Food Chemistry 112 (2009) 239–245

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Headspace, volatile and semi-volatile patterns of *Paliurus spina-christi* unifloral honey as markers of botanical origin

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ARTICLE INFO

Article history:
Received 16 November 2007
Received in revised form 9 April 2008
Accepted 26 May 2008

Keywords:
Paliurus spina-christi honey
Volatile compounds
Headspace solid-phase microextraction (HS-SPME)
Ultrasonic solvent extraction (USE)
Gas chromatography–mass spectrometry (GC–MS)

ABSTRACT

The samples of unifloral *Paliurus spina-christi* honey were analysed by means of headspace solid-phase microextraction (HS-SPME) and ultrasonic solvent extraction (USE) followed by gas chromatography and mass spectrometry (GC, GC–MS) in order to obtain complete patterns of headspace, volatile and semi-volatile compounds. In headspace pattern the most abundant compounds and possible markers were nonanal, four isomers of lilac aldehyde, decanal, methyl nonanoate, hexanoic and 2-ethylhexanoic acids. Although the main components of USE extracts were higher saturated aliphatic hydrocarbons, higher aliphatic alcohols and acids, they can not be considered reliable biomarkers due to their probable origin from bee wax or bee cuticle. Although present in small quantities, the more reliable markers in the extracts were benzene derivatives (particularly 4-hydroxy-3,5-dimethylbenzaldehyde, 4-hydroxybenzoic acid and 4-methoxybenzoic acid) along with lower aliphatic acids (butanoic, hexanoic, octanoic and nonanoic).

1. Introduction

Paliurus spina-christi unifloral honey is a product with limited production and distinct fragrance characteristics in comparison with other ubiquitous unifloral honeys from Croatia (sage, rosemary and others). Little information about this honey is available, and there are only a few papers on its floral source. *P. spina-christi* Mill. (Rhamnaceace) is a traditional Mediterranean and Asiatic medicinal plant (syn. Christ's thorn) commonly used as diuretic and against diarrhoea and rheumatism (Grlić, 1986). It blossoms at the beginning of summer and can be found particularly in large areas of sub Mediterranean part of Croatia. The flavonoid glycosides and tannins are present in different plant parts (Brantner & Males, 1999), while amino acids, alkaloids and sterols were found in the bark and fruits (Velcheva, 1986; Velcheva, 1993). The main flavonoid compounds were isoquercitrin, rutin, hyperoside and a quercetin-3-*O*-triglycoside (Brantner & Males,1990, 1999; Kustrak, Males, Brantner, & Pitarevic, 1990).

Since one of the most typical features of honey is its aroma profile, the gas chromatographic pattern of honey volatiles can be considered as a characteristic chemical "fingerprint". Although honeys of different botanical origin have been previously characterised on the basis of their volatile fraction (Cuevas-Glory, Pino, Santiago, & Sauri-Duch, 2007), to our knowledge there are no reports on *Paliurus* honey volatiles. Different methods can be used for honey volatiles isolation. Hydrodistillation (HD) and micro-simultaneous steam distillation – solvent extraction (MSDE) are the most common techniques for volatiles isolation, but for honey samples many artefacts can be generated by these methods due to the effect of heat on sugars and amino acids (Alissandrakis, Tarantilis, Harizanis, & Polissiou, 2005; Bonvehi & Coll, 2003). Nowdays, alternative to these classical methods that may overcome their disadvantages are used (Alissandrakis, Daferera, Tarantalis, Polissiou, & Harizanis, 2003; Pawliszyn, 1997) such as solid-phase microextraction (SPME) or ultrasonic solvent extraction (USE). SPME can be used in three basic modes: direct extraction, headspace extraction and extraction with membrane protection (Pontes, Marques, & Câmara, 2007).

In this research, five selected Croatian *P. spina-christi* monofloral honeys were analysed for volatiles using headspace solid-phase microextraction (HS-SPME) and ultrasonic solvent extraction (USE) followed by gas chromatography and mass spectrometry (GC, GC–MS). The aim of this study is to obtain the patterns of headspace, volatile and semi-volatile compounds of *Paliurus* honey as possible markers of the floral source.

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doi:10.1016/j.foodchem.2008.05.080

2. Materials and methods

2.1. Reagents

The solvents used were Et_2O and pentane purchased from Kemika (HR-Zagreb). Et_2O was distilled immediately before usage to remove stabilizer (2,6-di(tert-butyl)-4-methylphenol) that can interfere during GC and GC–MS analyses. Anhydrous $MgSO_4$ and NaCl were obtained from Fluka Chemie (CH-Buchs). All reference compounds (Tables 1 and 2) were purchased from Sigma-Aldrich (D-Steinheim).

2.2. Honey samples

This study was carried out on five unifloral Christ's thorn honeys from the same geographical area of sub Mediterranean part of Croatia (near Drniš). To be certain of the botanical origin, bees were placed in the middle of a large area of predominant wild growing *P. spina-christi*. The honey was harvested by pressing the combs. No mechanical treatment or heat was used. Conformation of honey unifloral origin was based on the pollen analysis. Melissopalynological analysis was performed by the methods recommended by the International Commission for Bee Botany (Louveaux, Maurizio, & Vorwohl, 1978) and *P. spina-christi* pollen percentage in the samples was in the range of 72–75% with minor percentage of other pollen species (each max 1–2%). All the samples were stored in hermetically closed glass bottles at 4 °C until the volatiles isolation.

2.3. Headspace solid-phase microextraction (HS-SPME)

The isolation of headspace volatiles were performed in duplicate for each honey sample using manual SPME fibre with the layer of carbowax/divinylbenzene (CAR/DVB) and SPME fibre with the layer of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) obtained from Supelco Co., (Bellefonte, PA, USA). The coatings were 1 cm long. Each fibre was conditioned prior to use according to the manufacturer instructions by inserting into the GC injector port.

