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Characterisation of volatile compounds in Tunisian fenugreek seeds

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ABSTRACT

In this study, we intend to develop a simple and fast analytical procedure to identify the volatile compounds implicated in the odour of Tunisian fenugreek (*Trigonella foenum-graceum* L.) seeds. Two procedures, solvent extraction and static headspace solid-phase microextraction (SHS-SPME), have been used under different conditions. The volatile compounds extracted were systematically identified using gas chromatography–mass spectrometry, based on their mass spectrum and Kovats index on two columns of different polarity. A total of 67 compounds were identified, some of them being reported for the first time in fenugreek seeds (e.g. several pyrazines, 2,5-dimethyl-4-hydroxy-3(2H)-furanone or 1-epi-cubenol). Methanol was found to be the preferred solvent for high and medium boiling point volatile compounds, such as sotolone and nitrogen compounds. For SHS-SPME, the fibre coated with divinylbenzene/carboxen/polydimethylsiloxane 2 cm was the most suitable for extracting volatile compounds from ground seeds. The efficiency of this fibre was confirmed by direct gas chromatography–olfactometry, with a global odour similar to that of fenugreek seeds.

1. Introduction

Fenugreek (*Trigonella foenum-graceum* L.) is an annual herbaceous aromatic leguminous, widely cultivated in Mediterranean countries and Asia, as it is a popular food (home remedies) consumed in various ways (Billaud & Adrian, 2001a). The pods contain about 10–20 yellowish seeds rich in proteins (30% dry matter) and with a pleasing appetizing aroma (Blank, Lin, Devaud, Fumeaux, & Fay, 1997). Besides, the seeds contain sapogenins (particularly diosgenin) used for medicinal steroids synthesis, steroidal saponins which are responsible for the hypocholesterolemic activity of fenugreek, as well as the free amino acid 4-hydroxyisoleucine (near 80% of free amino acids present in fenugreek seeds) that is responsible for the hypoglycemiant activity of fenugreek (Billaud & Adrian, 2001b; Pau, Petit, Sauvaire, & Ribes, 2003; Sharma et al., 1996; Taylor, Elder, Chang, & Richards, 2000; Taylor et al., 1997). As a spice, fenugreek adds nutritive value to foods as well as flavours; thus, it is used as a seasoning ingredient in products like artificial maple syrup and rum (Shankaracharya, Anandaraman, & Natarajan, 1973). However, despite its interesting properties, fenugreek use remains limited because, after ingestion, this plant leads to bad taste in cattle's meat and milk, as well as a strong odour in human's sweat and urine (odour of "maple syrup" urine disease) (Bartley, Hiltry, Andreson, Clairemont, & Maschke, 1981; Korman, Cohen, & Preminger, 2001; Mazza, Di Tommaso, & Foti, 2002; Sewell, Mosandl, & Bohles, 1999). Up to date sotolone (3-hydroxy-4,5-dimethyl-2(5H)-furanone) has been established as the main impact odour compound from fenugreek seeds responsible of this strong unpleasant odour, on the basis of gas chromatography–mass spectrometry (GC–MS) and gas chromatography–olfactometry (GC–O) (Blank et al., 1997; Girardon, Sauvaire, Baccou, & Bessiere, 1986; Podebrad et al., 1999; Sewell et al., 1999). This molecule has great flavouring potential, with an estimated threshold value of 0.02 ng L^{-1} in air and of 0.3 μg kg^{-1} in water, which are quite low (Blank & Schieberle, 1993; Blank et al., 1997). Its precursors in the seeds are suspected to be 4-hydroxyisoleucine, which is the major amino acid present and whose content decreases during germination, and its lactone [3-amino-4,5-dimethyl-3,4-dihydro-2(5H)-furanone] (Blank, Lin, Fumeaux, Welti, & Fay, 1996; Blank et al., 1997; Girardon et al., 1986). However, other molecules are also involved in the strong odour characteristic of fenugreek, as other potent aroma compounds have been reported in fenugreek using different modes of

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extraction: headspace vacuum entrainment, steam distillation at atmospheric pressure, water extraction, organic solvent (diethyl ether, methanol or dichoromethane) extraction and static headspace solid-phase microextraction (Blank et al., 1997; Girardon, Bessiere, Baccou, & Sauvaire, 1985; Mazza et al., 2002). Hence, Blank et al. (1997) reported 17 odor-active compounds in an aroma extract of fenugreek based on GC–O. Besides sotolone, the most potent odorant compound, diacetyl, acetic acid, linalool, butanoic acid, isovaleric acid, caproic acid, eugenol and 3-amino-4,5-dimethyl-3,4-dihydro-2(5H)-furanone could be identified using GC–MS. In the study of Mazza et al. (2002), 71 compounds were tentatively assigned in a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre extract, with additional 11 compounds still unidentified, while 77 compounds were reported in organic solvent extracts with near 40 additional unknown compounds. Differences in the volatile profiles were noted between Sicilian and Turkish fenugreek seeds. Indeed, fenugreek may be cultivated in different countries, so its constituents may vary depending on the nature of the seeds under investigation. The content of sotolone may, in particular, vary with estimated concentration in the seeds ranging from 3 to 25 mg kg^{-1} depending on the geographical origin of the fenugreek (Blank et al., 1997).

To our knowledge, nothing has been reported until now with regards to Tunisian fenugreek despite its broad use in that country. So, the aim of this work was to study the effect of different extraction conditions using solvent maceration and static headspace solid-phase microextraction (SHS-SPME) on the profile of extracted compounds, in order to determine the most suitable conditions for further determination of odorant compounds involved in the characteristic aroma of Tunisian fenugreek seeds. For this purpose several solvents and SPME fibre coatings were tested. The identification of compounds was carried out by gas chromatography-ion-trap mass spectrometry analysis performed on two types of columns of different polarities, enabling the estimation of relative retention indices using a modified Kovats method. For several compounds of interest, authentic compounds were used to confirm identification. Similarity of the SPME extracts with the odour of fenugreek was finally estimated based on direct gas chromatography–olfactometry.

2. Materials and methods

All experiments were done in duplicate and results are expressed as mean values; in addition, blank experiments were conducted to check for memory effects.

2.1. Fenugreek samples

Fresh fenugreek seeds were purchased from a regional producer, located at La Marsa, north of Tunisia. Samples (1.5 kg) were kept in screw-cap polypropylene containers and stored at −27 °C until the day before analysis, and then placed in the refrigerator at +4 °C until extraction the following day. Just before extraction, seeds were ground in a mortar as the use of an electronic mixer has been reported to induce loss of the more volatile compounds (namely hexanal and 2-methyl-2-butenal) and artifacts (such as production of carvone from limonene) (Mazza et al., 2002).

