Screening of Biotransformation Products of Carvone Enantiomers by Headspace-SPME/GC-MS[¥]

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In the course of our continuing work on transformation of monoterpenes by microorganisms, the biotransformation of (+)- and (–)-carvone was carried out. The metabolites formed during microbial transformations were screened using a simple, rapid and efficient technique: Headspace-solid phase microextraction (SPME)/GC-MS. The results as well as the application of this technique are described.

Introduction

A revival of interest has occurred recently on the microbial biotransformations of monoterpenoids (Kieslich, 1976; Kieslich *et al.*, 1986; Krasnobajew, 1984; Berger, 1995; Noma and Asakawa, 1995; van der Werf *et al.*, 1997; Azerad, 2000). Monoterpenes consist basically of two isoprene units resulting in a C-10 skeleton, a range of configurations and stereochemisty (Wise and Croteau, 1999). Due to these diverse features, monoterpenoids possess different flavor and fragrance properties and interesting bioactivity. Other important features of these compounds are as follows: they are highly abundant in nature, they are in **G**enerally **R**ecognized **A**s **S**afe (*abr.*= GRAS) category, easily available from diverse natural sources, they have broad applications in basic chemistry and chemicals, pharmaceuticals, and food additives and fragrance materials (Stammati *et al.*, 1999). The selection of the monoterpene used in this study was also targeted due to the vast use of carvone enantiomers.

Beside the use of carvone in flavor and fragrances, its biological activities can be summarized as: acetylcholine esterase inhibitor (Grundy and Still, 1985), antimicrobial agent (Knobloch *et al.*, 1989; Hinou *et al.*, 1989), antispasmodic (Evans *et al.*, 1978), insecticide (Bestmann *et al.*, 1984), hyperlipidemic and hypercholesterolemic (Imaizumi *et al.*, 1985), CNS depressant (Le Bourhis and Soenen, 1973), convulsant (Wenzel and Ross, 1957). However, the most practical use for (+)-carvone is its use as a natural sprouting inhibitor for potatoes (Bouwmeester *et al.*, 1995).

Carvone enantiomers have been used as starting material in the total synthesis of many natural products and intermediates (Srikrishna and Vijaykumar, 2000; Shimoda and Hirata, 2000; Mazzega *et al.*, 1999). Several biotransformation studies have also been conducted and reported (Noma 1977; Noma and Asakawa, 1995 and 1998; Nishimura and Noma, 1996; van Dyk *et al.*, 1998). More recently, the stereoselective metabolism of carvone has been investigated (Jager *et al.*, 2000).

Problems associated with the isolation and subsequent identification of metabolites formed by microbial transformation of monoterpenes and other substrates are well defined (Job and Blass, 1990; van der Werf *et al.*, 1997; Lye and Woodley, 1999).

In this paper, we demonstrate the extraction of metabolites formed by the biotransformation of carvone enantiomers using a modern technique, namely headspace-solid phase microextraction

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(SPME). Headspace-SPME/GC-MS analysis of the metabolites of carvone enantiomers formed by four different microorganisms were carried out. The efficiency and the advantages of headspace-SPME/GC-MS analysis of microbial biotransformation metabolites are discussed.

Materials and Methods

Materials

(−)-Carvone (Aldrich 12493−1), (+)-carvone (Nippon Terpene Chemical Co. Ltd., Japan).

Microorganisms

All stock cultures were maintained on agar slants (SDA, Acumedia, Baltimore, Maryland, USA), stored at 4 °C and refreshed in every 6 months.

