sensitive method with the advantage of being fast and solvent-free. The

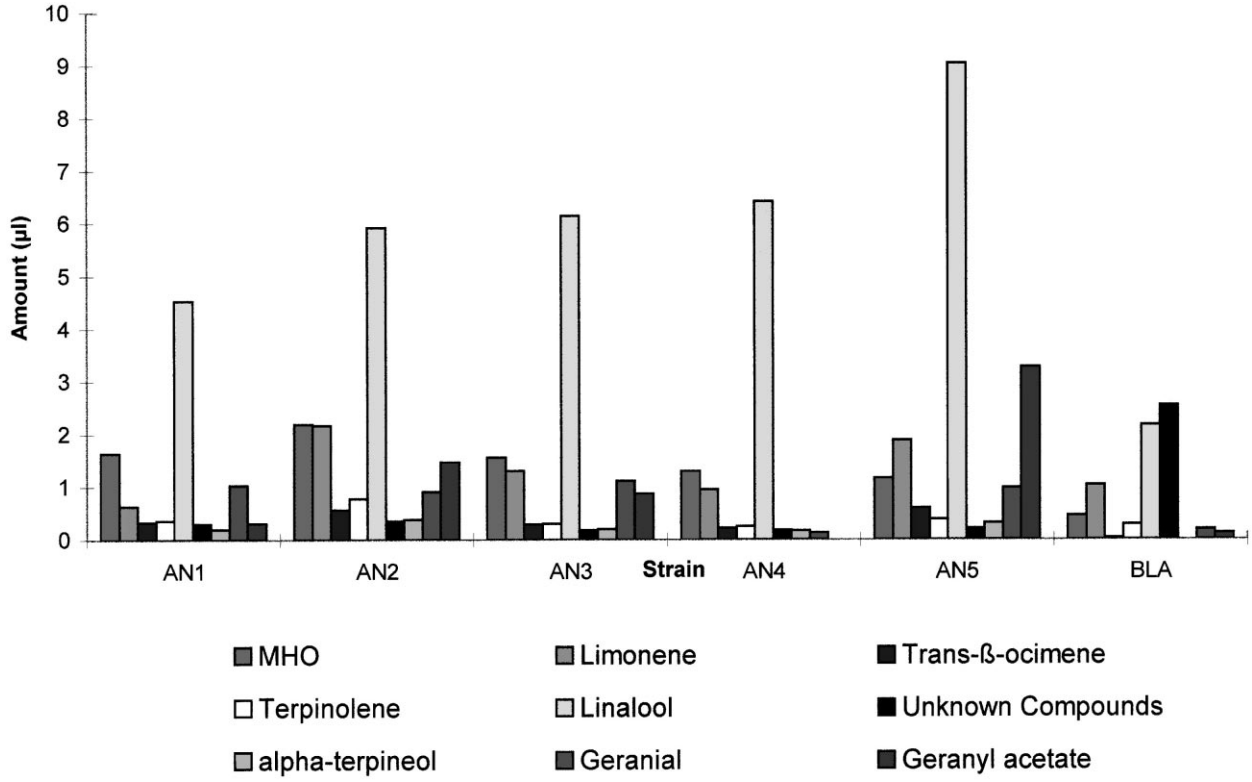


Fig. 4. Products recovered from the bioconversion of 200 µl geraniol by sporulated surface cultures of *Aspergillus niger*. MHO = 6-methyl-5-hepten-2-one; AN1, AN2, AN3, AN4, AN5 = *A. niger*; BLA = Blanco.

Table 3
Amounts (µl) of conversion products recovered from control media at different pH containing 200 µl geraniol during SDSE and headspace

Medium	pH	Sampling method	Geraniol recovered	Linalool recovered	α-Terpineol recovered	Nerol recovered
MEA	6.01	Headspace	5.75	n.d.[a]	n.d.	n.d.
		SDSE	69.88	1.03	n.d.	0.38
MEA	5	Headspace	5.36	n.d.	n.d.	n.d.
		SDSE	113.23	0.86	n.d.	0.30
MEA	3.5	Headspace	6.77	n.d.	n.d.	n.d.
		SDSE	111.41	13.29	0.68	0.57
AGAR	7.3	Headspace	5.31	n.d.	n.d.	n.d.
		SDSE	80.09	0.01	n.d.	0.08
AGAR	5	Headspace	5.42	n.d.	n.d.	n.d.
		SDSE	74.85	0.97	n.d.	0.28
AGAR	3.5	Headspace	2.59	n.d.	n.d.	n.d.
		SDSE	73.37	23.50	0.53	0.40

