A Variety of Volatile Compounds as Markers in Unifloral Honey from Dalmatian Sage (*Salvia officinalis* L.)

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Volatile compounds of unifloral *Salvia officinalis* L. honey has been investigated for the first time. The botanical origin of ten unifloral *Salvia* honey samples has been ascertained by pollen analysis (the honey samples displayed 23–60% of *Salvia* pollen). Fifty-four volatile compounds were identified by GC and GC/MS in ten *Salvia* honey extracts obtained by ultrasound-assisted extraction (USE) with pentane/Et_2O 1:2. The yield of isolated volatiles varied from 25.7 to 30.5 mg kg^{-1}. *Salvia* honey could be distinguished on the basis of the high percentage of benzoic acid (6.4–14.8%), and especially phenylacetic acid (5.7–18.4%). Minor, but floral-origin important volatiles were identified such as shikimate pathway derivatives, 'degraded-carotenoid-like' structures (3,5,5-trimethylcyclohex-2-ene derivatives) and 2,6,6-trimethylcyclohex-2-ene derivatives. Compounds from other metabolic pathways such as aliphatic acids and higher linear hydrocarbons, as well as heterocycles (pyrans, furans, and pyrroles), were also present. Most of the identified compounds do not constitute specific *Salvia* honey markers, due to their presence in honeys of other botanical origins; however, their ratio in different honeys could be useful to distinguish floral origin. *Salvia*-honey volatile markers were: benzoic acid, phenylacetic acid, *p*-anisaldehyde, α-isophorone, 4-ketoisophorone, dehydrovomifoliol, 2,6,6-trimethyl-4-oxocyclohex-2-ene-1-carbaldehyde, 2,2,6-trimethylcyclohexane-1,4-dione, and coumaran.

Introduction. – In Dalmatian region of Croatia, the famous unifloral honey is made from *Salvia officinalis* L., and it is used in traditional medicine for treatment of respiratory problems, as an antiseptic, and others. Dalmatian sage (*Salvia officinalis* L.) grows spontaneously on the sunny hillsides of the Dalmatian islands and adjacent coast zones (800–5000-m broad) of the Adriatic Sea [1]. It was previously considered mainly for its essential oil content [2][3], and *Guenther* [4] noted that the best type of aromatic sage plant is produced in the district of Dubrovnik, South Croatia. The yield and composition of Croatian *Salvia officinalis* L. essential oil have been determined with special reference to the content of α/β-thujones, 1,8-cineole, and camphor [1][5]. In the past few decades, sage has been the subject of an intense study for its phenolic antioxidant components [6–9]. The antioxidant properties have been related to carnosic acid, carnosol, and rosmarinic acid [10]. The phenol carbonic acids (rosmarinic, caffeic, chlorogenic, and ferulic acid) and oligomers of caffeic acid with multiple catechol groups are all constituents of *Salvia officinalis* [11]. Quercetin, luteolin, apigenin, and kaempferol contents of *Salvia officinalis* were also determined, including *Salvia* honey [12].

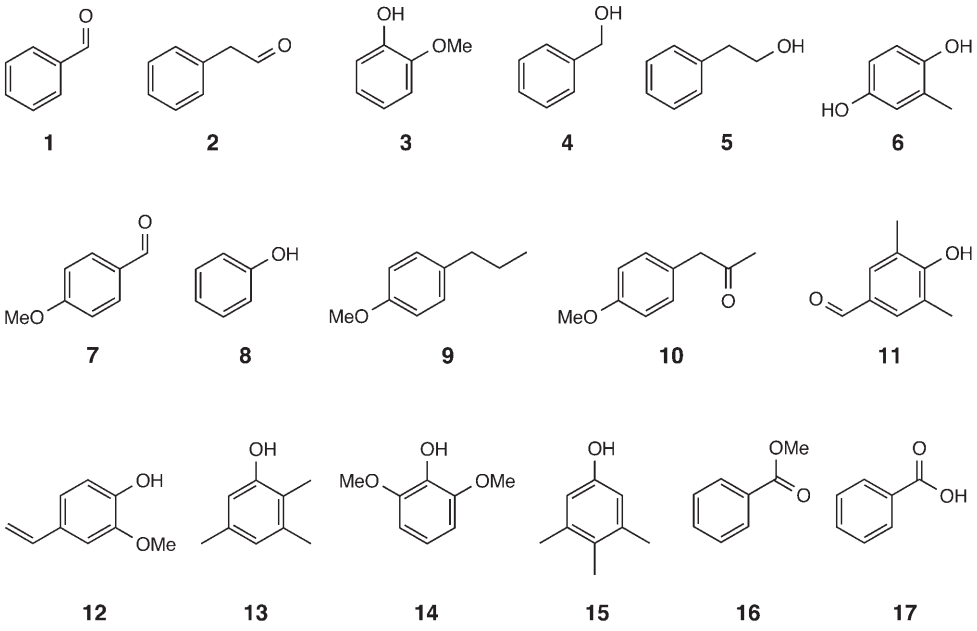
Since one of the most typical features of honey is its aroma profile, it is more recently used to characterize volatile marker compounds (chemical 'fingerprint')