Absidia glauca ATCC 22752 (I), *Cunninghamella echinulata* ATCC 9244 (II), *Penicillium claviforme* MR 376 (III), *Pseudomonas putida* NRRL-13 (IV) were cultivated at room temperature in 250 ml culture flasks containing 100 ml sterile (121 °C, approx. 0.11 MPa , 20 min, autoclave) α-medium [consisting of 20 g glucose; 5 g NaCl; 5 g K_2HPO_4; 5 g yeast extract (Acumedia, Baltimore, Maryland, USA); 5 g peptone (Sigma, St. Louis, MO, USA) per liter of distilled water adjusted to pH 7.0]. Culture flasks were shaken at 140 rpm. After 24−48 h of sufficient growth of the microorganisms, 50 μl of substrate [(−)-carvone or (+)-carvone] was added and incubated for 7 days. The biotransformation products were screened both by headspace-SPME for GC/MS analysis and liquid-liquid extraction of the withdrawn broth (3 ml) which was extracted exhaustively by ethylacetate, concentrated and evaluated by TLC. Substrate controls were composed of sterile medium to which the substrate (50 μl) was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without the addition of substrate. After ten days of incubation, controls were also harvested and analysed by TLC. Routine analyses were performed on pre-coated Silicagel G-25 UV_{254} plates (0.25 mm, Machery-Nagel) using *n*-hexane:acetone (75:25 v/v) as solvent system. Visualization was under UV (254/366 nm) and/or by spraying with anisaldehyde/H_2SO_4 spray reagent.

Headspace-SPME

The SPME fibre (Supelco, Bellefonte, PA, USA) precoated with a 100 μm layer of polydimethylsiloxane (PDMS − red) was used. The coated fibre is accommodated in a hollow, stainless steel needle which allows for an easy injection. The Erlenmayer flask containing the biotransformation medium was kept under sterile conditions in the laminar air flow cabinet and capped with a plastic film and then shaken gently. The fibre was pushed through the plastic film, for exposure to the headspace of the biotransformation broth for 15 min at room temperature. The fibre was then inserted into the injection port of the GC/MS for desorption of the absorbed metabolites for analysis. The SPME experiment was repeated three times to confirm its reproducibilty.

GC/MS *conditions*

The samples were analyzed by GC/MS using a Hewlett Packard GCD system. Innowax FSC column (60 m × 0.25 mm *i.d.*, 0.25 μm film thickness) was used with helium as a carrier gas (1.5 ml/min). GC oven temperature was kept at 100 °C for 5 min and programmed to 220 °C at a rate of 20 °C/min, then kept constant at 220 °C for 11.5 min. Injection was carried out in splitless mode. The injector temperature was at 250 °C. MS were recorded at 70 eV. Mass range was from *m/z* 35 to 425. Library search was carried out using "Wiley GC/MS Library", "TBAM Library of Essential Oil Constituents", and by comparison with authentic samples. Relative percentage amounts were calculated from TIC by the computer.

Results and Discussion

Comprehensive research on monoterpenoid biotransformations and especially on carvone enantiomers have previously been performed. A recent investigation of the biotransformation of carvones and other ketones using various kinds of organisms was reported (Shimoda and Hirata 2000; van Dyk *et al.*, 1998). Plant cell culture biotransformations have also been carried out (Hirata *et al.*, 1990; Hamada *et al.*, 1998). Major biotrans-

Table Ia. (−)-Carvone biotransformation using *Absidia glauca* (I)#.

No	Rt	Compound	1.	2.	3.	4.	5.	6.	7.
1	11.27	(−)-carvone	45.05	24.2	9.71	2.54	0.40	0.34	0.37
2	10.28	(+)-dihydrocarvone	44.42	55.96	60.86	62.80	51.46	40.96	32.63
3	10.46	(+)-isodihydrocarvone	0.74	1.31	2.05	2.75	2.79	3.42	3.04
4	11.04	(+)-neodihydrocarveol	9.23	17.64	25.93	30.38	43.31	52.64	61.29
5	11.23	(−)-dihydrocarveol	0.45	0.81	1.31	1.39	1.93	2.45	2.67
7	11.64	(+)-neoisodihydrocarveol	–	–	–	0.05	0.10	0.09	–

Table Ib. (+)-Carvone biotransformation using *Absidia glauca* (I).

