Communication

Headspace Solid-Phase Microextraction and Ultrasonic Extraction with the Solvent Sequences in Chemical Profiling of *Allium ursinum* L. Honey

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Abstract: A volatile profile of ramson (wild garlic, *Allium ursinum* L.) honey was investigated by headspace solid-phase microextraction (HS-SPME) and ultrasonic solvent extraction (USE) followed by gas chromatography and mass spectrometry (GC-FID/GC-MS) analyses. The headspace was dominated by linalool derivatives: cis- and trans-linalool oxides (25.3%; 9.2%), hotrienol (12.7%), and linalool (5.8%). Besides direct extraction with dichloromethane and pentane/diethyl ether mixture (1:2, v/v), two solvent sequences (I: pentane → diethyl ether; II: pentane → pentane/diethyl ether (1:2, v/v) → dichloromethane) were applied. Striking differences were noted among the obtained chemical profiles. The extracts with diethyl ether contained hydroquinone (25.8–36.8%) and 4-hydroxybenzoic acid (11.6–16.6%) as the major compounds, while (*E*)-4-(r-1′,t-2′,c-4′-trihydroxy-2′,6′,6′-trimethylcyclohexyl)-but-3-en-2-one predominated in dichloromethane extracts (18.3–49.1%). Therefore, combination of different solvents was crucial for the comprehensive investigation of volatile organic compounds in this honey type. This particular magastigmane was previously reported only in thymus honey and hydroquinone in vipers bugloss honey, while a combination of the mentioned predominant compounds is unique for *A. ursinum* honey.

Keywords: *Allium ursinum* L. honey; headspace solid-phase microextraction (HS-SPME); ultrasonic solvent extraction (USE) with the solvent sequence; (*E*)-4-(r-1′,t-2′,c-4′-trihydroxy-3′,6′,6′-trimethylcyclohexyl)-but-3-en-2-one; hydroquinone; methyl syringate; 4-hydroxybenzoic acid

1. Introduction

Ramson (wild garlic, *Allium ursinum* L.) is a perennial plant, widely distributed in Europe. Phytochemical investigations of this plant revealed the presence of S-alk(en)yl-L-cysteine-sulfoxides (methiin, alliin, isoalliin, propiin, and ethiin) and their degradation products ((poly)sulfides, dithiins, or ajoenes) [1,2]. Apart from the abovementioned, various sulphur compounds have also been detected as constituents of its essential oil, e.g., disulfides, trisulfides, and tetrasulfides [3,4]. *A. ursinum* has also been reported to be a rich source of phenolic compounds (up to 27.9 g GAE (gallic acid equivalent)/100 g) [5]. Similar to organosulfur compounds, it was found to contain steroidal saponins that are also commonly found in the *Allium* genus [1,6]. Other identified constituents of interest include lectins, polysaccharides, and fatty acids [1]. A great number of in vitro and in vivo experiments showed that *A. ursinum* is a plant with antimicrobial, cytotoxic, antioxidant, and cardio-protective effects [1,7].

*A. ursinum* provides excellent spring bee pasture with a good nectar flow [8,9]. *Allium* species tend to secrete highly concentrated nectar, and the daily nectar production of *A. ursinum* ranged from 0.1 to 3.8 µL per flower, with sugar concentrations of 25% to 50%. However, the floral nectar volume
and concentration varies in different populations of *A. ursinum* which can be also strongly affected by the varying conditions in different natural habitats. Nevertheless, the honey cannot be produced on a regular basis and its production is limited [8].

In continuation of the chemical fingerprinting of different unifloral honey types in search of specific or nonspecific chemical markers of botanical origin, the focus of this work was on not yet investigated volatile organic compounds (VOCs) of *Allium ursinum* L. honey of Croatian origin (a very rare sample). Headspace solid-phase microextraction (HS-SPME) followed by gas chromatography and mass spectrometry (GC-FID/GC-MS) analysis was applied to investigate its headspace chemical profile. To complement the honey profiling with data on less volatile organic compounds, ultrasonic solvent extraction (USE) was applied with solvents of different polarities, and the obtained extracts were analysed by GC-FID/GC-MS.

2. Results

A rare sample of *A. ursinum* honey from Croatia was confirmed to be unifloral according to performed mellisopalynological analysis. It contained 58% of *Allium ursinum* L. pollen grains accompanied by the pollen from *Prunus* spp. (19%), *Acer* spp. (14%), and a minor contribution from the grains of *Salix* spp. (2%), *Fraxinus excelsior* (1%), *Tilia* spp. (1%), Asteraceae (1%), Ericaceae (1%), and Brassicaceae (1%).