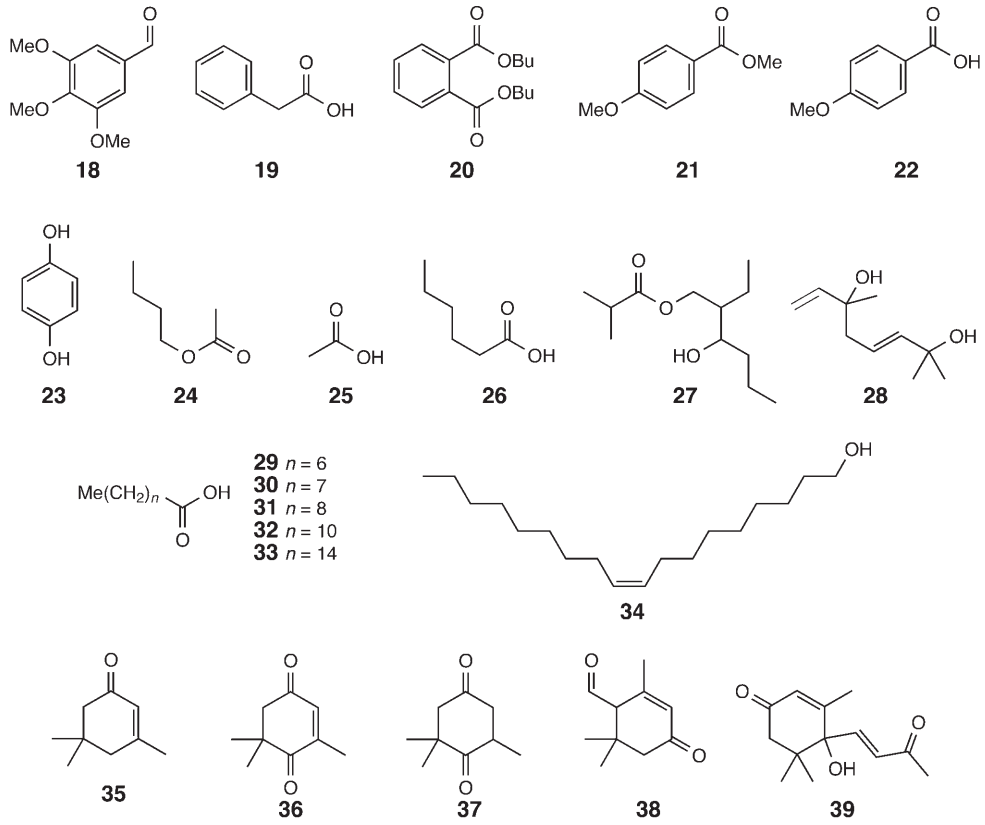
specific for botanical origin of given unifloral honey [13–15]. Different sources of aroma compounds in honey have been proposed, like plant constituents, transformation of plant constituents by honeybee, direct generation of constituents by the honeybee, generation of aroma compounds by thermal processing of honey, and others. Flavor qualities of honey particularly depend on the volatile and semivolatile organic compounds present in sample matrix and headspace aroma.