2.2. Reagents and chemicals

Reagents were all used in the form purchased without additional purification or alteration. Standards of *n*-alkanes (C7–C30) of purity higher than 99% and all reference compounds were from Sigma–Aldrich. HPLC-grade solvents were used (methanol, dichloromethane, ethanol), supplied by Carlo-Erba (Val de Reuil, France). Anhydrous sodium sulfate (analysis grade) was obtained from Merck (Darmstadt, Germany). Deionised water was produced with a Milli-Q system from Millipore (Saint-Quentin-en-Yvelines, France).

Standard solutions of individual compounds were prepared by diluting the pure compounds in methanol to obtain the desired concentration (i.e. 50 mg L^{-1}). All solutions were stored at 4 °C in the dark for less than 5 weeks.

2.3. Solvent extraction

About 10 g of fenugreek seeds were crushed in a mortar before being soaked in 30 ml solvent with agitation at 22 °C, as previously reported (Mazza et al., 2002). The extract was filtered and dried over anhydrous sodium sulfate and finally reduced to a volume of 1 ml by concentration with a Vigreux column. Several solvents (dichloromethane, methanol and ethanol) and various periods of maceration (2, 24 and 48 h) were tested. These solvents were chosen based on the previous study of Mazza et al. (2002) who tested methanol, dichloromethane and water for extracting compounds from fenugreek seeds. In our experiments, problems were encountered using water as the seeds contain galactomannanes which polymerise in presence of water (Billaud & Adrian, 2001a), so it was decided to not study this solvent, and also to change slightly the extraction procedure for methanol so as to avoid the use of water. Ethanol was also tested in our study as it is less toxic than methanol, and so it could be an interesting solvent for the extraction of polar volatile compounds from fenugreek seeds.

2.4. Solid-phase microextraction

The solid-phase microextraction holders for manual sampling and the fibres used for this study were obtained from Supelco. Several fibres were tested, with different stationary phases, various film thicknesses and lengths: polyacrylate (PA) 85 μm film thickness (1 cm long); polydimethylsiloxane (PDMS) 100 μm film thickness (1 cm long), carboxen/polydimethylsiloxane (CAR/PDMS) 75 μm film thickness (1 cm long); and divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm film thickness (1 and 2 cm long). These fibres were chosen to cover a wide range of polarities: apolar (PDMS), intermediate polarity (CAR/PDMS, DVB/CAR/PDMS), polar (PA). The PDMS fibre was chosen with the highest thickness as this should favour the retention of volatile compounds. The DVB/CAR/PDMS fibre was investigated for two lengths: 1 cm to enable comparison with the other fibre coatings tested, and 2 cm to see the influence of the coating volume on the quantities extracted. Indeed, previous results from Mazza et al. (2002) showed the DVB/CAR/PDMS fibre 2 cm to be suitable for the extraction of several volatiles from Sicilian to Turkish fenugreek seeds.

About 17 g of fenugreek seeds were ground in a mortar and quickly conditioned in a 40 ml screw-cap glass vial fitted with silicone-PTFE septum. The vial was then placed in an ultrasonic bath for 30 min at 40 ± 1 °C to help in volatilisation of the compounds as previously recommended for wine samples (Câmara, Arminda Alves, & Marques, 2006). Finally it was placed in a water bath at 22 ± 2 °C for 15 min to equilibrate the headspace, and the fibre was then exposed to the headspace during a sampling period of 60 min at 22 ± 2 °C. A long period of exposure was chosen to favor the adsorption of the polar compounds with low volatility as previously reported for beer samples (Jelen, Wlazky, Wasowicz, & Kaminski, 1998). The SHS-SPME experimental conditions were chosen based on a previous study reporting satisfactory results under these conditions for the extraction of volatile compounds from fenugreek (Mazza et al., 2002). We only changed the equilibration

conditions, keeping the same overall time as in that study (i.e. 45 min), but performing ultrasonication for the first 30 min under moderate heating (i.e. 40 °C) to favour volatilisation of the compounds as already reported for wine samples (Câmara et al., 2006).

Before extraction, all fibres were preconditioned in the injector at the temperature and for the time suggested by the manufacturer, and were cleaned between analyses to prevent cross-contamination. Blank tests and experiments were also performed to check that no carry-over occurred.

2.5. Analysis and identification of compounds

The identification of compounds was carried out by gas chromatography coupled to ion-trap mass spectrometry (GC-IT-MS) using a Trace GC Ultra gas chromatograph coupled to a Polaris Q ion-trap spectrometer (Thermo-Finnigan). Data analysis was performed using the Xcalibur® software version 1.4 SR1. Two fused silica capillary columns were used: the apolar RTx-5MS [30 m × 0.25 mm i.d., 0.25 μm, Restek, oven conditions: 50 °C (held for 5 min), then at 2 °C/min to 100 °C (held for 5 min) and finally at 5 °C/min to 300 °C] and the polar DB-FFAP [30 m × 0.25 mm i.d., 0.25 μm, J&W Scientific, oven conditions: 50 °C (held for 5 min), then at 2 °C/min to 100 °C (held for 5 min) and finally at 5 °C/min to 250 °C]. Other conditions were transfer line maintained at 300 °C for RTx-5MS and at 250 °C for DB-FFAP, and helium carrier gas at constant flow-rate of 1 mL min^{-1}.

A programmed temperature vaporisation (PTV) injector was used. For solvent extracts, 1 μL was injected in splitless mode. The injector temperature was moderate during injection of the sample to avoid possible degradation inside the injector chamber of some thermolabile odorant compounds (the possible partial degradation of sotolone and other furan compounds has already been suspected to occur during injection in gas chromatography as reported by Mazza et al. (2002)). Thus, the injector was initially set at 40 °C for 0.1 min, and then the temperature was raised at 14.5 °C/s to 250 °C (held for 1 min). Cleaning phase was performed by raising the injector temperature at a rate of 10 °C/s to 300 °C (held for 3 min). A solvent delay time of 5 min was used for the detector to avoid overloading the mass spectrometer with solvent. For SPME extracts, fibres were desorbed at 250 °C by keeping them in the PTV injector over 2 min (splitless mode).

Mass spectra in the electron impact (EI) mode (ionisation energy 70 eV; temperature source 200 °C) were generated. Spectra were collected from *m/z* 33 to 350 at 0.63 scan s^{-1}. Mass spectral matches were done by comparison of experimental mass spectra with those of the NIST library. Linear Kovats indices of authentic *n*-alkanes (C7–C30) were estimated for each compounds on both chromatographic columns and compared to the values given in the literature to confirm identification of compounds (Flavornet; Sewell & Clarke, 1987, chap. 4; The Pherobase). For some odorant compounds of potential interest, the comparison of retention times and mass spectra obtained from the samples with those from the pure standards injected under the same conditions were also used for peak confirmation of assignment.