[a] n.d. = Not detected.

chromatograms obtained after SPME were comparable with those obtained after dynamic headspace adsorption on Tenax. The best fibre was found to be polydimethylsiloxane. In all GC analyses obtained after thermal desorption of the polyacrylate fibre (three fibres were tested), a very high peak of 2,4-diisocyanato-1-methylbenzene was obtained, even after conditioning the fibre for more than 2 h, and when blanco runs were performed. This is an artefact and it is assumed that it originates from the glue of the SPME-fibre.

The main bioconversion products from nerol were linalool and α-terpineol; minor products were limonene, terpinolene, neral and geranial, geraniol and TMVP.

The main product obtained from geraniol was linalool; minor products were limonene, α-terpineol, terpinolene, 6-methyl-5-hepten-2-one and nerol (see Scheme 3).

Table 4
Amounts (μl) of conversion products recovered from control media at different pH containing 200 μl nerol during SDSE and headspace

Medium	pH	Sampling method	Nerol recovered	Linalool recovered	α-Terpineol recovered
MEA	6.01	Headspace	12.48	n.d.[a]	n.d.
		SDSE	67.30	1.73	2.40
MEA	5	Headspace	13.54	n.d.	n.d.
		SDSE	61.06	0.29	0.32
MEA	3.5	Headspace	12.17	n.d.	n.d.
		SDSE	49.61	3.22	6.45
AGAR	7.3	Headspace	6.26	n.d.	n.d.
		SDSE	17.02	0.05	0.07
AGAR	5	Headspace	2.63	n.d.	n.d.
		SDSE	136.32	0.33	0.41
AGAR	3.5	Headspace	10.76	n.d.	n.d.
		SDSE	72.75	20.67	41.95

[a] n.d. = Not detected.

Table 5
Total yields of bioconversion products from 100 μl nerol by spore suspensions of *Aspergillus niger*[a]

Total yield (%)	AN1	AN3
α-Terpineol	23.34	23.06
Linalool	3.74	4.88
Geraniol	0.51	1.34
Unidentified	4.79	5.25

[a] AN1 and AN3 = *A. niger* strains.

The best strains were AN2 and AN3, while AN4 gave poor yields.

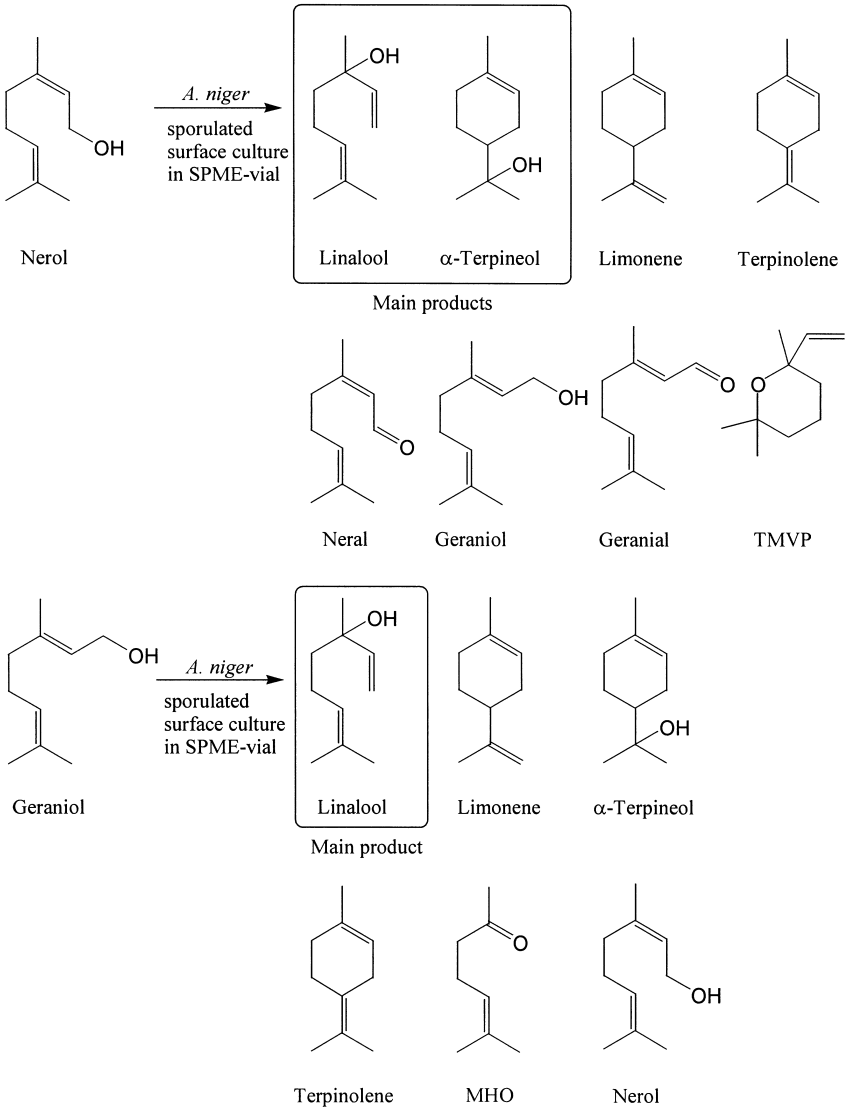
The bioconversion was also tested with *P. chrysogenum* and *P. lividum*. The first strain gave only poor yields, whereas the latter transformed nerol mainly to neryl acetate.