In general, literature data about chemical composition of *Salvia* honey is limited. The honey from flowering plants of *Salvia officinalis* L. in June is considered unifloral when pollen analysis confirmed the pollen percentage of at least 20% [16]. Although there are reports about honey volatiles of different botanical origin, to our knowledge, nothing is mentioned about *Salvia officinalis* L. honey. Therefore, the aim of this work is to investigate, for the first time, the presence of volatiles that can be useful as markers of Dalmatian sage unifloral honey. They were isolated by ultrasound-assisted extraction (USE) in order to avoid thermal artefacts. The qualitative and quantitative composition of isolated volatiles was determined by GC and GC/MS analyses.

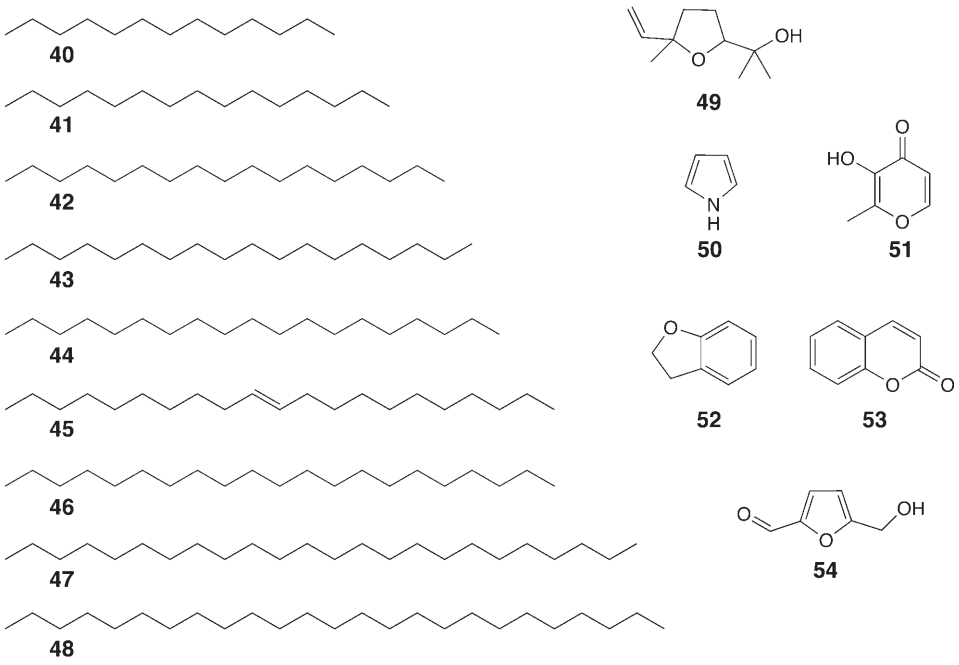
Results and Discussion. – The botanical origin of unifloral *Salvia* honey samples has been ascertained by pollen analysis, and it showed that the honey samples in this study displayed 23–60% of *Salvia* pollen (*Table*), in accordance with regulations [16] for *Salvia* unifloral honey.

Fifty-four volatile compounds, **1**–**54**, were identified by GC/MS in *Salvia officinalis* L. honey USE extracts (*Table*), representing 77.8–85.3% of total peak area. The yield of isolated volatiles varied among the investigated samples from 25.5 to 30.5 mg kg^{-1}.





USE is performed at room temperature so the formation of thermal artefacts is avoided in comparison with hydrodistillation methods. In addition, USE enables significant reduction of the extraction time [17] in comparison with traditional methods (exp. shake-flask extraction). The compounds identified cover a range of chemical classes including carbonyl compounds, acids, phenols, hydrocarbons, alcohols, and others. Most of the observed peaks in the chromatograms do not constitute specific *Salvia* honey markers, due to their presence in honeys of other botanical origins, but their ratio in different honeys could be useful to distinguish different floral origin. It is difficult to compare our results with the results of other published honey volatiles due to different extraction techniques applied, so general similarities/differences were noted. Namely, frequently used *Likens–Nickerson* steam distillation/solvent extraction (SDE) with its modifications usually does not lead to isolation of important low-molecular high-boiling compounds (such as benzoic acid and similar compounds) that are isolated by USE, and SDE isolate obtained usually contains thermal artefacts. In addition, many researchers performed solid-phase micro extraction (SPME) with different fibres for headspace flavor isolation and consequently did not detect semivolatiles.