No	Rt	Compound	1.	2.	3.	4.	5.	6.	7.
1′	11.27	(+)-carvone	0.31	–	–	–	0.09	0.09	–
2′	10.28	(−)-dihydrocarvone	8.87	12.91	18.29	18.92	22.87	24.64	26.05
3′	10.46	(−)-isodihydrocarvone	86.5	81.11	73.43	70.80	65.12	62.34	61.25
4′	11.04	(−)-neodihydrocarveol	1.69	1.56	1.96	2.08	2.52	2.58	2.72
5′	11.23	(+)-dihydrocarveol	–	0.11	0.24	0.27	0.39	0.46	0.51
6′	11.46	(−)-isodihydrocarveol	1.81	3.06	4.22	5.57	5.94	6.89	6.31
7′	11.64	(−)-neoisodihydrocarveol	0.82	1.26	1.86	2.36	3.09	3.01	3.17

Table IIa. (−)-Carvone biotransformation using *Cunninghamella echinulata* (II).

No	Rt	Compound	1.	2.	3.	4.	5.	6.	7.
1	11.27	(−)-carvone	0.34	0.12	–	–	–	–	–
2	10.28	(+)-dihydrocarvone	81.57	78.89	77.88	76.84	76.23	73.75	70.97
3	10.46	(+)-isodihydrocarvone	2.95	3.27	3.11	2.65	3.55	4.02	4.78
4	11.04	(+)-neodihydrocarveol	14.90	17.48	18.71	20.16	19.84	21.78	23.74
5	11.23	(−)-dihydrocarveol	0.07	0.12	0.10	0.11	0.12	0.14	0.21
7	11.64	(+)-neoisodihydrocarveol	–	–	0.19	0.24	0.25	0.31	0.31

Table IIb. (+)-Carvone biotransformation using *Cunninghamella echinulata* (II).

No	Rt	Compound	1.	2.	3.	4.	5.	6.	7.
1′	11.27	(+)-carvone	3.48	0.38	0.16	–	–	–	–
2′	10.28	(−)-dihydrocarvone	7.55	11.26	14.19	14.90	18.66	19.64	19.98
3′	10.46	(−)-isodihydrocarvone	87.22	86.20	82.29	80.57	74.97	70.20	67.08
4′	11.04	(−)-neodihydrocarveol	1.29	1.48	1.56	1.58	1.66	1.80	2.14
5′	11.23	(+)-dihydrocarveol	–	–	tr	0.32	0.77	1.54	2.06
6′	11.46	(−)-isodihydrocarveol	0.16	0.24	0.54	0.78	1.14	1.78	2.18
7′	11.64	(−)-neoisodihydrocarveol	0.29	0.45	1.30	1.86	2.81	5.04	6.56

Table IIIa. (−)-Carvone biotransformation using *Penicillium claviforme* (III).

No	Rt	Compound	1.	2.	3.	4.	5.	6.	7.
1	11.27	(−)-carvone	17.24	10.81	2.40	2.04	1.86	1.76	1.74
2	10.28	(+)-dihydrocarvone	53.52	48.04	49.92	46.22	39.91	41.81	38.65
3	10.46	(+)-isodihydrocarvone	1.00	1.13	1.41	1.66	1.77	1.85	2.00
4	11.04	(+)-neodihydrocarveol	27.93	39.47	45.49	49.14	55.50	53.59	56.62
5	11.23	(−)-dihydrocarveol	0.22	0.43	0.42	0.50	0.60	0.56	0.60
6	11.46	(+)-isodihydrocarveol	–	0.12	0.14	0.18	0.15	0.25	0.18
7	11.64	(+)-neoisodihydrocarveol	–	–	0.08	0.10	0.09	0.10	0.11

Table IIIb. (+)-Carvone biotransformation using *Penicillium claviforme* (III).

No	Rt	Compound	1.	2.	3.	4.	5.	6.	7.
1′	11.27	(+)-carvone	44.28	39.74	18.0	11.40	5.02	4.12	4.29
2′	10.28	(−)-dihydrocarvone	1.53	2.63	5.78	8.97	13.87	14.40	14.83
3′	10.46	(−)-isodihydrocarvone	50.38	52.96	70.83	73.69	74.59	75.46	74.49
4′	11.04	(−)-neodihydrocarveol	0.76	0.79	0.90	0.96	1.09	0.99	1.01
6′	11.46	(−)-isodihydrocarveol	1.19	1.78	2.20	2.42	2.67	2.51	2.59
7′	11.64	(−)-neoisodihydrocarveol	1.87	2.11	2.30	2.56	2.76	2.52	2.80

Table IVa. (−)-Carvone biotransformation using *Pseudomonas putida* (IV).