For each HS-SPME extraction 5 mL of honey water solution (1:1 v/v; the ionic strength was increased using saturated NaCl water solution) was placed in 15 mL amber glass vial (volume ratio headspace/solution was 1:1) and hermetically sealed with PTFE/silicone septa. The vial was maintained in a water bath at 60 °C during equilibration (15 min) and extraction (40 min) and was partially submerged so that the liquid phase of the sample was in the water. All the experiments were performed under constant stirring velocity (750 rpm) by magnetic stirrer. After sampling, the SPME fibre was withdrawn into the needle, removed from the vial and inserted into the injector (250 °C) of the GC and GC–MS system for

Table 1
Volatile compounds identified in *P. spina-christi* honey samples after headspace solid-phase microextraction (HS-SPME) using the fibres with different coatings

No.	Compound	RI_1	RI_2	SPME coating							
				PDMS/DVB				DVB/CAR/PDMS			
				Peak area (%)							
				Min.	Max.	Av.	SD	Min.	Max.	Av.	SD
1	Undecane[a]	<1200	/	1.2	2.2	1.70	0.50	10.5	10.6	10.57	0.06
2	Dodecane[a]	1200	/	0.0	4.5	1.50	2.60	–	–	–	–
3	Methyl octanoate	1397	/	0.4	2.5	0.97	1.34	–	–	–	–
4	Nonanal[a]	1400	1086	18.7	23.7	21.23	2.50	13.1	17.1	15.17	2.00
5	*Cis*-linalool oxide	1452	1063	0.0	1.5	1.00	0.87	–	–	–	–
6	Acetic acid[a]	1472	/	0.0	1.4	0.47	0.81	2.9	5.8	4.73	1.59
7	2-Furancarboxaldehyde (furfural)[a]	1492	874	–	–	–	–	3.5	5.1	4.37	0.81
8	Methyl nonanoate	1495	1491	1.6	8.0	4.73	3.20	-	–	–	–
9	Pentadecane[a]	1500	1500	0.0	1.0	0.63	0.55	–	–	–	–
10	Decanal[a]	1504	1181	8.1	8.9	8.43	0.42	4.1	5.9	5.27	1.01
11	Benzaldehyde[a]	1540	952	1.5	2.6	1.93	0.59	9.4	9.8	9.63	0.21
12	Lilac aldehyde (isomer I)	1551	1101	4.4	9.0	6.43	2.35	3.6	6.5	4.93	1.46
13	Lilac aldehyde (isomer II)	1564	1117	4.2	8.6	5.90	2.36	3.3	6.3	4.40	1.65
14	Lilac aldehyde (isomer III)	1572	1126	2.6	5.0	3.53	1.29	2.0	3.9	2.83	0.97
15	Lilac aldehyde (isomer IV)	1594	1142	3.6	7.4	4.93	2.14	2.5	4.7	3.67	1.11
16	Hotrienol	1614	1100	1.0	3.4	2.47	1.29	0.7	1.9	1.30	0.60
17	Phenylacetaldehyde[a]	1652	1038	5.6	7.7	6.83	1.10	5.1	7.8	6.10	1.48
18	2-Furanmethanol[a]	1666	/	–	–	–	–	0.0	1.5	0.50	0.87
19	4-Hydroxybenzaldehyde (salicyl aldehyde)[a]	1690	/	0.0	0.7	0.23	0.40	0.0	1.6	0.53	0.92
20	*o*-Ethylanisole	1766	/	0.0	4.3	1.43	2.48	–	–	–	–
21	5-Methyl-1,4-benzenedione	1779	/	0.0	2.1	0.77	1.16	–	–	–	–
22	Hexanoic acid[a]	1852	/	1.2	1.4	1.27	0.12	0.6	1.0	0.83	0.21
23	Benzyl alcohol[a]	1891	/	0.0	0.8	0.40	0.40	0.0	0.9	0.30	0.52
24	2-Phenylethanol[a]	1927	1165	0.0	1.8	0.87	0.90	0.5	0.9	0.67	0.21
25	2-Ethylhexanoic acid	1954	1211	2.7	14.2	6.77	6.45	1.6	5.6	3.13	2.16
26	Octanoic acid (caprylic acid)[a]	2066	1280	0.6	4.5	2.43	1.96	0.6	1.7	1.20	0.56
27	Heneicosane[a]	2100	/	0.0	0.4	0.13	0.23	–	–	–	–
28	Nonanoic acid[a]	2169	/	0.0	4.4	2.20	2.20	0.9	3.0	2.23	1.16
Compounds identified only on HP-101 column											
29	Heptanal[a]	/	875	0.9	1.0	0.93	0.06	1.0	5.1	3.20	2.07
30	Octanal[a]	/	973	1.3	2.9	2.00	0.82	–	–	–	–
31	Methylbenzene (toluene)[a]	/	1053	–	–	–	–	0.0	3.5	1.97	1.79
32	Tridecane[a]	/	1300	0.0	3.6	1.20	2.08	–	–	–	–
33	Nonadecane[a]	/	1900	0.0	0.5	0.17	0.29	–	–	–	–
Total identified (%)				89.5–97.8%				85.3–91.4%			

RI_1, retention indices on HP-FFAP column; RI_2, retention indices on HP-101 column; –, not identified by HS-SPME; Min., minimal percentage; Max., maximal percentage; Av., average percentage; SD, standard deviation; /, not detected on this column; [a], identification confirmed with reference compound.

Table 2
Volatile compounds of honey from *P. spina-christi* extracted by ultrasonic solvent extraction (USE) with different polarity solvents