2.6. Sensory evaluation of SPME extracts

The Direct-Gas-Chromathography (D-GC-O) method was applied to the five SPME fibre extracts in order to evaluate their representativeness relative to the original fenugreek sample, following Rega, Fournier, and Guichard (2003). This technique is currently applied to estimate the sensory quality of the global odour of solventless extracts and is based on the direct olfaction of the extract at the sniffing port of a modified GC-Olfatometry device without any chromatographic separation of the odorants in the extract. A Hewlett-Packard 5890 gas chromatograph equipped with a short capillary of untreated silica (80 cm × 0.32 mm i.d) and a sniffing port was used. The flow-rate of the carrier gas (H_2) was 25 mL min^{-1}, and the oven temperature was 200 °C. Each SPME fibre was desorbed in the injection port at 250 °C, and kept into the GC injector only until the end of the sensorial stimulus. A time delay of 4 min was maintained between two fibres. At the sniffing port, the global odour of each SHS-SPME extract was perceived and evaluated by a trained panel of seven assessors. Prior to sensory evaluation, training sessions were performed: panelists were first familiarized with the odour of fenugreek seeds, before being familarized with the D-GC-O device.

A similarity test was performed in triplicate by the seven trained assessors (three sessions of five minutes for each assessor) on the five SHS-SPME odors issued from the headspace of the same fenugreek. Extracts were presented in Latin square order. Sniffers were asked to smell the reference (i.e. fenugreek seeds contained in a sealed glass vial at 22 °C). They had to memorize the odour, and then evaluate with the output of the D-GC-O device for the different SHS-SPME extracts. In each case they had to rate the extract similarity to the reference using a scale ranging from 0 (far from the reference) to 10 (close to the reference). Between two sample evaluations, assessors had to smell the reference again.

2.7. Statistical analysis

Statistical analyses of results from sensory evaluation were done using Statgraphics Centurion® software version 15.1.0.2. Multiple range tests using Fisher's least significant difference (LSD) procedure were performed (at a risk of 5%) to discriminate among the means of similarity values obtained for the different fibres. A three-way ANOVA was also performed on the similarity values in order to study the contribution of each effect (i.e. the type of fibre, the judge, and the repetition effects). Then, factorial discriminant analysis was carried out to discriminate among the five types of fibre, based on the similarity values given by the seven assessors.

3. Results

3.1. Solvent extraction

A total of 45 compounds were identified in solvent extracts as indicated in Table 1, most of them playing a role in the odour of natural extracts. Some of these compounds are reported for the first time in fenugreek seeds: pyridine, *m*-xylene, furfuryl alcohol, β-picoline (i.e. 3-methylpyridine), 2,3-dimethylpyrazine, 2-ethylpyrazine, 5-methylfurfural, 2-ethyl-6-methylpyrazine, trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, methyl nicotinate, 2,4-bis(1,1-dimethylethyl)phenol, 1-epi-cubenol. Differences in the extracted compounds are noted with the solvent used in Table 2. In particular, the presence of sotolone was observed only in alcoholic extracts, which is consistent with previous results reporting its presence in methanolic extracts and its non-detection in dichloromethane extracts (Mazza et al., 2002). Similarly, the presence of nitrogen compounds is noticeable mainly in methanolic extract.

Considering the total area counts of detected peaks in the extracts, ethanol appeared as the most efficient solvent compared to methanol and dichloromethane as indicated in Fig. 1a, significantly higher total peak areas being observed with ethanol. However, as observed in the chromatograms, this solvent extracts higher levels of compounds of low and medium volatility such as fatty acids and unidentified sapogenins which are not thought to be involved in the odour of fenugreek. This effect was more pronounced for long extraction times. On the opposite, despite its chemical similarity with ethanol, methanol extraction was not significantly affected by the soaking time. As well, this solvent was

Table 1
Compounds identified in solvents (soaking time: 48 h) and in fibre extracts.