No	Rt	Compound	1.	2.	3.	4.	5.	6.	7.
1	11.27	(−)-carvone	100	98.67	41.25	41.22	41.54	41.58	44.12
2	10.28	(+)-dihydrocarvone	–	1.33	57.15	57.11	56.74	56.77	54.14
3	10.46	(+)-isodihydrocarvone	–	–	1.01	1.04	0.99	1.04	1.02
4	11.04	(+)-neodihydrocarveol	–	–	0.34	0.38	0.41	0.35	0.37
5	11.23	(−)-dihydrocarveol	–	–	0.18	0.17	0.26	0.21	0.28

Table IVb. (+)-Carvone biotransformation using *Pseudomonas putida* (IV).

No	Rt	Compound	1.	2.	3.	4.	5.	6.	7.
1′	11.27	(+)-carvone	100	98.43	8.51	6.80	6.44	6.45	6.02
2′	10.28	(−)-dihydrocarvone	–	–	4.05	4.80	5.69	5.86	5.80
3′	10.46	(−)-isodihydrocarvone	–	1.57	87.44	87.67	87.28	87.10	87.59
6′	11.46	(−)-isodihydrocarveol	–	–	–	0.20	0.15	0.18	0.17
7′	11.64	(−)-neoisodihydrocarveol	–	–	–	0.53	0.45	0.42	0.43

tr: trace <0.01. -= not present/ not dedected.
No: compound number.
Rt: Retention time (minute) on a polar column.
all figures represent **relative percentages** (%) between day 1 to day 7.
Day 1= 24 h (after 24 h)
2= 48 h
3= 72 h
4= 96 h
5= 120 h
6= 144 h
7= 168 h

formation routes of carvone enantiomers are well established and comparatively investigated and reported in detail (Noma and Asakawa, 1995, 1998; Nishimura and Noma, 1996).

In this study we have focused more on the screening, isolation and identification of the biotransformation products of carvone. A recent technique, headspace-SPME, was successfully applied to this work. In previous biological and microbiological studies, the application of this technique for the identification of volatile microbial metabolites was used as an aid for specimen distinction and chemotaxonomy (Nilson *et al.*, 1996; Vergnais *et al.*, 1998). A comprehensive review on the utilisation of SPME in biological materials has recently been published (Theodoridis *et al.*, 2000).

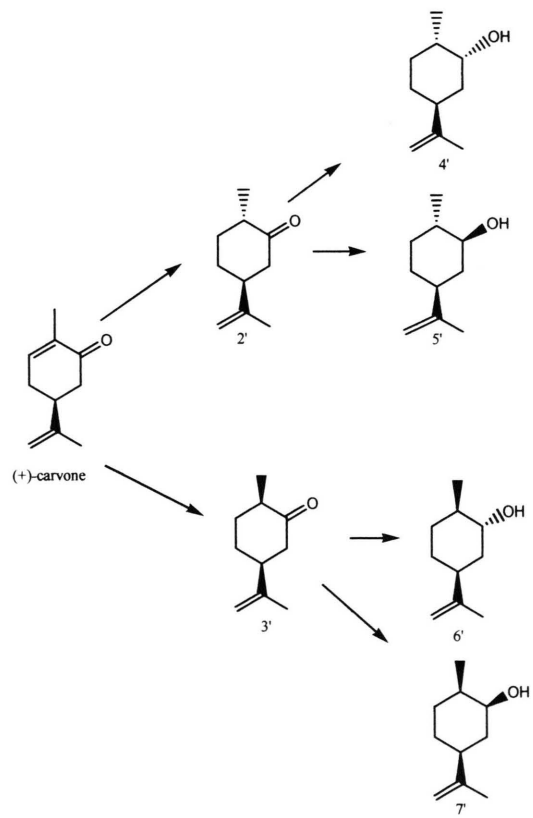
The headspace-SPME technique was useful in rapid extraction of the volatile metabolites which were formed by microbial transformation of the carvone enantiomers. Conventional extraction methods using solvents are laborious and time consuming especially when dealing with small amounts of extractable matter. With such techniques microbial metabolites are also extracted alongside the biotransformation metabolites. Headspace-SPME selectively recovers the volatile metabolites of carvone formed during incubation. Alternatively, recovery of the volatile metabolites