No.	Compound	RI_1	RI_2	Solvent A				Solvent B				Solvent C			
				Peak area (%)											
				Min.	Max.	Av.	SD	Min.	Max.	Av.	SD	Min.	Max.	Av.	SD
1	Dodecane[a]	1200	1200	0.0	0.3	0.17	0.15	–	–	–	–	–	–	–	–
2	Tetradecane[a]	1300	/	0.2	0.2	0.20	0.00	0.0	0.4	0.13	0.23	0.0	0.3	0.10	0.17
3	Nonanal[a]	1400	1086	0.1	0.1	0.10	0.00	0.0	0.6	0.37	0.32	0.0	0.3	0.13	0.15
4	*Cis*-linalool oxide	1452	1063	–	–	–	–	–	–	–	–	0.0	0.1	0.03	0.06
5	Acetic acid[a]	1472	/	0.5	2.8	1.53	1.17	–	–	–	–	4.1	4.8	4.37	0.38
6	2-Furancarboxaldehyde[a]	1492	874	–	–	–	–	–	––	–	–	0.0	0.2	0.13	0.12
7	Benzaldehyde[a]	1540	952	–	–	–	–	0.0	0.1	0.03	0.06	0.0	0.2	0.07	0.12
8	Propanoic acid[a]	1547	/	0.0	0.1	0.07	0.06	–	–	–	–	0.2	0.3	0.23	0.06
9	1,3-Butanediol*	1557	1046	0.0	0.8	0.27	0.46	–	–	–	–	0.0	0.3	0.10	0.17
10	2-Methylpropanoic acid	1575	/	–	–	–	–	–	–	–	–	0.0	0.4	0.13	0.23
11	2,3-Butanediol*	1588	/	0.0	0.2	0.07	0.11	–	–	–	–	0.0	0.3	0.10	0.17
12	Hexadecane[a]	1600	1600	0.0	0.2	0.07	0.11	0.0	0.6	0.20	0.35	–	–	–	–
13	Hotrienol[a]	1614	1100	–	–	–	–	–	–	–	–	0.0	0.3	0.10	0.17
14	Butanoic acid[a]	1632	/	0.3	0.5	0.37	0.12	–	–	–	–	0.3	1.2	0.63	0.49
15	3-Methylbutanoic acid	1672	/	–	–	–	–	––	–	–	–	0.0	0.5	0.30	0.26
16	Pentanoic acid[a]	1742	/	0.0	0.1	0.07	0.06	0.0	0.1	0.03	0.06	0.3	0.3	0.30	0.00
17	3-Pyridinecarbonitrile	1760	/	0.0	0.1	0.03	0.06	–	–	–	–	0.0	0.2	0.07	0.12
18	3-Methylpentanoic acid	1792	/	0.0	0.3	0.10	0.17	–	–	–	–	0.0	0.5	0.17	0.29
19	Octadecane[a]	1800	/	0.0	0.2	0.13	0.12	0.0	0.2	0.07	0.12	0.0	0.2	0.07	0.12
20	2,4-Dimethylbenzaldehyde	1824	/	–	–	–	–	–	–	–	–	0.0	1.3	0.43	0.75
21	Hexanoic acid[a]	1852	/	0.0	0.3	0.17	0.15	0.0	0.2	0.07	0.12	0.6	1.0	0.80	0.20
22	Benzyl alcohol[a]	1891	/	0.0	0.1	0.07	0.06	–	–	–	–	0.0	0.4	0.20	0.20
23	Nonadecane[a]	1900	1900	0.0	0.2	0.13	0.11	0.5	1.5	1.07	0.51	–	–	–	–
24	2-Phenylethanol[a]	1927	1165	0.0	0.3	0.17	0.15	0.0	0.2	0.13	0.12	0.5	1.3	0.83	0.42
25	3,7-Dimethyl-1,5-octadien-3,7-diol	1948	1101	0.0	0.6	0.33	0.31	–	–	–	–	0.0	2.5	0.8	1.44
26	2-Hydroxy-3-methyl-4H-pyran-4-one (maltol)	1984	1178	0.0	0.9	0.50	0.46	–	–	–	–	1.4	1.7	1.60	0.17
27	2-Methoxy-6-methylpyrazine	1999	/	0.0	0.2	0.07	0.12	–	–	–	–	0.0	0.7	0.43	0.38
28	Methyl 2-furancarboxylate	2025	/	0.0	0.6	0.20	0.35	–	–	–	–	0.3	0.8	0.60	0.26
29	Octanoic acid[a]	2066	/	0.6	1.0	0.73	0.23	0.0	0.9	0.30	0.52	0.0	0.8	0.50	0.44
30	Heneicosane[a]	2100	2100	3.3	4.0	3.57	0.38	4.9	14.9	8.33	5.70	0.0	0.6	0.20	0.35
31	Phytol**	2120	1644	0.0	0.6	0.30	0.30	–	–	–	–	–	–	–	–
32	Nonanoic acid[a]	2169	/	0.4	0.8	0.57	0.21	1.2	2.0	1.53	0.40	0.9	1.3	1.10	0.20
33	Docosane[a]	2200	2200	0.0	1.4	0.63	0.71	0.0	1.7	1.07	0.93	–	–	–	–
34	4-Vinyl-2-methoxyphenol	2211	1372	0.0	0.2	0.07	0.12	–	–	–	–	0.5	1.2	0.80	0.36
35	Decanoic acid[a]	2293	/	–	–	–	–	–	–	–	–	0.0	0.8	0.27	0.46
36	Tricosane[a]	2300	2300	5.2	7.3	6.17	1.06	5.2	7.8	6.17	1.42	–	–	–	–
37	2,3-Dihydro-3,5-dimethyl-4H-pyran-4-one	2309	1306	0.0	0.1	0.03	0.06	–	–	–	–	1.6	3.	2.20	0.95
38	3,5-Dihydroxy-2-methyl-4H-pyran-4-one	2344	1355	–	–	–	–	–	–	–		1.0	2.7	1.67	0.91
39	Tetracosane[a]	2400	2400	10.0	16.4	12.40	3.49	9.1	10.8	9.90	0.85	0.0	0.5	0.17	0.29
40	1H-Pyrrole-2-carboxylic acid	2427	/	0.0	0.8	0.27	0.46	–	–	–	–	–	–	–	–
41	1-Hexadecanol[a]	1452	1893	2.0	3.2	2.53	0.61	3.6	4.0	3.77	0.21	0.0	6.0	3.60	3.17
42	2,3-Dihydrobenzofuran	2503	/	0.0	0.4	0.27	0.23	–	–	–	–	0.0	1.2	0.63	0.60
43	Benzoic acid[a]	2583	1410	1.6	2.1	1.83	0.25	0.0	1.6	0.70	0.82	8.2	14.9	11.03	3.47
44	4-Methoxybenzoic acid	2599	/	0.1	1.0	0.37	0.64	–	–	–	–	–	–	–	–
45	Hexacosane[a]	2600	2600	1.1	2.9	1.83	0.95	0.0	1.8	0.80	0.92	–	–	–	–
46	1H-Indole	2619	/	–	–	–	–	–	–	–	–	0.0	0.3	0.10	0.17
47	Benzofuran (Coumarone)	2628	/	–	–	–	–	–	–	–	–	0.0	0.6	0.20	0.35
48	Dodecanoic acid[a]	2699	/	–	–	–	–	–	–	–	–	0.0	0.5	0.17	0.29
49	Heptacosane[a]	2700	2700	8.3	11.7	10.57	1.96	4.5	18.8	11.83	7.16	-	–	–	–
50	3-Metyl-1H-indole	2729	1492	–	–	–	–	–	–	––	–	0.0	2.4	1.40	1.25
51	5-Hydroxymethylfurfural	2798	1458	0.3	0.6	0.47	0.15	–	–	–	–	1.3	4.6	2.47	1.85
52	Diethyl phthalate	2861	/	–	–	–	–	–	–	–	–	0.0	3.2	1.07	1.85
53	Phenylacetic acid[a]	2928	1493	1.3	1.9	1.53	0.32	0.0	4.9	1.63	2.83	3.3	3.8	3.57	0.25
54	Hexadecanoic acid[a]	>3000	/	5.5	11.1	8.43	2.81	7.9	8.7	8.23	0.42	5.3	11.7	7.87	3.82
55	(*Z*,*Z*)-9,12-Octadeca-dienoic acid	>3000	/	0.0	7.3	2.43	4.21	–	–	–	–	0.0	9.1	4.17	4.60
56	(*Z*)-9-Octadecen-1-ol (oleil alcohol)[a]	>3000	/	3.6	5.9	4.63	1.17	5.4	9.3	7.73	2.06	2.4	5.1	3.63	1.37
57	Octadecanoic acid (stearic acid)[a]	>3000	/	0.0	1.1	0.70	0.61	–	–	–	–	0.6	2.2	1.57	0.85
58	3-Pyridinecarboxylic acid	>3000	>3000	–	–	–	–	0.0	0.3	0.10	0.17	–	–	–	–
Compounds identified only on HP-101 column															
59	Octanal[a]	/	973	0.0	0.1	0.03	0.06	0.0	0.6	0.20	0.35	–	–	–	–
60	3-Methyl-2-butanol	/	1012	0.0	0.2	0.07	0.12	–	–	–	–	–	–	–	–
61	Toluene[a]	/	1053	–	–	–	–	0.0	0.1	0.03	0.06	–	–	–	–
62	Phenylacetaldehyde[a]	/	1049	0.0	0.2	0.07	0.12	0.2	0.5	0.37	0.15	0.2	0.3	0.23	0.06
63	Decanal[a]	/	1181	0.0	0.6	0.27	0.31	0.0	0.5	0.27	0.25	–	–	–	–
64	2-(*p*-Methoxyphenyl)-ethanol	/	1319	0.4	0.9	0.57	0.29	–	–	–	–	0.0	0.8	0.27	0.46
65	2,6-Dimethyl-1,5,7-octatrien-3-ol	/	1440	–	–	–	–	–	–	–	–	0.0	0.6	0.20	0.35
66	2-Methyl-1H-indole*	/	1491	–	–	–	–	–	–	–	–	0.0	2.1	0.70	1.21
67	4-Methoxybenzoic acid[a]	/	1683	0.0	0.9	0.47	0.45	–	–	–	–	0.0	1.0	0.57	0.51
68	Heptadecane[a]	/	1700	0.0	0.4	0.23	0.21	0.0	1.3	0.83	0.72	–	–	–	–
69	1,4-Benzenediol	/	1734	1.1	1.5	1.30	0.20	–	–	–	–	1.7	2.4	2.07	0.35
70	4-Hydroxy-3,5-dimethyl-benzaldehyde	/	1786	1.3	2.4	1.97	0.59	0.0	0.9	0.37	0.47	1.4	4.0	2.97	1.38
71	Eicosane[a]	/	2000	–	–	–	–	0.3	1.3	0.73	0.51	–	–	–	–