As a control, two vials were filled with agar solution to which a solution of nerol in EtOH was sprayed. In the headspace SPME-extracts of the two control vials without fungal spores, almost pure nerol (95.8 and 94%, respectively) was recovered, in addition to small traces of neral (3.1 and 3.2%, respectively), geraniol (0 and 1.0%, respectively) and geranial (1.0 and 0.9%, respectively).

Another control consisted of two surface cultures of *P. digitatum* in SPME-vials to which nerol was added by spraying. In the headspace SPME-extract of the *P. digitatum* controls, treated with 5 and 2.5 μl of nerol respectively, 80 and 83% of 6-methyl-5-hepten-2-one respectively, 9 and 6% of 6-methyl-5-hepten-2-ol (sulcatol) respectively, and only 2 and 1% respectively, of unchanged substrate, i.e. nerol, were found (and traces of unidentified terpenes). This means that SPME is a very powerful screening technique for biotransformation of volatile terpenoids.

Finally the possible conversion of geraniol and nerol due to acid conditions was monitored by SPME. Therefore, the agar suspension and the solidified MEA-medium were acidified to pH 3.5 and 5 with HOAc. The possible rearrangement of the substrates, nerol and geraniol, was monitored by headspace SPME in 40-ml SPME vials. The first headspace sample was taken during the first day after substrate addition, the second headspace sample after one week. The main conversion product of geraniol and nerol due to acid conditions (pH 3.5) was α-terpineol, contributing to up to 23% of the SPME headspace composition of the control vials. After 1 week, also linalool was formed from geraniol and nerol at pH 3.5 (contributing to up to 7% of the SPME headspace composition of the control vials).

It is very interesting to note that there are only few examples in the literature on the conversion of geraniol and nerol to linalool. It is known that geraniol can be biotransformed to nerol by immobilised grapevine cells (Guardiola et al., 1996). Also the hydroxylation of geraniol and nerol in the allylic position by suspension cultured cells of *Catharanthus roseus* has been reported (Hamada et al., 1997). It was also found that the main product from geraniol during acid-catalysed transformation was linalool and that the main products from nerol were linalool and α-terpineol in 'shochu' model solutions during distillation (Ohta et al., 1991). More recently, the formation of α-terpineol from linalool and from (+)-limonene in citrus juices due to acid conditions was reported (Haleva-Toledo et al., 1999). The formation of α-terpineol was investigated in juice, buffer and model solution. It was found that the pH had a strong effect on α-terpineol formation either from linalool or (+)-limonene in buffer solutions stored at 4 and 35°C. In both cases, the formation of α-terpineol was highest at pH 2.8. At pH 3.8, the rate of formation of α-terpineol from limonene was five times slower and its formation from linalool was reduced by half. The pathways for α-terpineol formation from linalool and (+)-limonene are given in Scheme 4. The conversion of



Scheme 3. Bioconversion of nerol and geraniol by *Aspergillus niger* (SPME-headspace extract); TMVP = 2,6,6-trimethyl-2-vinyltetrahydropyran; MHO = 6-methyl-5-hepten-2-one.

linalool to α-terpineol by spore suspensions of *A. niger* has been published (Demyttenaere and Willemen, 1998). However, no chemical conversion of linalool to α-terpineol was observed at pH 3.5.