The total ion current GC/MS chromatogram (*Fig.*) of *Salvia* honey was dominated by benzoic acid (**17**), resulting mainly from cinnamic acid degradation [18]. This compound was present in our samples at percentages ranging from 6.4 to 14.8%. With its flavor threshold around 85 μg g^{-1} [18], benzoic acid (**17**) could contribute to the aroma of *Salvia* honey. Heather honeys displayed also **17** [13] that was isolated by a CH_2Cl_2 solubilization, followed by *Likens–Nickerson* steam distillation/solvent extraction. It should be taken into account that **17** has high boiling point and high solubility in boiling water, and it was not previously quantitatively isolated [13]. In addition, SPME with different fibres usually does not detect benzoic acid (**17**). In comparison with our unpublished results, the content of benzoic acid (**17**) in *Salvia* honey is much higher in comparison with USE extracts from honeys of *Pseudoacacia robinia* L., *Castanea sativa* L., and *Satureja montana* L.

The second quantitatively important aromatic acid was 2-phenylacetic acid (**19**; 5.7–18.4%, *Table*). Previously, it was found exclusively in *Calluna vulgaris* honeys with approximate concentration varying from 7.5 to 16.8 μg g^{-1} [13]. Derived from the shikimate pathway, 2-phenylacetic acid (**19**) exhibits a flavor threshold of 2.5 μg g^{-1} [18] and is currently described as displaying 'honey-like' notes. Its high content could be valuable as *Salvia* honey marker. High content of **19** is also found in *Castanea sativa* honey USE extract (our unpublished results). Structurally related 2-phenylacetaldehyde (**2**) was also found in *Salvia* USE honey isolates with percentages of 1.1 up to 4.2%. It is not valuable as sage marker since 2-phenylacetaldehyde (**2**) has been found to participate in the aroma of many types of honeys, like benzaldehyde (**1**) and 2-

Table. *USE Extracted Volatile Compounds of Honey from* Salvia officinalis L.

Compound	Name	*RI*[a])		Peak area [%][b])			
		(*HP-20 M*)	(*HP-101*)	Min.	Max.	Av.	σ
Phenols, Phenylpropane Derivatives, and Related Compounds							
1	Benzaldehyde	1472	952	0.0	0.3	0.20	0.17
2	2-Phenylacetaldehyde	1591	1040	1.1	4.2	2.73	1.57
3	2-Methoxyphenol (=Guaiacol)	1789	–	0.3	0.6	0.36	0.21
4	Benzyl alcohol	1805	1106	0.3	0.7	0.33	0.35
5	2-Phenylethanol	1844	1165	0.9	2.1	1.70	0.69
6	2-Methylbenzene-1,4-diol	1905	–	0.0	0.8	0.26	0.46
7	4-Methoxybenzaldehyde (=*p*-Anisaldehyde)	1953	1271	1.0	3.9	2.20	1.51
8	Phenol	1926	1152	0.7	3.8	2.10	1.57
9	1-Methoxy-4-propylbenzene	1992	–	0.0	0.8	0.26	0.46
10	1-(4-Methoxyphenyl)propan-2-one (=*p*-Methoxyphenylacetone)	1996	1312	0.1	5.8	2.96	2.90
11	4-Hydroxy-3,5-dimethylbenzaldehyde	2092	1784	0.0	5.1	0.00	0.00
12	4-Ethenyl-2-methoxyphenol	2114	1365	1.2	3.0	2.30	0.96
13	2,3,5-Trimethylphenol	2142	–	0.0	0.5	0.16	0.29
14	2,6-Dimethoxyphenol	2184	–	0.3	3.0	1.60	1.35
15	3,4,5-Trimethylphenol	>2200	–	0.1	2.1	1.13	1.06
16	Methyl benzoate	-	1388	0.1	0.5	0.16	0.29
17	Benzoic acid	>2200	1402	6.4	14.8	11.0	4.25
18	3,4,5-Trimethoxybenzaldehyde	>2200	–	0.0	0.7	0.23	0.40
19	2-Phenylacetic acid	>2200	1486	5.7	18.4	10.4	6.94
20	Dibutyl phtalate	>2200	–	0.0	0.7	0.23	0.40
21	Methyl 4-methoxybenzoate	–	1594	0.0	2.5	0.83	1.44
22	4-Methoxybenzoic acid	>2200	1664	0.0	2.9	1.90	1.65
23	Benzene-1,4-diol (=Hydroquinol)	–	1720	0.0	4.1	1.63	2.17
Aliphatic Acids, Carbonyl Compounds, and Alcohols:							
24	Butyl acetate	1062	–	0.0	7.9	2.63	4.56
25	Acetic acid	1397	–	0.6	1.1	0.76	0.29
26	Hexanoic acid	1727	–	0.1	0.7	0.23	0.40
27	2-Ethyl-3-hydroxyhexyl 2-methylpropanoate	1820	–	0.0	4.3	1.43	2.48
28	3,7-Dimethylocta-1,5-diene-3,7-diol[c])	1892	–	0.1	1.5	0.50	0.86
29	Octanoic acid (=Caprylic acid)	1986	–	0.0	1.7	0.56	0.98
30	Nonanoic acid	2096	–	0.7	1.4	1.06	0.35
31	Decanoic acid (=Capric acid)	>2200	1454	0.0	1.5	0.93	0.81
32	Dodecanoic acid (=Lauric acid)	>2200	–	0.0	0.8	0.26	0.46
33	Hexadecanoic acid (=Palmitic acid)	–	2029	0.0	1.4	0.46	0.81
34	(*Z*)-Octadec-9-en-1-ol	>2200	2058	0.0	1.3	0.83	0.72
Derivatives of 3,5,5-Trimethylcyclohex-2-ene and 2,6,6-Trimethylcyclohex-2-ene and Similar Structures							
35	3,5,5-Trimethylcyclohex-2-en-1-one (=α-Isophorone)	1546	1108	0.1	0.4	0.26	0.23
36	3,5,5-Trimethylcyclohex-2-ene-1,4-dione (=4-Ketoisophorone)	1636	1132	0.5	0.6	0.56	0.06
37	2,2,6-Trimethylcyclohexane-1,4-dione	1717	–	0.1	1.3	0.56	0.66
38	2,6,6-Trimethyl-4-oxocyclohex-2-ene-1-carbaldehyde	2001	1319	5.4	9.5	6.90	2.26