(*continued on next page*)

Table 2 (*continued*)

No.	Compound	RI_1	RI_2	Solvent A				Solvent B				Solvent C			
				Peak area (%)											
				Min.	Max.	Av.	SD	Min.	Max.	Av.	SD	Min.	Max.	Av.	SD
72	Octanedioic acid	/	2027	0.0	1.4	0.47	0.81	–	–	–	–	0.0	1.8	0.60	1.04
73	4-Hydroxybenzoic acid[a]	/	2122	3.0	3.9	3.50	0.46	–	–	–	–	5.1	6.4	5.50	0.78
74	Decanedioic acid	/	2209	3.7	6.2	4.97	1.25	–	–	–	–	6.1	11.0	7.93	2.67
75	4-Hydroxy-3,5-dimethoxy- benzoic acid	/	2220	0.1	1.5	0.50	0.87	0.0	10.1	5.73	5.19	0.0	6.1	4.00	3.47
76	(*Z*,*Z*)-9,12-Octadecadienal	/	2233	0.0	12.2	7.40	6.50	0.0	10.3	6.07	5.39	0.0	11.3	4.47	6.01
77	1H-Indole-3-acetic acid	/	2426	0.0	0.6	0.20	0.35	–	–	–	–	–	–	–	–
78	Octacosane[a]	/	2800	0.0	1.7	0.57	0.98	1.5	12.0	6.97	5.26	–	–	–	–
79	Nonacosane[a]	/	2900	3.2	5.6	4.60	1.25	8.3	13.1	10.00	2.69	–	–	–	–
Total identified (%)				88.8–99.7%				92.3–99.2%				88.3–95.5%			

Solvent A, mixture pentane: diethyl ether = 1:2 v/v; Solvent B, pentane; Solvent C, diethyl ether after extraction with pentane; Min., minimal percentage; Max., maximal percentage; Av., average percentage; SD, standard deviation; *, tentatively identified; **, correct isomer not identified; /, not detected; RI_1, retention indices on HP-FFAP column; RI_2, retention indices on HP-101 column; [a], identification confirmed with reference compound.

6 min where the extracted volatiles were thermally desorbed directly to the GC column.

2.4. Ultrasonic solvent extraction (USE)

Ultrasound-assisted extraction (USE) was performed in an ultrasound cleaning bath (Transsonic Typ 310/H, Germany) by the mode of indirect sonication, at the frequency of 35 kHz at 25 ± 3 °C. Each batch of five honey samples was extracted in duplicate as further described. Forty grams of each honey sample were dissolved with 22 mL of distilled water in a 100 mL flask. Magnesium sulphate (1.5 g) was added and each sample was extensively vortexed (Jerković, Mastelić, & Marijanović, 2006; Jerković, Mastelić, Marijanović, Klein, & Jelić, 2007). Different extraction solvents were separately used for USE: (1) a mixture pentane: diethyl ether 1:2 (v/v), (2) pentane and (3) diethyl ether (applied on the samples after sonication with pentane and after removing the pentane extract). Sonication was held for 30 min. After sonication, the organic layer was separated in a separation funnel and filtered over anhydrous $MgSO_4$. Aqueous layer was returned to the flask and another batch of the same extraction solvent (20 mL) was added and extracted by ultrasound for 30 min. Organic layer was separated in the separation funnel, filtered over anhydrous $MgSO_4$ and aqueous was sonicated third time for 30 min with another batch (20 mL) of the extraction solvent. Joined organic extracts were concentrated up to 0.2 mL by fractional distillation, and 1 μL was used for GC and GC–MS analyses.