No.	Identified compounds	Retention indices on RTx-5MS (experiment/ literature)		Retention indices on DB-FFAP (experiment/ literature)		Relative area (%) in the extracts								Odorant notes according to literature	Ref.[a]
		Exp.	Lit.	Exp.	Lit.	MeOH	EtOH	CH_2Cl_2	DVB/CAR/ PDMS 2 cm	DVB/CAR/ PDMS 1 cm	CAR/ PDMS	PDMS	PA		
46	Dimethyl sulfide	< 800	515	<1000	716	nd	nd	nd	29.15	38.07	68.12	2.47	3.27	Fruity, sulfury	NI
1	Acetic acid*	< 800	606	1456	1454	1.14	0.11	Trace	0.85	0.67	0.68	nd	0.17	Sour, pungent	1,2
2	Propanoic acid	< 800	668	1526	1523	0.03	nd	Trace	0.16	0.14	nd	nd	nd	Rancid	1,2
3	2-Methyl-2-butenal	< 800	715	1091	1090	0.32	nd	0.20	3.88	4.66	0.39	0.13	Trace	Coffee-like	1,2
4	Pyridine*	< 800	753	1209	1193	0.16	0.03	nd	nd	nd	nd	nd	nd	Fat-like	NI
47	2-Methyl-2-buten-1-ol	< 800	766	1308	1316	nd	nd	nd	3.11	3.66	0.56	0.21	0.19	–	1,2
5	Dimethyl sulfoxide*	843	843	1553	1549	0.03	0.02	0.01	0.16	0.28	0.89	nd	nd	—	2
6	*m*-Xylene	864	866	1135	1132	nd	0.03	Trace	0.18	0.06	Trace	nd	nd	Plastic	NI
7	Furfuryl alcohol	882	NF	1632	1661	0.10	nd	nd	nd	nd	nd	nd	nd	Burnt sugar	NI
8	2-Heptanone*	886	888	1178	1177	0.01	0.03	Trace	0.25	0.10	0.05	nd	nd	Soap	2,3,4
9	β-Picoline	919	NF	1301	1319	0.02	Trace	nd	nd	nd	nd	nd	nd	Green earthy	NI
10	2,3-Dimethylpyrazine	926	920	1336	1315	0.03	Trace	0.01	nd	nd	nd	nd	nd	Caramel	NI
11	2-Ethylpyrazine	928	906	1317	1314	0.26	nd	nd	nd	nd	nd	nd	nd	Nutty	NI
48	Valeric acid	928	911	1740	1721	nd	nd	nd	0.05	nd	nd	nd	nd	Sweaty, pungent	2
49	Dimethyl sulfone	931	925	1887	1890	nd	nd	nd	0.04	0.02	Trace	nd	nd	Sulfur,burnt	2
12	5-Methylfurfural	960	964	1544	1560	0.01	nd	nd	nd	nd	nd	nd	nd	Burnt sugar	NI
50	Phenol	987	980	1996	1998	nd	nd	nd	0.02	0.03	0.02	0.02	0.05	Medicinal	2,3,4
13	6-Methyl-5-hepten-2-one*	986	987	1320	1320	Trace	Trace	Trace	0.19	0.22	0.01	Trace	0.03	Vinyl, mushroom	2
14	2-Pentylfuran*	989	993	1213	1220	nd	Trace	0.02	0.97	0.58	Trace	0.07	Trace	Buttery	1,2
51	6-Methyl-5-hepten-2-ol*	995	992	1443	1443	nd	nd	nd	0.58	0.50	Trace	nd	nd	Oily-green	2
15	δ-3-Carene*	1006	1004	1129	1130	0.01	0.02	nd	0.36	0.50	0.06	Trace	nd	Lemon	1
16	2-Ethyl-6-methylpyrazine	1011	1003	1364	1381	0.02	nd	nd	nd	nd	nd	nd	nd	Roasted	NI
17	Trimethylpyrazine	1013	999	1387	1387	0.07	nd	nd	nd	nd	nd	nd	nd	Potato-like	NI
18	1,4-Dichlorobenzene*	1014	1016	1420	1434	nd	Trace	nd	0.08	0.07	Trace	Trace	nd	—	2
19	Caproic acid	1017	1019	1845	1846	0.59	0.48	0.13	0.71	0.30	0.06	0.16	0.15	Sweaty, cheese	1,2
52	*p*-Cymene*	1022	1025	1245	1248	nd	nd	nd	0.46	0.36	Trace	0.07	nd	Herbal, spicy	2
20	Limonene*	1025	1031	1171	1178	Trace	nd	nd	1.54	1.20	Trace	0.24	Trace	Citrus, fruity	2
53	Benzyl alcohol	1027	1032	1862	1897	nd	nd	nd	0.04	0.02	Trace	nd	Trace	Floral, fruity	2
54	3-Octen-2-one*	1040	1040	1390	1388	nd	nd	nd	0.42	0.15	0.01	Trace	Trace	Spicy	1,2,3,4
55	E,E-3,5-octadien-2-one	1073	1068	1549	1563	nd	nd	nd	0.51	0.19	Trace	0.28	0.03	Fresh, sweet	2
56	E,Z-3,5-octadien-2-one	1104	1098	1504	1515	nd	nd	nd	0.36	0.09	Trace	0.05	0.04	Synthetic, plastic	2
21	3-Ethyl-2,5-dimethylpyrazine	1085	1079	1421	1429	0.16	nd	nd	nd	nd	nd	nd	nd	Roasty	NI
22	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	1108	1090	1998	2016	0.20	0.02	Trace	nd	nd	nd	nd	nd	Burnt sugar	NI
23	Sotolone*	1118	1120	2193	2196	0.09	0.06	Trace	0.02	0.02	nd	nd	nd	Burnt	1,2
57	2-Phenylethyl alcohol	1120	1122	1892	1903	nd	nd	nd	0.06	0.08	Trace	0.08	0.05	Sweet, floral	2
24	Methyl nicotinate	1149	1137	1739	NF	0.03	0.01	nd	nd	nd	nd	nd	nd	Tobacco	NI
58	Menthone*	1153	1154	1427	1427	nd	nd	nd	0.14	0.17	Trace	0.02	nd	Herbaceous	NI
25	Naphthalene*	1181	1179	1699	1694	nd	nd	0.02	0.20	0.10	Trace	0.21	0.05	Medicinal	2
26	Coumaran	1239	1268	2359	NF	0.84	0.13	nd	nd	nd	nd	nd	nd	—	4
59	Carvone*	1242	1249	1695	1693	nd	nd	nd	0.12	Trace	Trace	nd	Trace	Spicy	1
60	Benzoic acid	1262	1276	2469	NF	nd	nd	nd	0.07	0.03	0.02	nd	Trace	Balsamic	2
27	Non-anoic acid	1278	1275	2165	NF	0.06	0.07	0.08	0.07	0.02	0.02	0.20	nd	Green, fat	2
61	Trans-anethol	1290	1289	1803	1808	nd	nd	nd	Trace	nd	nd	nd	Trace	Sweet, anise	2
28	*p*-Vinylguaiacol	1329	1323	2158	2185	0.06	Trace	nd	nd	nd	nd	nd	nd	Clove, curry	2
29	Eugenol*	1362	1351	2159	2153	0.01	nd	Trace	nd	nd	nd	nd	nd	Spicy, honey	1,2
62	γ-Nonalactone	1374	1366	2002	2005	nd	nd	nd	0.04	0.05	Trace	0.19	0.05	Coconut, peach	2,3
63	α-Copaene	1379	1376	1525	1536	nd	nd	nd	0.12	0.14	0.03	0.32	Trace	Woody	NI
30	2-Butyl-2-octenal	1381	1378	1640	1659	nd	0.03	Trace	0.15	nd	Trace	Trace	Trace	—	2
64	Decanoic acid	1397	1380	2273	2274	nd	nd	nd	0.05	0.01	Trace	0.16	0.10	Rancid, fat	2,3,4
65	α-Ionone*	1433	1426	1819	1809	nd	nd	nd	0.01	nd	nd	0.03	nd	Floral	NI

Table 1 (*continued*)

No.	Identified compounds	Retention indices on RTx-5MS (experiment/literature)		Retention indices on DB-FFAP (experiment/literature)		Relative area (%) in the extracts								Odorant notes according to literature	Ref.[a]
		Exp.	Lit.	Exp.	Lit.	MeOH	EtOH	CH_2Cl_2	DVB/CAR/PDMS 2 cm	DVB/CAR/PDMS 1 cm	CAR/PDMS	PDMS	PA		
66	Trans-geranyl-acetone*	1459	1455	1836	1840	nd	nd	nd	0.01	Trace	Trace	0.08	Trace	Fresh, floral	NI
31	α-Muurolene	1480	1480	1719	1718	0.03	0.01	0.07	0.40	0.16	nd	0.69	Trace	Woody	2,3,4
67	γ-Cadinene	1510	1512	1757	1752	nd	nd	nd	0.02	nd	nd	Trace	nd	Herbaceous	NI
32	2,4-Bis(1,1-dimethylethyl)-phenol	1519	NF	2312	NF	Trace	Trace	Trace	0.01	Trace	nd	Trace	nd	—	NI
33	cis-Calamenene	1529	1523	1795	1807	0.16	0.04	0.03	0.04	0.03	0.01	0.19	Trace	Spicy	2,3,4
34	Dihydroactinidiolide	1537	1539	2316	2294	0.01	Trace	Trace	0.17	0.13	Trace	0.82	0.21	Sweet	2,3,4
35	Dodecanoic acid*	1582	1580	2457	2458	nd	nd	Trace	0.56	0.22	0.06	2.27	1.38	Metallic	2,3,4
36	1-epi-Cubenol	1622	1620	2075	2080	1.74	1.79	3.26	2.32	1.58	0.13	7.84	2.99	Herbal	NI
37	Pantolactone	1679	1685	2034	2033	0.10	0.08	nd	0.03	0.05	0.01	nd	nd	Cotton candy	2
38	Tetradecanoic acid*	1777	1780	2701	2724	0.34	0.25	0.38	1.21	0.47	0.18	4.10	3.09	Waxy	2
39	Pentadecanoic acid	1876	1878	2809	NF	Trace	0.23	Trace	0.91	0.34	0.28	3.15	1.70	—	2
40	Hexadecanoic acid*	1984	1984	2871	2871	5.70	9.35	14.1	2.10	1.45	0.6	15.42	9.35	Oily	2,4
41	Heptadecanoic acid	2077	2022	2968	NF	0.14	0.64	0.73	nd	nd	nd	nd	nd	—	2
42	Linoleic acid*	2097	2078	>3000	NF	10.35	15.95	22.8	nd	nd	nd	nd	nd	—	2
43	Octadecanoic acid*	2128	2124	>3000	3181	1.06	3.02	3.33	1.19	0.31	0.08	3.53	1.22	—	2,4
44	Linolenic acid	2162	NF	>3000	3292	3.18	4.42	5.62	nd	nd	nd	nd	nd	—	2,4
45	Oleic acid*	2173	2161	>3000	3184	3.69	6.97	8.76	1.61	0.4	0.10	4.14	1.50	Fatty	2,4