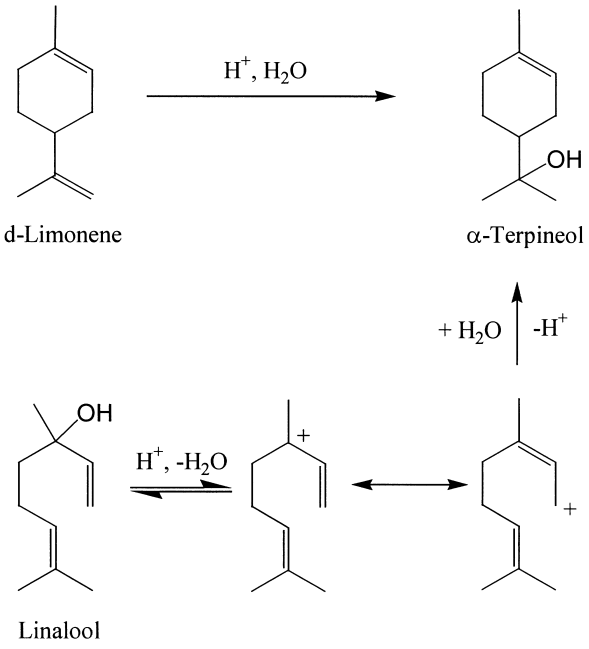
Only one example of the microbial conversion of geraniol and nerol to linalool is known, namely the biotransformation by the fungus *Botrytis cinerea*, yielding a complex mixture of metabolites, including ω-hydroxylation and isomerisation products (Bock et al., 1988). It was also found that linalool was formed from geraniol by isomerisation, induced by the acidity of grape must, and that nerol was converted to α-terpineol by acid catalysis (di Stefano et al., 1992). Only one example is known of the opposite reaction: regioselective isomerisation of linalool to geraniol without nerol formation by an isolated denitrifying bacterial strain 47Lol (Foß and Harder, 1997).

A possible pathway for the acid catalysed conversion of nerol to linalool, α-terpineol, limonene and ocimene is given in Scheme 5.

3. Experimental

3.1. Cultivation of the fungi

Four strains of *Penicillium* and five strains of *A. niger* were used. The *Penicillium* strains were identified by the MUCL (Mycothèque de l'Université Catholique de Louvain, Laboratoire de Mycologie Systématique et Appliquée, Belgium). A strain of *P. chrysogenum* Thom (marked PCT) was isolated from a contaminated agar medium, a strain of *P. lividum* Westling (marked PLW) was isolated from a branch of a Pinus tree, and a strain



Scheme 4. Pathways for α-terpineol formation from linalool and *d*-limonene (after Haleva-Toledo et al., 1999).

of *P. rugulosum* Thom (marked PRT) was isolated from a spoiled orange. The *A. niger* strains were marked as follows: AN1 (*A. niger* isolated from a Cypress branch), AN2 (*A. niger* DSM 821, obtained from Deutsche Sammlung von Mikroorganismen), AN3 (isolated from an African *Welwitschia* plant), AN4 (isolated from a coriander branch), AN5 (*A. niger* ATCC 9142 obtained from the American Type Culture Collection). 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%).