2.5. Gas chromatography (GC)

Gas chromatography analysis was performed on a Hewlett–Packard Model 5890 Series II gas chromatograph equipped with flame ionisation detector and capillary column HP-FFAP (crosslinked polyethylene glycol TPA phase, Hewlett–Packard, Vienna, Austria), 50 m × 0.32 mm i.d., film thickness 0.52 μm and column HP-101 (methyl silicone fluid, Hewlett–Packard, Vienna, Austria), 25 m × 0.2 mm i.d., coating thickness 0.2 μm. Chromatographic conditions were as follows: helium as carrier gas at 1 mL min^{-1}; injector and detector temperatures, 250 °C and 300 °C. HP-FFAP column temperature was programmed from 70 °C isothermal for 4 min, then increased to 180 °C at a rate of 4 °C min^{-1} and for HP-101 column temperature was isothermal at 70 °C for 2 min, then increased to 200 °C, at a rate of 3 °C min^{-1} and held isothermal for 15 min. The injected volume was 1 μL and split ratio was 1:50.

2.6. Gas chromatography–mass spectrometry (GC–MS)

The samples were analysed by gas chromatography–mass spectrometry (Hewlett–Packard, model 5890, with a mass selective detector, model 5971A) on two columns. GC operating conditions were: column HP-FFAP (crosslinked polyethylene glycol TPA phase, Hewlett–Packard, Vienna, Austria), 50 m × 0.32 mm i.d., film thickness 0.52 μm; column temperature programmed from 70 °C isothermal for 4 min, then increased to 180 °C at a rate of 4 °C min^{-1}; column HP-101 (methyl silicone fluid, Hewlett–Packard, Vienna, Austria), 25 m × 0.2 mm i.d., film thickness 0.2 μm; column temperature programmed from 70 °C isothermal for 2 min, then increased to 200 °C at a rate of 3 °C min^{-1}. Carrier gas was helium at flow rate 1 mL min^{-1}. Injector temperature was 250 °C. Volume injected was 1 μL and split ratio was 1:50. MS conditions were: ionisation voltage 70 eV; ion source temperature 280 °C; mass scan range: 30–300 mass unit.

2.7. Data analysis and data evaluation

The individual peaks were identified by comparison of their retention indices (relative to C_8-C_{30} *n*-alkanes for HP-FFAP and HP-101 column) to those of authentic samples and literature (El-Sayed, 2007 and references therein), as well as by comparing their mass spectra with the Wiley 275 MS library (Wiley, New York) and NIST98 (National Institute of Standards and Technology, Gaithersburg) mass spectral database. The percentage composition of the samples was computed from the GC peak areas using the normalisation method for both columns (without correction factors). The component percentages (Tables 1 and 2) and standard deviations were calculated from duplicate GC analysis for each batch of five honey samples on two columns.

3. Results and discussion

A total of five samples of unifloral *P. spina-christi* honeys were analysed by means of headspace solid-phase microextraction (HS-SPME) and ultrasonic solvent extraction (USE) followed by gas chromatography and mass spectrometry (GC, GC–MS) in order to obtain, for the first time, complete patterns of headspace, volatile and semi-volatile compounds. For better resolution and more complete analysis of the isolated volatile mixtures two GC columns with different polarities were used: HP-101 (apolar) and HP-FFAP (moderately polar). HS-SPME procedure enabled identification of high volatile compounds in the samples. The chemical composition of these compounds was remarkably different in comparison with USE procedure, and some low-molecular weight compounds were only identified using HS-SPME method (such as four isomers of lilac aldehyde) or were present in remarkably higher amounts in comparison with the extracts (particularly lower aliphatic aldehydes octanal, nonanal and decanal). On the other hand, USE procedure enabled isolation of semi-volatile and other compounds

(not identified by HS-SPME) that are also important for enquiring the complete aroma profile of this honey as well for identification of potential markers of botanical origin.

3.1. Headspace volatiles of Paliurus honey

The fibres with carbowax/divinylbenzene (CAR/DVB) coating and divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating were selected for HS-SPME in preliminary research taking into account the overall amount of the extracted volatiles. The operating conditions were selected in accordance with previously published optimisation procedure (Alissandrakis, Tarantilis, Harizanis, & Polissiou, 2007; Pontes et al., 2007) to avoid the formation of thermal artefacts.

Table 1 contains the minimum, maximum and mean percentages of identified compounds in headspace of *Paliurus* honey. A total of 33 compounds were identified from which 12 were detected only on HP-FFAP column and 5 were detected only on HP-101 column. Representative total ion chromatogram of *Paliurus* honey headspace volatiles is presented in Fig. 1.

Major constituents were aliphatic aldehydes, particularly nonanal (15.17–21.23%) followed by decanal (5.27–8.43%) and smaller quantities of octanal (0.00–2.00%) and heptanal (0.93–5.1%). Four isomeric lilac aldehydes were also found with high percentages (15.83–20.79%). Biogenetic studies have shown that lilac aldehydes are formed from linalool via (*E*)-8-hydroxylinalool and (*E*)-8-oxolinalool (Kreck, Püschel, Wüst, & Mosandl, 2003) or from linalool acetate (Wilkins, Lu, & Tan, 1993). Lilac aldehydes have been reported as characteristic compounds of nodding thistle honey (Wilkins et al., 1993) and citrus honey (Alissandrakis et al., 2007). Straight chain aliphatic acids were also present in headspace volatiles such as acetic (0.47–4.73%), hexanoic (0.83–1.27%), octanoic (1.20–2.43%) and nonanoic (2.20–2.23%) acids. 2-Ethylhexanoic acid (3.13–6.77%) was the only identified branched acid. Variable percentages of methyl esters, particularly methyl octanoate (0.00–0.97%) and methyl nonanoate (0.00–4.73%) were identified. Ubiquitous benzaldehyde (1.93–9.63%), 2-phenylethanol (0.67–0.87%), phenylacetaldehyde (6.10–6.83%) and benzyl alcohol (0.30–0.40%) were also found. Hotrienol was present with minor percentages (1.30–2.47%). It is known thermally generated product, but some quantity seems to exist in non-thermally treated honey (Alissandrakis et al., 2003).