Table (cont.)

Compound	Name	*RI*[a])		Peak area [%][b])			
		(*HP-20 M*)	(*HP-101*)	Min.	Max.	Av.	σ
39	4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-2-enyl)cyclohex-2-en-1-one (= Dehydrovomifoliol)	–	1885	0.0	3.2	1.06	1.85
Hydrocarbons							
40	Tridecane	1300	–	0.0	0.7	0.23	0.40
41	Pentadecane	1500	–	0.0	0.8	0.40	0.40
42	Heptadecane	1700	–	0.2	0.4	0.30	0.10
43	Octadecane	1800	–	0.0	0.6	0.20	0.35
44	Nonadecane	1900	–	0.0	1.5	0.50	0.86
45	Henicos-10-ene[c])	–	2059	0.0	1.2	0.40	0.69
46	Henicosane	2100	2100	0.7	1.3	1.00	0.30
47	Tetracosane	>2200	>2200	1.2	2.6	0.96	0.87
48	Pentacosane	>2200	>2200	0.1	1.4	0.46	0.81
Other Compounds							
49	*cis*-5-Ethenyltetrahydro-α,α,5-trimethylfuran-2-methanol (= Linalool oxide B)	1404	–	0.0	0.3	0.10	0.17
50	1*H*-Pyrrole	1467	–	0.0	0.5	0.16	0.29
51	3-Hydroxy-2-methyl-4*H*-pyran-4-one	1899	1169	0.0	4.8	1.60	2.77
52	2,3-Dihydrobenzofuran (= Coumaran)	>2200	1403	4.1	6.8	5.40	1.35
53	2*H*-1-Benzopyran-2-one	>2200	–	0.0	0.7	0.23	0.40
54	5-(Hydroxymethyl)furan-2-carbaldehyde	1899	1169	1.3	5.4	2.86	2.21
Yield [mg/kg]				25.7	30.5	28.3	2.42
Pollen percentage [%]				23	60	39.3	18.87

[a]) *RI* (*HP-20 M*): retention indices on *HP-20 M* column, *RI* (*HP-101*): retention indices on *HP-101* column, – : not detected on this column. [b]) Min.: minimal percentage, Max.: maximal percentage, Av.: average percentage, σ: standard deviation. [c]) Correct (*E*,*Z*)-isomer not identified.

phenylethanol (**5**) (also detected in *Salvia* honey, *Table*). *Strecker* degradation of amino acids, especially during heat treatment, can produce aromatic aldehydes, and 2-phenylacetaldehyde (**2**) is the *Strecker* aldehyde of phenylalanine. In our preliminary research, we performed hydrodistillation of *Salvia* honey and obtained an isolate containing mainly 2-phenylacetaldehyde (>90%).