Trace, ‹0.01%; nd, not detected; *, confirmation by standard analysis; NI, newly identified in fenugreek; NF, not found.

[a] The references are as numbered below: Blank et al. (1997); Mazza et al. (2002); Girardon et al. (1985); Nijssen, Visscher, Maarse, Willemsens, and Boelens (1996).

more efficient in extracting volatile compounds such as acetic acid, pyridine and 2-methyl-2-butenal (see Table 1). In the case of dichloromethane, quantities extracted decreased over time as shown in Fig. 1a. This is consistent with the high volatility of dichloromethane, resulting in an increased solvent evaporation as the soaking duration is longer, with possible entrainment of extracted compounds, especially volatiles such as 2-heptanone. Dichloromethane was also found to yield higher amounts of compounds with no relevance to fenugreek aroma, such as unidentified sesquiterpenes and long chain alkanes.

Finally, methanol was preferred because (i) it presents the least variation in the signals as a function of soaking time; (ii) it offers the highest level of some volatile compounds, in particular nitrogen compounds; (iii) it extracts significant amounts of sotolone. Repeatability of the experiments was satisfactory (most RSDs in the range 3–25%) considering the low levels analysed.

3.2. Fibre extraction

The volatile compounds identified after extraction of fenugreek seeds using the different fibres tested are presented in Table 1, with their relative contribution to the total area. Repeatability of the experiments was found to be acceptable (most RSDs in the range 1–30%) considering the low levels analysed and the technique used.

A total of 50 compounds were found, most of them with odorant notes. Some of them are reported for the first time in fenugreek seeds: dimethyl sulfide, *m*-xylene, menthone, α-copaene, α-ionone, trans-geranyl-acetone, γ-cadinene, 2,4-bis(1,1-dimethylethyl)phenol, and 1-epi-cubenol. The total area counts of the chromatograms obtained from SHS-SPME extracts are given in Fig. 1b. The DVB/CAR/PDMS coated fibre extracted the highest total amount of volatile compounds, followed by the CAR/PDMS coating, while the total amount of compounds extracted with the PDMS and PA coated fibres was much lower. In fact PDMS, as a non-polar coating, extracts non-heterocyclic volatile compounds such as α-muurolene, dodecanoic acid, tetradecanoic acid and hexadecanoic acid, which are not really relevant for the aroma of fenugreek seeds, whereas other volatile compounds previously pointed out as the character impact flavour compounds of fenugreek seeds (such as valeric acid and sotolone) were not extracted, as indicated in Table 2. The low extractive potential of PA, a polar crystalline polymer, may be due to the fact that this coating allows diffusion of analytes at a slower rate, so that longer extraction times are required to reach a correct enrichment capacity (Ho, Wan Aida, Maskat, & Osman, 2006).

The highest peak areas of *O*-heterocyclic compounds were obtained using the DVB/CAR/PDMS fibre coating (1 and 2 cm lengths). Indeed, this coating seems to be the most efficient for the analysis of aroma compounds in fenugreek seeds as it combines the characteristics of the CAR/PDMS coating with the addition of PDMS/DVB proprieties. Thus, the DVB layer has insaturations due to the aromatic ring, giving rise to π–π interactions with the double bonds of *O*-heterocyclic compounds (such as sotolone, 2-pentylfuran, γ-nonalactone, and pantolactone) as already observed for aroma compounds in palm sugar (Ho et al., 2006). On the other hand, CAR is a porous carbon with a high surface area (around 1200 $m^2 g^{-1}$) due to the presence of different types of pores (micro-, meso- and macropores). As already reported, the CAR/PDMS is efficient for extracting small molecules such as dimethyl sulfide, acetic acid, 2-methyl-2-butenal, 2-methyl-2-buten-1-ol, dimethyl sulfoxide, and 2-heptanone (Garcia-Esteban, Ansorena, Astiasaran, & Ruiz, 2004; Ho et al., 2006). The presence of the PDMS/DVB layer in the DVB/CAR/PDMS fibre favours the extraction of high boiling compounds such as long chain fatty acids as previously observed for dry cured ham samples (Garcia-Esteban et al., 2004). These characteristics explain the different amounts of compounds with low and high boiling points extracted with each type of fibre observed in this study. The DVB/CAR/PDMS fibre coating was also reported to be the most efficient for the extraction of several volatiles, such as in cocoa products or in a traditional Chinese medicine (Ducki, Miralles-Garcia, Zumbé, Tornero, & Storey, 2008; Zhang, Qi, Shao, Zhou, & Fu, 2007).

Table 2
Comparison of the compound profiles obtained with the different extracting media's.