3.2. Volatiles of Paliurus honey isolated by ultrasonic solvent extraction (USE)

Ultrasonic solvent extraction (USE) was previously used for the isolation of volatile compounds from other honeys without formation of thermal artefacts (Alissandrakis et al., 2003; Jerković et al., 2007). Firstly, solvent that was used in this research for USE procedure was the mixture pentane: ether 1:2 v/v. After the GC and GC–MS analyses large content of long chain saturated aliphatic hydrocarbons were found (particularly C_{21} (3.3–4.0%), C_{23} (5.2–7.3%), C_{24} (10.0–16.4%), C_{26} (1.6–2.1%), C_{27} (8.3–11.7%) and C_{29} (3.2–5.6%)), followed by higher aliphatic alcohols (1-hexadecanol (2.3–3.2%) and (*Z*)-9-octadecen-1-ol (3.6–5.9%)) and acids (particularly decanedioic (3.7–6.2%) and hexadecanoic acid (5.5–11.1%)). The aliphatic diterpenic alcohol phytol (0.0–0.6%) with a simple

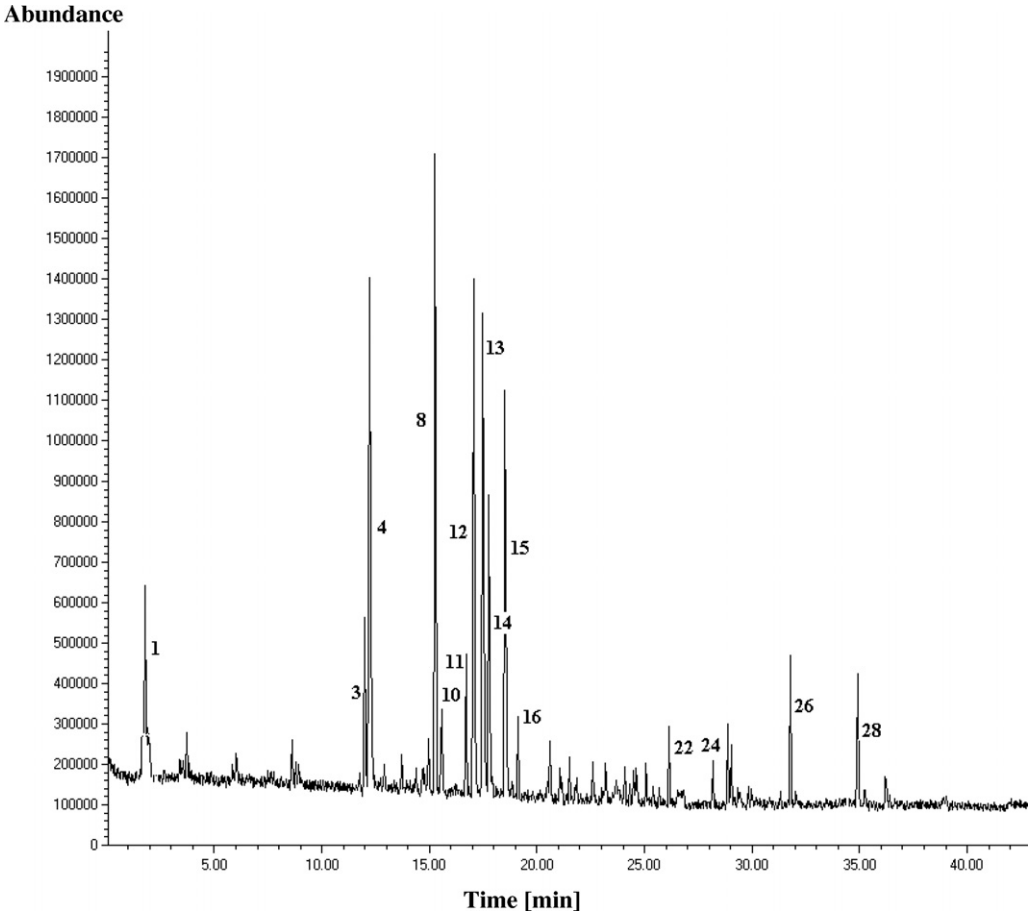

Fig. 1. Representative total ion chromatogram on HP-FFAP column of *Paliurus* honey headspace volatiles obtained by HS-SPME (PDMS/DVB fibre). Numbers refer to major compounds in Table 1.

structure that comprises about one-third of the chlorophyll molecule was also present in some samples. (*Z*,*Z*)-9,12-octadecadienal (0.0–12.2%) was the major aliphatic aldehyde, while only minor percentages of short chain aliphatic aldehydes were identified (nonanal (0.1%), octanal (0.0–0.1%) and decanal (0.0–0.6%)). The natural origin of all these compounds in the samples is probably from bee wax or plant wax which contains a variety of hydrophobic compounds that include very long chain hydrocarbons, alcohols, carbonyls, acids or others. In addition, the hydrocarbons were found on the cuticle of honeybees, but their role in nestmate recognition is more controversial in comparison with other insects (Châline et al., 2005). The products of Maillard reactions were not abundant, since no heat was applied: pyranones (such as 2-hydroxy-3-methyl-4H-pyran-4-one (0.0–0.9%) and 2,3-dihydro-3,5-dimethyl-4H-pyran-4-one (0.0–0.1%)), furans (5-hydroxymethylfurfural (0.3–0.6%), methyl 2-furancarboxylate (0.0–0.6%)), 1-H-pyrole-2-carboxylic acid (0.0–0.8%) and 2-methoxy-6-methylpyrazine (0.0–0.2%). Among other heterocycles the most abundant were 3-pyridinecabonitrile (0.0–0.1%) and 1-H-indol-3-acetic acid (0.0–0.6%). Shikimate pathway derivatives were found with small percentages such as cinnamic acid degradation products (benzoic (1.6–2.1%) and 4-methoxybenzoic (0.1–1.0%) acids), phenylacetic acid (1.3–1.9%) as well as structurally similar 4-hydroxybenzoic (3.0–3.9%) and 4-hydroxy-3,5-dimethoxybenzoic (0.1–1.5%) acids. Ubiquitous honey flavour volatiles where also found such as phenylacetaldehyde (Strecker aldehyde; 0.0–0.2%), 2-phenylethanol (0.0–0.3%) and benzyl alcohol (0.0–0.1%).

To obtain better separation and more complete analysis of the volatiles each sample was also extracted (USE) with pure pentane and then (after pentane extract was removed) diethyl ether was applied (USE) for the isolation of more polar constituents. The pentane extracts were mainly constituted of higher hydrocarbons (mainly C_{21} (4.9–14.9%), C_{23} (5.2–7.8%), C_{24} (9.1–10.8%), C_{27} (4.5–18.8%), C_{28} (1.5–12.0%) and C_{29} (8.3–13.1%)), oleil alcohol (5.4–9.3%), (*Z*,*Z*)-9,12-octadecadienal (0.0–10.3%), 1-hexadecanol (3.6–4.0%) and hexadecanoic acid (7.5–8.7%). These pentane ultrasonic extracts enabled extraction of apolar compounds, so the following diethyl ether extracts were free from the most abundant apolar constituents. Representative total ion chromatogram of diethyl ether extract is shown in Fig. 2.