From the *Table*, it can be seen that three 3,5,5-trimethylcyclohex-2-ene derivatives (= norisoprenoids) were present: 3,5,5-trimethylcyclohex-2-en-1-one (= α-isophorone; **35**) with a mean percentage of 0.26%, 3,5,5-trimethylcyclohex-2-ene-1,4-dione (= 4-ketoisophorone; **36**) with a mean percentage of 0.56%, and 4-hydroxy-3,5,5-trimethyl-4-(3-oxobut-2-enyl)cyclohex-2-en-1-one (= dehydrovomifoliol; **39**) with an average percentage of 1.06%. In general, they have attractive sensory properties and low odor thresholds. Norisoprenoids were already identified in honeys from different botanical origins such as strawberry tree [19], thyme [20], heather [13], and eucalyptus [21]. Despite their 'degraded-carotenoid-like' structure (*i.e.*, 3,5,5-trimethyl-cyclohex-

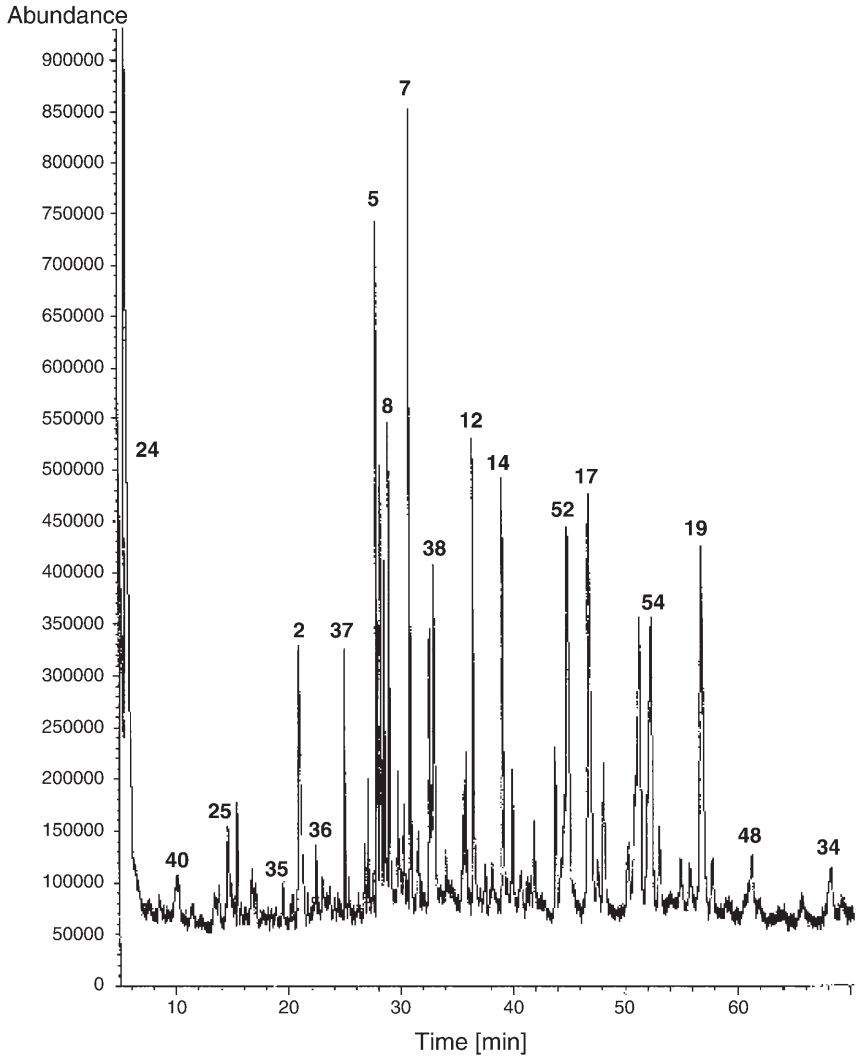


Figure. *Representative total ion current GC/MS chromatogram of* Salvia officinalis L. *honey volatiles* (isolated by USE) *on a* HP-20 M *column*. Numbers refer to major compounds in the *Table.*