3.2. Biotransformation of terpenes by sporulated surface cultures in conical flasks

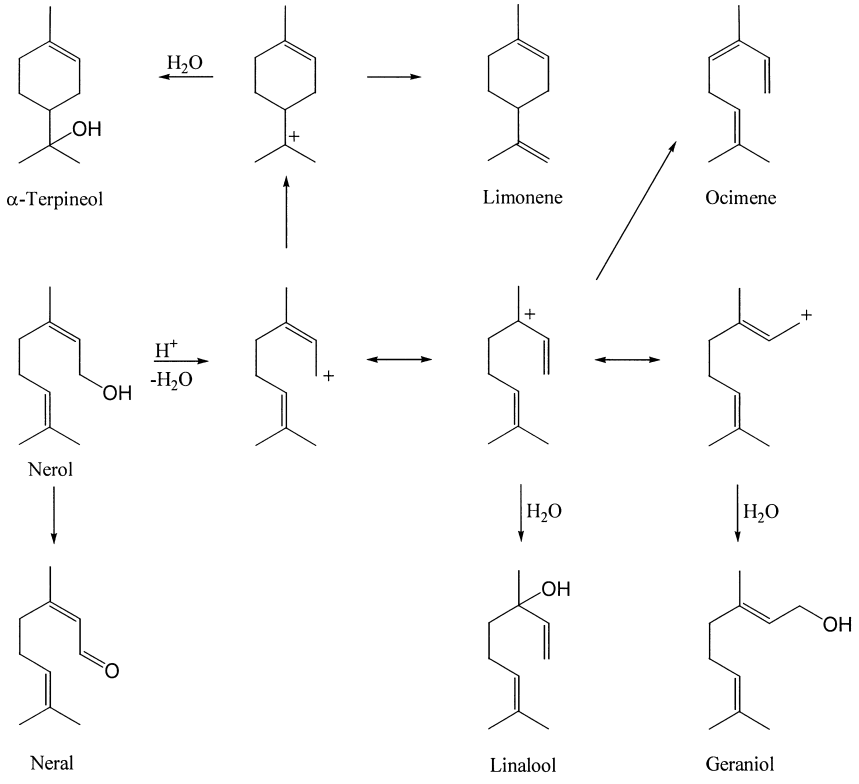
For these experiments 500-ml conical flasks were used, filled with 100 ml MEA and inoculated with the following strains: AN1, AN2, AN3, AN4, AN5, PCT, PLW and PRT. After incubation (1 day at 30°C, 6 days at room temperature), the cultures were grown and had sporulated. Then substrate (geraniol, nerol or citral) was added as a 50% (v/v) solution in EtOH. This was done by spraying 100 μl of a 50% solution of the terpene in EtOH onto the cultures. Control experiments with agar medium without fungi were also performed as described previously (Demyttenaere and De Pooter, 1998). The biotransformation was monitored by dynamic headspace sampling and by steam distillation solvent extraction as described earlier (Demyttenaere and Willemen, 1998).

3.3. Control experiments with different media at different pH and effect of heating

To test the possible acidic conversion of geraniol and nerol due to acid conditions and during heating (during the steam distillation solvent extraction) a control experiment was run. For each substrate (geraniol and nerol) six 500-ml control flasks with different medium at different pH were used, namely three flasks with MEA medium, and three with solidified agar medium. For each type of medium, three different pH conditions were used, namely without pH adjustment (pH MEA medium 6.01; pH agar medium 7.3), with pH adjustment to pH 5, and with pH adjustment to pH 3.5. The pH adjustment was done with HOAc. Substrate addition to the control media was performed by spraying twice 1 ml of a 10% (v/v) solution of the substrate in EtOH (absolut) with a time interval of 48 h. The conversion of the substrate was monitored by dynamic headspace (one headspace sample of 24 h was taken 24 h after the first addition of substrate and one headspace sample of 8 h was taken 48 h after the second substrate addition) and by SDSE, which was performed after 1 week, as described earlier (Demyttenaere and Willemen, 1998).

3.4. Biotransformation of nerol and geraniol by submerged liquid cultures

The biotransformation was run during 2 weeks with the following strains: AN1 and AN3. The fungi were cultivated in 500-ml conical flasks, filled with 100 ml



Scheme 5. Possible pathways for the acid catalysed conversion of nerol to linalool, α-terpineol, limonene and ocimene.