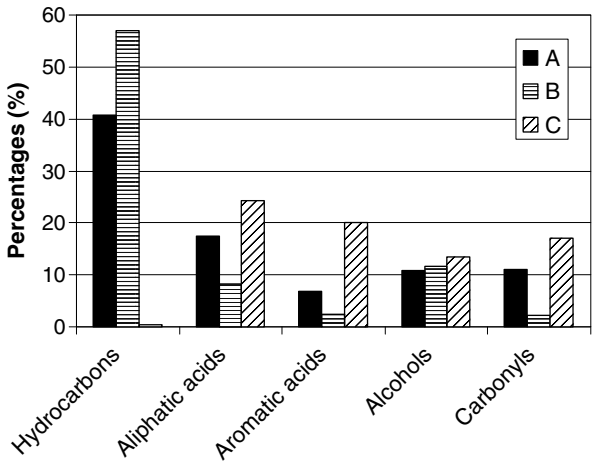

Fig. 3. Total average percentages of hydrocarbons, aliphatic and aromatic acids, alcohols and carbonyls from Table 2 isolated by ultrasonic solvent extraction (USE) with different solvents: A, mixture pentane:diethyl ether = 1:2 v/v; B, pentane; C, diethyl ether after the extraction with pentane.

The diethyl ether ultrasonic extracts exhibited two major compounds hexadecanoic (5.3–11.7%) and more polar decanedioic (6.1–11.0%) acids. Other abundant components were benzoic (8.2–14.9%), 4-hydroxybenzoic (5.1–6.4%), acetic (4.1–4.8%), phenylacetic (3.3–3.8%) and stearic acids (0.6–2.2%) with the most

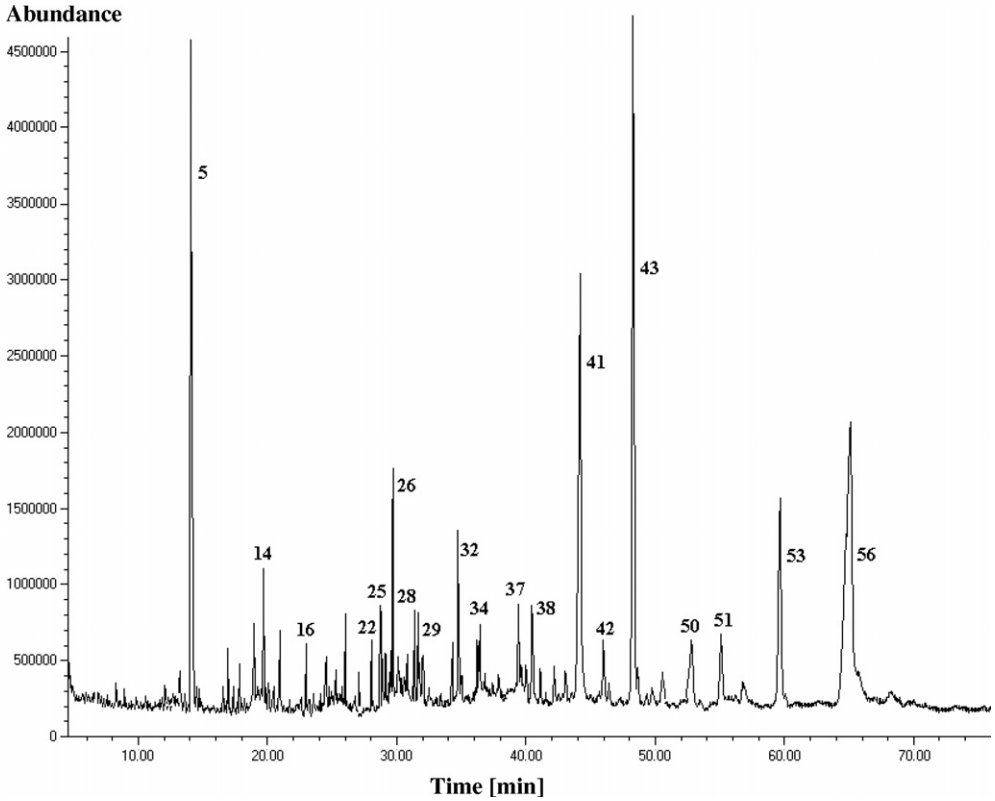

Fig. 2. Representative total ion chromatogram on HP-FFAP column of *Paliurus* honey diethyl ether extract. Numbers refer to major compounds in Table 2.

abundant percentages in all the extracts. Sixteen compounds were identified only in the diethyl ether extracts and therefore these extracts were useful for better identification of polar biomarker compounds that are not so abundant in other extracts.

Fig. 3 presents average percentages of hydrocarbons, aliphatic acids, aromatic acids, alcohols and carbonyls from Table 2 isolated by ultrasonic solvent extraction (USE) with different solvents.

3.3. Paliurus honey volatile biomarkers

Among the compounds identified by HS-SPME and USE, only a part of them could be used as floral markers of *Paliurus* honey as they are present in other honeys in significantly lower amounts. In headspace profile these were nonanal, decanal, four isomers of lilac aldehyde, methyl nonanoate, hexanoic and 2-ethylhexanoic acids. High content of three isomers of lilac aldehyde were previously found in citrus honey of Greek origin (Alissandrakis et al., 2007). Nonanal was identified in citrus, eucalyptus, lime tree, chestnut and thyme honeys (Piasenzotto, Gracco, & Conte, 2003; Radovic et al., 2001) and decanal is very abundant in chestnut and heather honeys (Radovic et al., 2001). Particularly interesting is the presence of 2-ethylhexanoic acid that was reported in Portugese multifloral honeys (Pontes et al., 2007), as well as methyl nonanoate and hexanoic acid, not so often in headspace honey volatiles.

Although the main components of USE extracts were higher saturated aliphatic hydrocarbons, higher aliphatic alcohols and acids, they can not be considered as reliable biomarkers due to their probably origin from bee wax or bee cuticle. Since they could also originate from plant wax and they were found in all the samples only the most abundant are mentioned: hydrocarbons C_{23}, C_{24}, C_{27}, C_{29}, hexadecanoic acid, decanedioic acid and (*Z*)-octedecen-1-ol. Although in smaller quantities, the more reliable markers were other classes of compounds such as benzene derivatives (particularly 4-hydroxy-3,5-dimethyl-benzaldehyde, 4-hydroxybenzoic acid, 4-methoxybenzoic acid) and lower aliphatic acids (butanoic, hexanoic, octanoic, nonanoic). 4-Methoxybenzoic acid was previously found as specific marker of *Erica arborea* honey (Guyot, Scheirman, & Collin, 1999). Small level of benzoic and phenylacetic acid were identified.