At the time of blooming, *A. ursinum* plants emit a strong garlic odour that can also be smelled in the nectar and in front of the beehives. However, it has been reported that the odour of the corresponding ripe honey is different, with a pleasant, particular aroma [8]. Therefore, significant differences among the chemical profiles obtained from *A. ursinum* honey VOCs and the corresponding plant VOCs were expected. To investigate in detail the headspace, volatile, and semi-volatile compounds from *A. ursinum* honey, up-to-date complementary methodologies were applied: headspace solid-phase microextraction (HS-SPME) and ultrasonic solvent extraction (USE) followed by GC-FID/GC-MS analyses. Striking differences were found among the chemical profiles obtained by those methods and the plant VOCs.

2.1. The Headspace Chemical Profile

The headspace of *A. ursinum* honey (Table 1) dominated with monoterpens—linalool derivatives such as *cis*- and *trans*-linalool oxides (9.2%; 25.3%), hotrienol (12.7%), and linalool (5.8%).

### Table 1. The Headspace volatiles of the sample determined by HS-SPME/GC-MS.

| No. | Compound                        | RI | RI | %  | No. | Compound                        | RI | RI | %  |
|-----|--------------------------------|----|----|----|-----|--------------------------------|----|----|----|
| 1   | Dimethyl disulfide              | <900 | 747 | 1.2 | 14 | Hotrienol                      | 1106 | 1110 | 12.7 |
| 2   | Butanoic acid                   | <900 | 763 | 0.7 | 15 | 2-Phenylethanol                 | 1116 | 1116 | 3.0  |
| 3   | 3-Methylbut-2-enal              | <900 | 781 | 1.0 | 16 | Phenylacetonitrile              | 1143 | 1141 | 1.9  |
| 4   | Octane                          | <900 | 800 | 0.1 | 17 | 4-Ketoisophorone               | 1147 | 1147 | 2.8  |
| 5   | 3-Methylbutanoic acid           | <900 | 888 | 1.7 | 18 | Octanoic acid                  | 1176 | 1179 | 1.7  |
| 6   | Benzoic acid                    | 965  | 966 | 1.1 | 19 | Nonan-1-ol                     | 1178 | 1171 | 1.4  |
| 7   | Hexanoic acid                   | 980  | 982 | 0.9 | 20 | *trans*-Linalool oxide (pyran type) | 1183 | 1183 | 1.5  |
| 8   | (E)-Hex-3-enoic acid            | 991  | /   | 0.7 | 21 | α-Terpinol                     | 1194 | 1191 | 0.8  |
| 9   | (Z)-Hex-3-enoic acid            | 1013 | 1013 | 0.8 | 22 | 5-Hydroxymethylfurural        | 1230 | 1226 | 4.0  |
| 10  | Phenylacetaldehyde              | 1048 | 1049 | 1.7 | 23 | 4-Methoxybenzaldehyde        | 1256 | 1258 | 1.1  |
| 11  | *cis*-Linalool oxide (furan type) | 1076 | 1075 | 25.3 | 24 | Nonanoic acid                  | 1273 | 1276 | 4.0  |
| 12  | *trans*-Linalool oxide (furan type) | 1091 | 1091 | 9.2 | 25 | 3,4,5-Trimethylphenol **       | 1336 | 1336 | 3.2  |
| 13  | Linalool                        | 1101 | 1101 | 5.8 | 26 | Hexadecanoic acid              | 1970 | 1977 | 1.7  |

1 RI—retention indices on HP-5MS column relative to C9-C25 alkanes; 2 RI from the literature (National Institute of Standards and Technology (NIST) Chemistry WebBook, NIST Standard Reference Database Number 69, http://webbook.nist.gov/chemistry/); 3 Area percentages (%); 4 identification confirmed with standard compound; *—tentatively identified; **—correct isomer is not identified.

Few benzene derivatives, often found in different honey types [10], were detected by HS-SPME with minor abundance, e.g., benzoic acid (1.1%), phenylacetaldehyde (1.7%), 2-phenylethanol (3.0%), 4-methoxybenzaldehyde (1.1%), and phenylacetonitrile (1.9%). 4-Ketoisophorone (2.8%) was the only
norisoprenoid detected in the headspace in distinction from the extracts. Dimethyl disulfide (1.2%) was the only headspace compound that could be connected to the plant VOCs (it was found in A. ursinum essential oil). The majority of the essential oil constituents, such as typical sulphides, disulfides, and trisulfides, were not present in the honey [3,4]. As was mentioned before, ripe ramson honey possesses a pleasant, particular aroma, and the probably typical sulfur volatile organic compounds were lost during the honey maturation in the hive. In addition, it is well known that honey VOCs usually significantly differ from the corresponding plant VOCs [11].