2-ene), these substances probably arise through degradation of abscissic acid, a well-known growth hormone [20]. α-Isophorone (**35**) is particularly abundant in *Calluna vulgaris* samples [13], and it was exceptionally observed in sunflower and eucalyptus honeys [21], thus restricting the use of this compound as specific floral marker. Two other 2,2,6-trimethylcyclohex-2-ene derivatives were also identified: 2,6,6-trimethyl-4-oxocyclohex-2-ene-1-carbaldehyde (**38**; average percentage 6.90%) and 2,2,6-trimethylcyclohexane-1,4-dione (**37**; average percentage 0.56%). These structures could be specific *Salvia* markers.

4-Methoxybenzaldehyde (= *p*-anisaldehyde; **7**) is another shikimate-pathway derivative. This compound was not detected in any of other honey samples, except *Erica arborea* honeys [13], and *Blank* and *Fischer* determined it to be one of the most powerful odorants [22]. This aroma compound might derive from cinnamic acids through cleavage of an acetate during formation of benzoic acids [18].

Phenol (**8**), 2-methoxyphenol (**3**), 4-ethenyl-2-methoxyphenol (= eugenol; **12**), 2,5-dimethylphenol (**14**), 2,3,5-trimethylphenol (**13**), and 3,4,5-trimethylphenol (**15**) were also isolated. Volatile phenols are mainly produced by biochemical degradation of phenolic acids in honey. Among them, the average percentages of eugenol (2.30%) and phenol (2.10%) were the highest. Eugenol is known as honeybee attractant [23]. The presence of phenol in honey is controversial because it was first used as a bee repellent, but an alternative theory suggests that phenol is a natural constituent of honey [24].

Hexanoic acid (**26**), octanoic acid (**29**), nonanoic acid (**30**), decanoic (**31**), dodecanoic (**32**), and hexadecanoic acid (**33**) were also identified. Decanoic acid (**31**) was previously exclusively detected in heather honeys [13], while linear fatty acids were found as honey constituents [20]. With a low flavor threshold, fatty acids should not contribute to *Salvia* honey aroma.

In addition, high-molecular-weight *n*-alkanes (particularly C_{21} and C_{24}) were identified, and, in general, the hydrocarbons were the largest single class of compounds contained in hexane extract of honey [25]. Their pattern was very similar to beewax, and, in some cases, wax may be transported by bees from some part of visited plants. It is assumed that they can also originate from a condensation–decarboxylation–reduction–elimination mechanism [26] or others.

Other chemical classes of identified compounds in the *Table* included mainly heterocycles like furans, pyrans, and pyrroles that could originate from *Maillard* reactions [27]. Among them, the percentage of 2,3-dihydrobenzofuran (= coumaran; **52**) was highest (4.1–6.0%), followed by 5-(hydroxymethyl)furan-2-carbaldehyde (1.3–5.4%).

Conclusions. – Due to the lack of current physicochemical determinations and literature data on *Salvia officinalis* L. honey chemical composition, volatiles identified in this work (for the first time) are important for its unifloral determination. Quite rapid USE procedure, easy to be carried out, enabled isolation of volatile and semivolatile honey compounds without thermal artifacts.

Salvia honey could be distinguished on the basis of its high content in benzoic acid, especially 2-phenylacetic acid (previously found exclusively in *Calluna vulgaris* honeys). Minor, but originally important volatiles such as shikimate-pathway derivatives, 'degraded-carotenoid-like' structures (*i.e.*, 3,5,5-trimethylcyclohex-2-ene derivatives), and 2,6,6-trimethylcyclohex-2-ene derivatives were identified. Compounds from other metabolic pathways were also present such as aliphatic and fatty acids, higher linear hydrocarbons (C_{13}, C_{15}–C_{19}, C_{21}, C_{24}, and C_{25}), and heterocycles (pyrans, furans, and pyrroles). Most of the observed peaks do not constitute specific *Salvia* honey markers, due to their presence in honeys of other botanical origins, but their ratio in different honeys could be useful to distinguish different floral origin. *Salvia*-honey volatile markers were: benzoic acid (**17**), 2-phenylacetic acid (**19**), *p*-anisaldehyde (**7**), α-isophorone (**35**), 4-ketoisophorone (**36**), dehydrovomifoliol (**39**),

2,6,6-trimethyl-4-oxocyclohex-2-ene-1-carbaldehyde, 2,2,6-trimethylcyclohexane-1,4-dione (**37**), and coumaran (**52**).