4. Conclusion

The results presented above indicate that there is a great variability in the identified volatiles, depending on the procedure employed that were complementary. The HS-SPME technique selectively isolated headspace volatiles, while USE method isolated volatiles and semi-volatiles. Different solvents applied for USE enabled obtaining of the extracts with components of different polarity that contributed to more complete overall analysis.

In headspace pattern of *Paliurus* honey biomarker compounds were nonanal, decanal, four isomers of lilac aldehyde, methyl nonanoate, hexanoic and 2-ethylhexanoic acids. Although the main components of USE extracts were higher saturated aliphatic hydrocarbons, higher aliphatic alcohols and acids, they can not be considered as reliable biomarkers, such as benzene derivatives (particularly 4-hydroxy-3,5-dimethyl-benzaldehyde, 4-hydroxybenzoic acid and 4-methoxybenzoic acid) and lower aliphatic acids (butanoic, hexanoic, octanoic and nonanoic). In further research, it would be interesting to investigate *Paliurus* honey volatiles of different geographical origin and volatiles from *Paliurus* nectar.

Acknowledgements

This research was supported by UKF Fund Evaluation of Unifloral Honeys – Chemical Fingerprinting and Nutritional Properties, Ministry of Science, Education and Sports of the Republic of Croatia. We are grateful to Dr. Dragan Bubalo, assistant Prof. (Faculty of Agronomy, University of Zagreb) for the pollen analyses on the honey samples and Mr. Miljenko Čavlina (“M&A”-Med, Marasovine) for providing the honey samples.

References

Alissandrakis, E., Daferera, D., Tarantalis, P. A., Polissiou, M., & Harizanis, P. C. (2003). Ultrasound-assisted extraction of volatile compounds from citrus flowers and citrus honey. *Food Chemistry, 82*(4), 575–582.

Alissandrakis, E., Tarantilis, P. A., Harizanis, P. C., & Polissiou, M. (2005). Evaluation of four isolation techniques for honey aroma compounds. *Journal of the Science of Food and Agriculture, 85*, 91–97.

Alissandrakis, E., Tarantilis, P. A., Harizanis, P. C., & Polissiou, M. (2007). Aroma investigation of unifloral Greek citrus honey using solid-phase microextraction coupled to gas chromatographic-mass spectrometric analysis. *Food Chemistry, 100*, 396–404.

Bonvehi, J. S., & Coll, F. S. (2003). Flavour index and aroma profiles of fresh and processed honeys. *Journal of the Science of Food and Agriculture, 83*, 275–282.

Brantner, A., & Males, Z. (1990). Investigations on the flavonoid glycosides of *P. spina-christi*. *Planta Medica, 56*, 582.

Brantner, A. H., & Males, Z. (1999). Quality assessment of *P. spina-christi* extracts. *Journal of Ethnopharmacology, 66*, 175–179.

Châline, N., Sandoz, J.-C., Stephen, J., Martin, S. J., Ratnieks, F. L. W., & Jones, G. R. (2005). Learning and discrimination of individual cuticular hydrocarbons by honeybees (*Apis mellifera*). *Chemical Senses, 30*, 327–335.

Cuevas-Glory, L. F., Pino, J. A., Santiago, L. S., & Sauri-Duch, E. (2007). A review of volatile analytical methods for determining the botanical origin of honey. *Food Chemistry, 103*, 1032–1043.

El-Sayed, A. M. (2007). *The Pherobase: Database of Insect Pheromones and Semiochemicals, www.pherobase.com*.

Grlić, Lj. (1986). Enciklopedija samoniklog bilja. *August Cesarec, Zagreb*, 165.

Guyot, C., Scheirman, V., & Collin, S. (1999). Floral origin markers of heather honeys: *Calluna vulgaris* and *Erica arborea*. *Food Chemistry, 64*, 3–11.

Jerković, I., Mastelić, J., & Marijanović, Z. (2006). A variety of volatile compounds as markers in unifloral honey from dalmatian sage (*Salvia officinalis* L. *Chemistry & Biodiversity, 3*, 1307–1316.

Jerković, I., Mastelić, J., Marijanović, Z., Klein, Ž., & Jelić, M. (2007). Comparison of hydrodistillation and ultrasonic solvent extraction for the isolation of volatile compounds from two unifloral honeys of *Robinia pseudoacacia* L and *Castanea sativa* L. *Ultrasonic Sonochemistry, 14*, 750–756.

Kreck, M., Püschel, S., Wüst, M., & Mosandl, A. (2003). Biogenetic studies in *Syringa vulgaris* L.: Synthesis and bioconversion of deuterium-labeled precursors into lilac aldehydes and lilac alcohols. *Journal of Agricultural and Food Chemistry, 51*, 463–469.

Kustrak, D., Males, Z., Brantner, A., & Pitarevic, I. (1990). Flavonoids of the leaves of Christ’s thorn (*P. spina-christi* Mill). *Acta Pharmaceutica Jugoslavica, 40*, 551–554.

Louveaux, J., Maurizio, A., & Vorwohl, G. (1978). Methods of melissopalynology. *Bee World, 59*, 139–157.

Pawliszyn, J. (1997). *Solid-phase microextraction. Theory and practice*. New York: Wiley–VCH.

Piasenzotto, L., Gracco, L., & Conte, L. (2003). Solid phase microextraction (SPME) applied to honey quality control. *Journal of the Science of Food and Agriculture, 83*, 1037–1044.

Pontes, M., Marques, J. C., & Câmara, J. S. (2007). Screening of volatile composition from Portuguese multifloral honeys using headspace solid-phase microextraction-gas chromatography-quadrupole mass spectrometry. *Talanta, 74*, 91–103.

Radovic, B. S., Careri, M., Mangia, A., Musci, M., Gerboles, M., & Anklam, E. (2001). Contribution of dynamic headspace GC–MS analysis of aroma compounds to authenticity testing of honey. *Food Chemistry, 72*, 511–520.

Velcheva, M. P. (1986). Studies of *P. spina-christi* Mill. Note 1. Composition of some lipid components isolated from light petroleum extracts of the fruits of *P. spina-christi* Mill. *Rivista Italiana delle Sostanze Grasse, 63*, 213–216.

Velcheva, M. P. (1993). Constituents of *P. spina-christi*. *Fitoterapia, 64*, 284–285.

Wilkins, A. L., Lu, Y., & Tan, S. T. (1993). Extractives from New Zealand honeys. 4. Linalool derivatives and other substances from nodding thistle (*Carduus nutans*) honey. *Journal of Agricultural and Food Chemistry, 41*, 873–878.