liquid medium (YMPG: yeast extract 0.5%, malt extract 1%, bacteriological peptone 0.5%, glucose 1%) as described earlier (Demyttenaere and Willemen, 1998). The test substrates (geraniol and nerol) were added as a 5% (v/v) solution in EtOH absolut. At different time intervals, 5-ml samples were taken and extracted with Et_2O. This experiment was also run with two control flasks, which contained sterile YMPG-medium that was not inoculated. To one control flask, nerol was added as substrate, while to the other control flask, geraniol was added. The conversion of the substrates (geraniol and nerol) due to acidic conditions was also tested with two acidified control flasks. Therefore, two flasks were filled with sterile YMPG-medium that was not inoculated and acidified to pH 3.5 by addition of HOAc. To one control, nerol was added as substrate, to the other control, geraniol was added. The control flasks were stirred at 150 rpm. After 1 week, 5-ml samples were taken and extracted with Et_2O and analysed by GC/MS.

3.5. Chemical compounds

The substrates used for the biotransformation experiments were: nerol (97%, Aldrich), geraniol (99%, Acros), citral (contg 38.2% neral, 63.2% geranial; Janssen Chimica).

3.6. Headspace analysis

The solid surface cultures were sampled by dynamic headspace purge and trap, as described earlier (Demyttenaere and De Pooter, 1998).

3.7. Biotransformation with spore suspensions

From 2-week-old surface cultures of *A. niger* on Petri dishes, spore suspensions were prepared. To the sporulated surface cultures, 15 ml of a sterile physiological Tween 80 solution (0.85% NaCl, 0.1% Tween 80 in distilled water) was added. The spores were brought into suspension with a sterile glass rod. It was found that the spore suspension thus obtained contained 10^8 spores ml^{-1}. For the preparation of 100 ml spore suspension, the spores of 10 Petri dish surface cultures (strains AN1 and AN3) were harvested. In a period of 2 weeks, four times 25 μl nerol was added to each suspension as a solution of 5% (v/v) nerol/EtOH. The flasks were shaken at 100 rpm and kept at 24°C. 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 analysed by GC and GC–MS.

3.8. SPME

The fungi were cultivated as small sporulated surface cultures in 40-ml SPME-vials (Supelco Inc., Bellafonte, USA). Therefore, the vials were filled with 10 ml medium (MEA), autoclaved, and inoculated with fresh spores of *A. niger* (strains AN1, AN2, AN3, AN4 and AN5), *P. chrysogenum* (PCT) and *P. lividum* (PLW). The vials were covered with cotton wool and the cultures were incubated at 30°C during 24 h and at room temperature during 48 h, after which complete sporulation had taken place. To each sporulated surface culture, 5 μl of a solution of nerol in EtOH (10%) or geraniol in EtOH (10%) was sprayed. The vials were covered with PTFE-silicone septa and open top phenolic closures (Supelco) and stored at room temperature until the start of the headspace SPME-extraction. During extraction, the SPME fibres (PDMS 100 μm and PA 85 μm, Supelco Inc., Bellafonte, USA) were exposed to the headspace of the cultures for 30 min at room temperature (Zhang et al., 1994; Steffen and Pawliszyn, 1996).

As a control, two 40-ml SPME-vials were filled with 10 ml agar medium (2% agar/water) to which 5 μl of the same solution of nerol in EtOH (10%) was sprayed. The control vials were subjected to the same sampling procedure as the fungal cultures.

Another control consisted of two cultures of *P. digitatum*, known for its capacity to biotransform nerol to 6-methyl-5-hepten-2-one (Demyttenaere and De Pooter, 1998). To one sporulated surface culture 50 μl nerol/EtOH (10%; = 5 μl of pure nerol) was sprayed and after 3 days a headspace SPME sample was taken (5 min at 30°C). To the other culture 25 μl nerol/EtOH (10%) was sprayed and after 2 days a headspace SPME sample was taken (5 min at 30°C).

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

GC- and GC–MS-analyses were performed as described earlier (Demyttenaere and Willemen, 1998). 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, hold 2 min; programmed from 40 to 150°C at 5°C min^{-1}, from 150 to 220°C at 10°C min^{-1} and from 220 to 250°C at 20°C min^{-1}, hold 5 min; carrier gas (He) 1.2 ml min^{-1}; split 1/10; ionisation: EI 70 eV; acquisition parameters: scanned m/z: 40–180 (5–20 min), 40–250 (>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 NIST Mass Spectral Library (Version 1.6 d, 1998).

Acknowledgements

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