No.	Compounds	Organic solvents			SHS-SPME fibres				
		MeOH	EtOH	CH_2Cl_2	DVB/CAR/PDMS 2 cm	DVB/CAR/PDMS 1 cm	CAR/ PDMS	PDMS	PA
Aldehydes									
3	2-Methyl-2-butenal	x		x	x	x	x	x	
12	5-Methylfurfural	x							
30	2-Butyl-2-octenal		x		x				
Ketones									
8	2-Heptanone*	x	x		x	x	x		
13	6-Methyl-5-hepten-2-one*								
22	2,5-Dimethyl-4-hydroxy-3(2H)-furanone				x	x	x		x
23	Sotolone*	x	x		x	x			
37	Pantolactone [or 4,5-dihydro-4,4-dimethyl-3-hydroxy-2(3H)-furanone]				x	x	x		
54	3-Octen-2-one*				x	x	x		
55	E,E-3,5-Octadien-2-one				x	x		x	x
56	E,Z-3,5-Octadien-2-one				x	x		x	x
58	Menthone*				x	x		x	
59	Carvone*				x				
62	γ-Nonalactone [or dihydro-5-pentyl-2(3H)-furanone]				x	x		x	x
65	α-Ionone*				x			x	
66	Trans-geranyl-acetone*				x			x	
Acids									
1	Acetic acid*	x	x		x	x	x		x
2	Propanoic acid	x			x	x			
19	Caproic acid	x	x	x	x	x	x	x	x
27	Non-anoic acid	x	x	x	x	x	x	x	
35	Dodecanoic acid*				x	x	x	x	x
38	Tetradecanoic acid*	x	x	x	x	x	x	x	x
39	Pentadecanoic acid		x		x	x	x	x	x
40	Hexadecanoic acid*	x	x	x	x	x	x	x	x
41	Heptadecanoic acid	x	x	x					
42	Linoleic acid*	x	x	x					
43	Octadecanoic acid*	x	x	x	x	x	x	x	x
44	Linolenic acid	x	x	x					
45	Oleic acid*	x	x	x	x	x	x	x	x
48	Valeric acid				x				
60	Benzoic acid				x	x	x		
64	Decanoic acid				x	x		x	x
Alcohols									
7	Furfuryl alcohol	x							
47	2-Methyl-2-buten-1-ol				x	x	x	x	x
51	6-Methyl-5-hepten-2-ol*				x	x			
53	Benzyl alcohol				x	x			
57	2-Phenylethyl alcohol				x	x		x	x
Sulfur compounds									
5	Dimethyl sulfoxide*	x	x	x	x	x	x		
46	Dimethyl sulfide				x	x	x	x	x
49	Dimethyl sulfone				x	x			
Nitrogen compounds									
4	Pyridine*	x	x						
9	β-Picoline	x							
10	2,3-Dimethylpyrazine	x		x					
11	2-Ethylpyrazine	x							
16	2-Ethyl-6-methylpyrazine	x							
17	Trimethylpyrazine	x							
21	3-Ethyl-2,5-dimethylpyrazine	x							
24	Methyl nicotinate	x	x						
Furans									
14	2-Pentylfuran*			x	x	x		x	
26	Coumaran	x	x						
Phenols									
28	*p*-Vinylguaiacol	x							
29	Eugenol*	x							
32	2,4-Bis(1,1-dimethylethyl)-phenol				x				
50	Phenol				x	x	x	x	x
Monoterpenes									
15	δ-3-Carene*	x	x		x	x	x		
20	Limonene*				x	x		x	
34	Dihydroactinidiolide	x			x	x		x	x
52	p-Cymene*				x	x		x	

Table 2 (*continued*)

No.	Compounds	Organic solvents			SHS-SPME fibres				
		MeOH	EtOH	CH_2Cl_2	DVB/CAR/PDMS 2 cm	DVB/CAR/PDMS 1 cm	CAR/PDMS	PDMS	PA
Sesquiterpenes									
31	α-Muurolene	x	x	x	x	x		x	
33	cis-Calamenene	x	x	x	x	x	x	x	
36	1-epi-Cubenol	x	x	x	x	x	x	x	x
63	α-Copaene				x	x	x	x	
67	γ-Cadinene				x				
Aromatic hydrocarbons									
6	*m*-Xylene		x		x	x			
25	Naphthalene*			x	x	x		x	x
Others									
18	1,4-Dichlorobenzene*				x	x			

3.3. Odour quality of SPME extracts

As our aim was to select the best SPME extraction conditions, not only from the analytical point of view but also for a further characterisation of the odour-active compounds in fenugreek, it was crucial to evaluate the odour representativeness of SPME extracts by a similarity test. The Box and Whiskers plot (Fig. 2) shows the resulting means of similarity values obtained by the seven assessors for each type of fibre coating. The PDMS and PA fibre coatings led to a poor extract from a sensory point of view (mean similarity values of 1.7 and 2.5, respectively, as indicated in Table 3), with a poor repeatability observed for the latter coating. The CAR/PDMS coating led to higher similarity values (mean value of 5.9), but again with a low repeatability. It appears that only the DVB/CAR/PDMS fibre coating made it possible to obtain a global

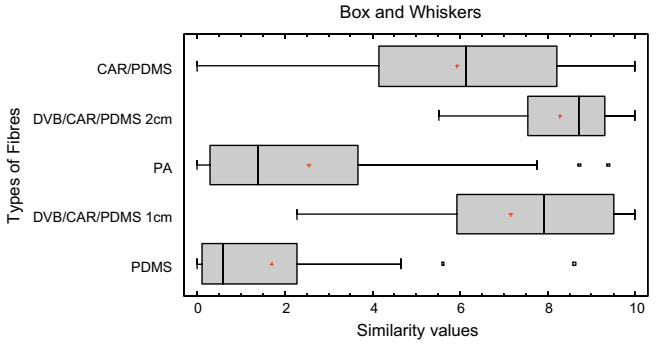

Fig. 2. Similarity values obtained for SHS-SPME extracts by the sensory panel of seven assessors; the scale ranges from 0 (far from the reference) to 10 (close to the reference).

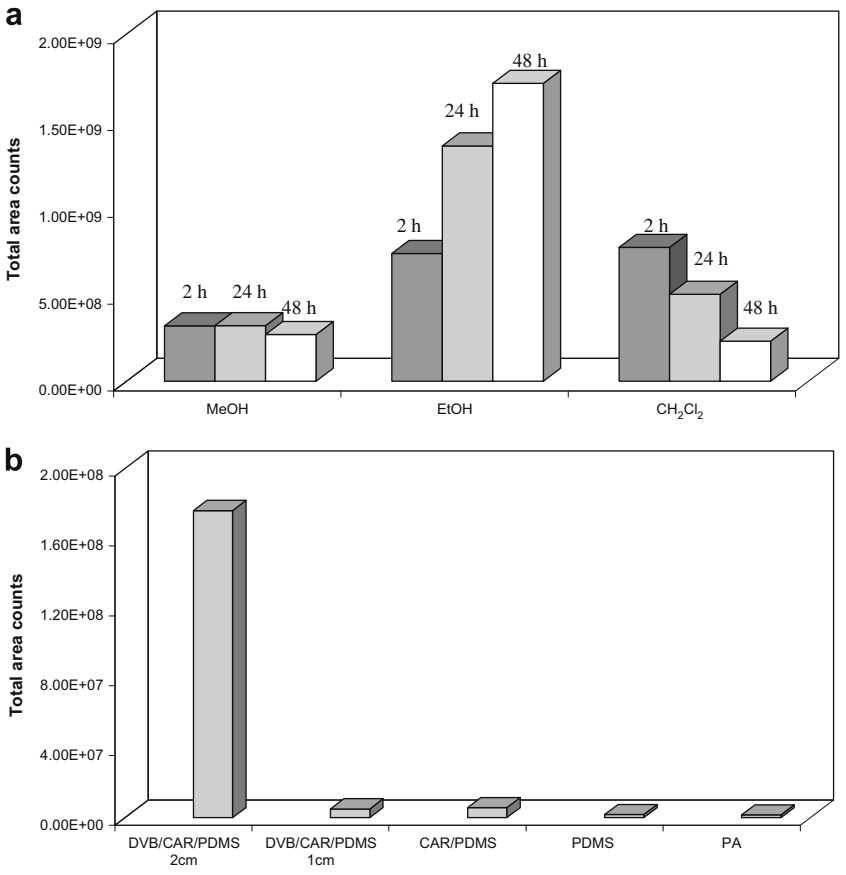

Fig. 1. Total area counts of detected peaks extracted from fenugreek seeds. (a) Solvent extraction (with varying soaking times); (b) SHS-SPME (with different fibre coatings).