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Experimental Part

Honey Samples and Reagents. Ten unifloral honey samples of *Salvia officinalis* L. were selected from various honey producers in South Croatia. Screening of honey unifloral origin was based on pollen analysis and sensory test. Melissopalynological analysis was performed by the methods recommended by the *International Commission for Bee Botany* [28]. All honey samples met Croatian requirements [16] for unifloral origin (*Salvia* pollen percentage of at least 20%). All samples were stored in hermetically closed glass jars at 4° until analyzed.

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. Anh. $MgSO_4$ and menthol were obtained from *Fluka Chemie* (CH-Buchs).

Ultrasound-Assisted Extraction (USE). 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°. Each sample was extracted in duplicate as described below. 40 g of each honey sample were dissolved with 22 ml of dist. H_2O in a 100-ml flask. $MgSO_4$ (1.5 g) was added, and each sample was extensively vortexed. A mixture (20 ml) of pentane/Et_2O 1 : 2 was used as the extraction solvent. Sonication was held for 30 min. After sonication, the org. layer was separated in a separation funnel and filtered over anh. $MgSO_4$. The aq. layer was returned to flask, and another batch of extraction solvent (20 ml) was added, and the mixture was extracted by ultrasound for 30 min. The org. layer was separated in the separation funnel, filtered over anh. $MgSO_4$, and the aq. layer was sonicated a third time for 30 min with another batch (20 ml) of the extraction solvent. Collected org. extracts were concentrated up to 0.2 ml by fractional distillation, and 1 µl was used for GC/MS analysis.

Gas Chromatography (GC). GC Analysis was performed on a *Hewlett-Packard* model *5890 Series II* gas chromatograph equipped with flame ionization detector and capillary column *HP-101* (methyl silicone fluid, *Hewlett-Packard*, A-Vienna), 25 m × 0.2 mm i.d., coating thickness 0.2 µm. Chromatographic conditions: He as carrier gas at 1.0 ml min^{-1}; injector and detector temp., 250° and 300°. Oven temp. was isothermal at 70° for 2 min, then increased to 200°, at a rate of 3° min^{-1}, and held isothermal for 15 min; volume injected 1 µl; split ratio 1 : 50.

Gas Chromatography Mass Spectrometry (GC/MS). The samples were analyzed by GC/MS (*Hewlett-Packard*, model *5890*, with a mass selective detector, model *5971A*) on two columns. GC Operating conditions [29][30]: column *HP-20 M* (*Carbowax 20 M*, *Hewlett-Packard*, A-Vienna), 50 m × 0.2 mm i.d., film thickness 0.2 µm; column temp. programmed from 70° isothermal for 4 min, then increased to 180° at a rate of 4° min^{-1}; column *HP-101* (methyl silicone fluid, *Hewlett-Packard*, A-Vienna), 25 m × 0.2 mm i.d., film thickness 0.2 µm; column temp. programmed from 70° isothermal for 2 min, then increased to 200° at a rate of 3° min^{-1}; carrier gas He, flow rate 1 ml min^{-1}; injector temp. 250°; volume injected 1 µl; split ratio 1 : 50. MS Conditions: ionization voltage: 70 eV; ion-source temp. 280°; mass range: 30–300 mass units.

Quantization and Identification. The individual peaks were identified by comparison of their retention indices (relative to C_8–C_{22} *n*-alkanes) with those of authentic samples and literature values [31], as well as by comparing their mass spectra with the *Wiley 6.0* library (John Wiley & Sons) and *NIST98* (National Institute of Standards and Technology, Gaithersburg) mass-spectral database. The percentage composition of the samples was calculated from the GC-peak areas using the normalization

method (without correction factors). For quantization, internal standard (menthol) was added prior to extraction. Preliminary GC/MS analysis showed the absence of menthol among the *Salvia officinalis* L. honey volatiles.

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