by distillation requires a special set up and may result in loss and modification of compounds due to heat treatment. Whereas, headspace-SPME is a very clean method and can give the results within 15 min. For optimising the conditions for headspace-SPME various suggestions have been considered (Ibañez *et al.*, 1998).

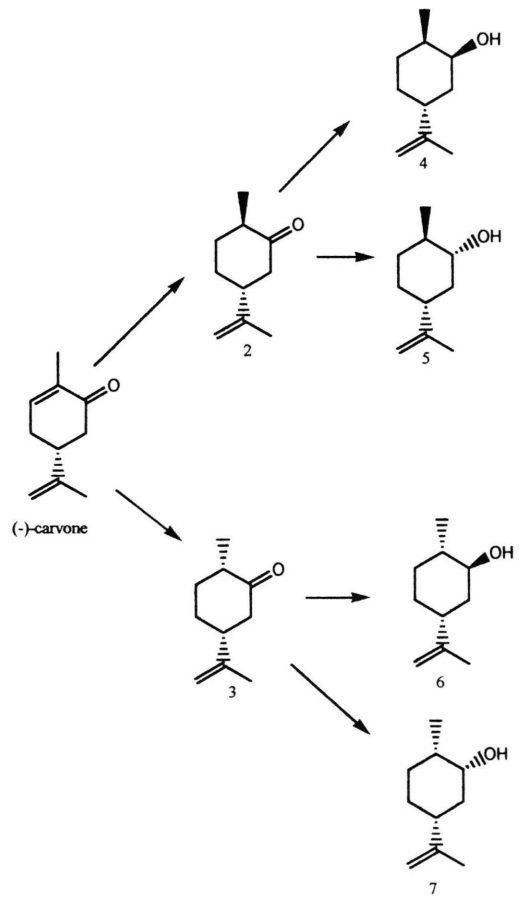
The metabolites of carvone enantiomers transformed by four different microorganisms are shown in Tables I–IV. Interestingly the conversion of (+)-carvone and (−)-carvone leads to different metabolites. Both undergo reduction of the C=C double bond first and then the ketone was reduced in quite good yields. Although this phenomenon is known and reported previously, no previous work was carried out using headspace-SPME for screening such reactions. Routine sample screenings of the extracted (EtOAc) broth by TLC indicated the transformations and their subsequent metabolites compared with authentic samples. Comparable results were also obtained by GC/MS analysis of both, the headspace-SPME and solvent extraction of the culture broth.

With all of the microorganisms tested, the rate of conversion of (−)-carvone was less than the other (+)-enantiomer, the major products being different in both. (+)-Dihydrocarvone (**2**) was the major metabolite **1**, where as (−)-isodihydrocarvone (**3′**) was the main metabolite in **1′**. Except for *P. putida*, (although a different strain) all other microorganisms were used for the first time in the biotransformation of carvone. *P. claviforme* and *P. putida* converted (+)-carvone almost quantitatively in a period of 7 days. Scheme I and II show the possible routes of the metabolites as screened by headspace-SPME.

Finally, it is worthwhile to use this technique in biotransformation experiments, especially for screening of the biotransformation products of terpenoids and volatile compounds. Further experi-



Scheme I. Biotransformation pathways for (+)-carvone.



Scheme II. Biotransformation pathways for (−)-carvone.

ments are under way for the use of SPME to recover nonvolatile metabolites formed by microbial transformations.

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