Table 3
Multiple range tests for means of similarity values obtained by the seven assessors.

Fibres	Count	LS mean value	Homogeneous groups[A]		
PDMS	21	1.7	a		
PA	21	2.5	a		
CAR/PDMS	21	5.9		b	
DVB/CAR/PDMS 1 cm	21	7.2		b	c
DVB/CAR/PDMS 2 cm	21	8.3			c

[A] a, b and c refer to homogeneous groups: group means are not statistically different.

odour very close to that of fenugreek seeds (mean similarity value of 7.2 for the 1 cm length), which is consistent with a previous study reporting this fibre coating to afford a global odour most resembling that of the reference for orange juice (Rega et al., 2003) and sponge cake (Rega, Guerard, Delarue, Maire, & Giampaoli, 2009). In the case of fenugreek, this may be partially explained by the better extraction of sotolone with this fibre. Moreover, by increasing the DVB/CAR/PDMS fibre length from 1 to 2 cm, a more intense odour was obtained without changing the odour profile and the odour quality (mean similarity value of 8.3), with a better repeatability. Consequently, the DVB/CAR/PDMS fibre coating with 2 cm length will be particularly suitable for obtaining highly concentrated and representative fenugreek extracts.

The three-way ANOVA on similarity values obtained by the seven assessors showed a significant "fibre coating" effect, as well as a strong "assessor" effect (see Table 4). On the other hand, the repetition factor did not affect the variance. Interestingly, the "fibre coating" × "assessor" and "fibre coating" × "repetition" interactions also have an effect. Indeed, the "fibre coating" effect means that the type of coating greatly influences the odour quality of SHS-SPME extracts, as the nature and contents of the volatile compounds extracted greatly differ depending on the fibre coating. The "assessor" effect is very common in sensory analyses and it is mainly due to interindividual differences in odour perception. Interestingly, the length of the DVB/CAR/PDMS fibre seems to affect judges' repeatability: doubling the fibre length makes it possible to highly increase repeatability. This is probably due to a more intense odour of the corresponding SHS-SPME extracts which makes the similarity rating task easier.

Results of the factorial discriminant analysis are presented in Fig. 3. Two groups are clearly discriminated along the first axis: PA and PDMS on the one hand; CAR/PDMS, DVB/CAR/PDMS 1 cm and DVB/CAR/PDMS 2 cm on the other. This suggests the importance of the adsorbent CAR on the retention of volatiles characteristic of fenugreek aroma. The second axis discriminates between CAR/PDMS and both fibres of DVB/CAR/PDMS, showing that the presence of DVB in the fibre coating is important to obtain an odour highly representative of fenugreek. A much lower dispersion of the results can be achieved by increasing the amount of DVB/CAR/PDMS on the fibre; this can be explained by larger amounts of volatile extracted, resulting in a more intense odour that is more easily evaluated by the assessors, as previously said.

4. Discussion

Our results show that SHS-SPME enables the detection of some volatile odorant compounds that were absent or only detected at low levels in organic solvent extracts, such as dimethyl sulfide, 2-methyl-2-butenal, 3-octen-2-one, menthone, carvone, α-muurolene as well as a number of sesquiterpenes that could not be precisely identified. The amounts of 2-methyl-2-butenal and α-muurolene are clearly higher with this technique using the DVB/CAR/PDMS fibre coating 2 cm long as seen for 2-methyl-2-butenal in Fig. 4. As mentioned previously, SHS-SPME, as with other head-

Table 4
Three-way ANOVA of the similarity values obtained by D-GC-O for SHS-SPME extracts.

Factors and interactions	Degrees of freedom	ANOVA sum of square	Mean square	*F* ratio	*P* value[a]
Fibre coating	4	699.77	174.94	45.91	0.000
Assessor	6	104.90	17.48	4.59	0.001
Repetition	2	3.41	1.71	0.45	0.641
Fibre coating X assessor	24	212.70	8.86	2.33	0.006
Fibre coating X repetition	8	100.20	12.52	3.29	0.004
Assessor X repetition	12	15.81	1.32	0.35	0.975
Residual	48	182.91	3.81		
Total (corrected)	104	1319.70			

[a] The *P* value indicates the statistical significance of each factor or interaction.

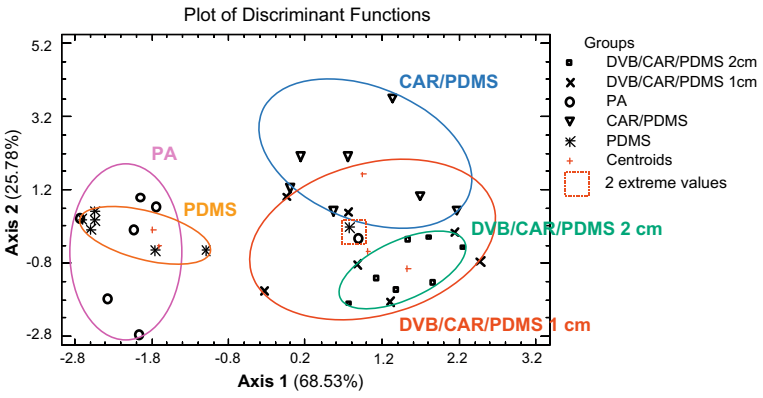

Fig. 3. Results of the factorial discriminant analysis performed on the similarity values given for the different fibre coatings by the seven assessors.

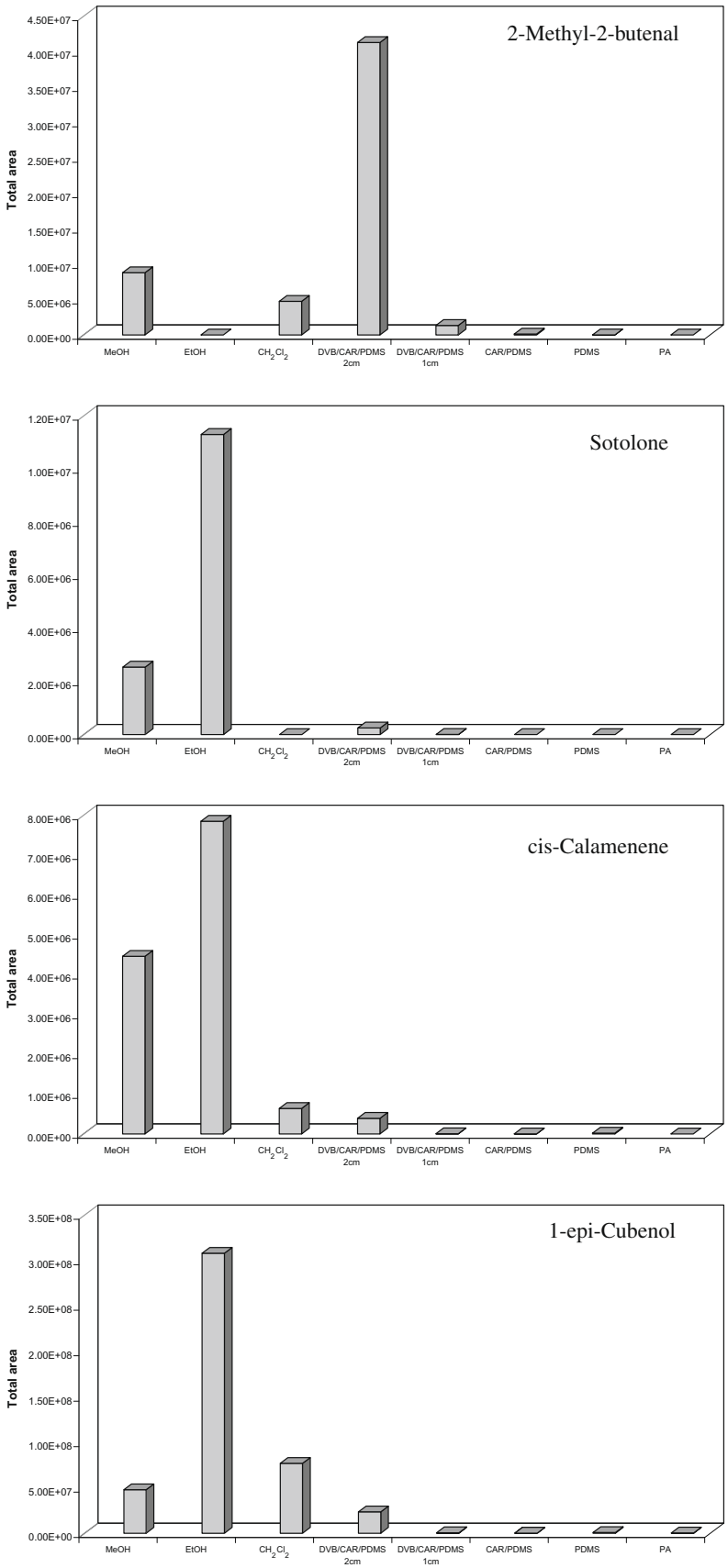

Fig. 4. Total area counts (per gram of fenugreek) for selected volatile compounds extracted from fenugreek seeds under different conditions (organic solvents – 48 h; SHS-SPME – different fibres).

space techniques, leads to discrimination during the extraction, as highly volatile compounds are preferentially volatilised compared to medium and non-volatile compounds. SHS-SPME favours the extraction of volatile compounds (such as 2-methyl-2-butenal) that are not recovered or only extracted at trace levels using organic solvents (Mazza et al., 2002). Consequently it is possible that SPME leads to extracts with poor sensory quality (having an odour different from that of the initial fenugreek seeds). This explains the need to assess the sensory representativeness of such extracts.

Among the fibre coatings tested, the DVB/CAR/PDMS fibre coating extracts a higher amount of compounds with different polarities and volatilities as shown in Table 2, in particular some heterocyclic compounds which are known to be involved in the odour of fenugreek seeds, such as sotolone (Blank et al., 1997). Such results are consistent with a previous study reporting the efficiency of this fibre for extracting volatiles from fenugreek seeds, some of them being particularly involved in the aroma (carbonyls, sesquiterpenes, alcohols, heterocyclic compounds) (Mazza et al., 2002). However, differences in the volatile profiles are evident among varieties. In particular 2-methyl-2-butenal, 2-heptanone, 3-octen-2-one and γ-nonalactone were observed in our Tunisian fenugreek seeds as reported in a Sicilian variety, while these compounds were absent or present at low levels in a Turkish variety despite similar extraction conditions (Mazza et al., 2002). On the other hand, α-muurolene, present in the Turkish variety and not detected in the Sicilian one, was observed in our SPME extracts. Also sotolone could be quantified in our fibre extracts, when only traces were reported in the Sicilian variety along with no detection in the Turkish variety. On the other hand, 1-octen-2-ol was not detected, even though its presence in Sicilian fenugreek seeds was reported.

Despite a more limited range of volatile compounds being extracted, organic solvents (especially alcoholic solvents) enabled the extraction of some compounds hardly or not at all extracted by SHS-SPME. This is particularly evident for nitrogen compounds as indicated in Table 2. Eight new molecules were thus, identified in fenugreek seeds: pyridine, β-picoline, 2,3-dimethylpyrazine, 2-ethylpyrazine, 2-ethyl-6-methylpyrazine, trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine and methyl nicotinate. Such a result was surprising as pyridine and pyrazines could be extracted from palm sugar, dry cured ham and cocoa products by SHS-SPME using CAR/PDMS or DVB/CAR/PDMS under similar conditions (Ducki et al., 2008; Garcia-Esteban et al., 2004; Ho et al., 2006). The lower levels of these nitrogen compounds in fenugreek seeds may explain these differences. Similarly, alcoholic solvents offered better extraction of sotolone and cis-calamenene as shown in Fig. 4. These molecules are known to be key aroma compounds of fenugreek seeds and could be considered as markers of a good extraction. However, it should be stressed that the overall odour of a complex sample is due to the presence of numerous volatile odour-active compounds in specific proportions. A good aroma extract should thus, assure a good balance of several odour-active compounds. This seems to be achieved with the DVB/CAR/PDMS extracts. Our D-GC-O experiments clearly indicate that SHS-SPME extraction with the DVB/CAR/PDMS fibre made it possible to obtain extracts with an overall odour very similar to that of genuine fenugreek seeds and that doubling the amount of solid phase (1 cm up to 2 cm) made it possible to increase the odour intensity without modifying the odour profile. In fact, traces of sotolone were found in DVB/CAR/PDMS extracts; so, even when present in very low amounts, this compound seems to participate in the overall odour quality of the SPME extract, probably thanks to its very low perception threshold. These results confirm the potential of SHS-SPME to give an aroma profile as perceived by the human nose. Further analysis by GC-O will be considered in a future study for the comprehensive identification of the key aroma compounds really involved in the strong aroma of fenugreek seeds.

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