2.2. The Extracts Chemical Profile

Ultrasonic extraction (USE) of the honey was first performed separately with two solvents: (a) the mixture of pentane and diethyl ether (1:2, v/v) (A), and (b) dichloromethane (B), as in our previous research [12,13]. Significant differences were found among chemical profiles of the extracts (Table 2). The extract A contained 1,4-benzenediol (25.8%) as the major compound followed by a variety of benzene derivatives, particularly benzoic acid and its p-substituted derivatives: 4-hydroxybenzoic acid (16.4%), benzoic acid (4.4%), and 4-methoxybenzoic acid (3.7%). 4-Hydroxybenzaldehyde (10.3%) and methyl syringate (9.8%) were also quite abundant. In contrast, the extract B contained as the major compound C_{13}-norisoprenoid (E)-4-(r-1′,t-2′,c-4′-trihydroxy-3′,6′,6′-trimethylcyclohexyl)-but-3-en-2-one (18.3%), which was present only with 3.1% in the extract A. Aromatic compounds were present among the major compounds (similar as in the extract A: methyl syringate (12.2%), 4-hydroxybenzaldehyde (9.9%), 4-methoxybenzoic acid (4.2%), and benzoic acid (3.1%)). However, the major difference in 4-hydroxybenzoic acid and hydroquinone abundance was noted among two extracts (dominated in A). Both of them also contained other C_{13}-norisoprenoids (solvent A; solvent B): 3-oxo-α-ionone (1.8%; 1.5%), vomifoliol (1.1%; 2.6%), and 3-oxo-7,8-dihydro-α-ionone (0.5%; 0.1%). Higher aliphatic compounds were present among minor constituents in both extracts as well as trans- or cis-linalool oxides (furan type).

Table 2. The volatile organic compounds composition of the sample determined by ultrasonic solvent extraction (USE)/GC-FID; GC-MS.

| No. | Compound                                      | RI 1 | RI 2  | A % | B % | C % | D % | E % | F % |
|-----|----------------------------------------------|------|-------|-----|-----|-----|-----|-----|-----|
| 1   | 2-Furancarboxaldehyde                        | <900 | 835   | -   | -   | -   | -   | 0.6 | 0.1 |
| 2   | 4-Methylacetate                               | <900 | /     | 0.1 | -   | 0.1 | 0.1 | -   | -   |
| 3   | 1,3-Dimethylbenzene                          | <900 | 864   | 1.5 | -   | 0.6 | 0.7 | 0.6 | -   |
| 4   | 2-Furanmethanol                               | <900 | 866   | -   | -   | -   | -   | 0.1 | -   |
| 5   | Ethylbenzene                                  | <900 | 868   | 0.2 | -   | 0.6 | 0.2 | 0.1 | -   |
| 6   | 3-Methylbutanoic acid (Isovaleric acid)       | <900 | 888   | -   | -   | -   | -   | 0.1 | -   |
| 7   | 3-Methylbut-2-enoic acid *                   | <900 | /     | 0.1 | 0.2 | -   | 0.1 | 0.1 | -   |
| 8   | 1,2-Dimethylbenzene                          | <900 | 892   | 0.1 | -   | 0.1 | -   | -   | -   |
| 10  | Methoxybenzene                                | 912  | /     | 0.1 | -   | 0.2 | -   | -   | -   |
| 11  | 2-Acetylfuran                                 | 918  | 914   | -   | -   | -   | -   | 0.1 | -   |
| 12  | Benzaldehyde 4                                | 965  | 966   | 0.1 | 0.2 | 0.7 | 0.1 | -   | -   |
| 13  | 3-Methylfurfural 4                           | 970  | 966   | -   | -   | -   | -   | 0.1 | -   |
| 15  | (E)-Hex-3-enoic acid 4                       | 991  | /     | 0.5 | 0.3 | -   | 0.2 | -   | -   |
| 15  | (Z)-Hex-3-enoic acid 4                       | 1013 | 1013  | 0.1 | 0.2 | -   | 0.1 | 0.1 | -   |
| 16  | Pantolactone                                  | 1044 | /     | 0.1 | 0.2 | -   | 0.1 | 0.1 | 0.2 |
| 17  | Phenylacetaldehyde 4                         | 1048 | 1049  | 0.1 | 0.2 | 0.6 | 0.1 | -   | -   |
| 18  | Acetophenone 4                               | 1065 | 1065  | -   | -   | -   | 0.1 | -   | -   |
| 19  | cis-Linalool oxide (furan type)               | 1076 | 1075  | 0.7 | 0.3 | 3.3 | 0.2 | 1.3 | 0.1 |
| 20  | trans-Linalool oxide (furan type)             | 1091 | 1091  | 0.2 | 0.2 | 1.2 | -   | 0.4 | -   |
Table 2. Cont.

| No. | Compound | RI 1 | RI 2 | A % | B % | C % | D % | E % | F % |
|-----|----------|------|------|-----|-----|-----|-----|-----|-----|
| 21  | Linalool 4 | 1102 | 1101 | 0.1 | -   | -   | -   | -   | -   |
| 22  | Hotrienol  | 1106 | 1110 | 0.1 | 0.2 | 0.6 | -   | -   | -   |
| 23  | 2-Phenylethanol 4 | 1116 | 1116 | 0.7 | 0.7 | 1.6 | 0.3 | 0.4 | -   |
| 24  | 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one | 1143 | 1149 | 0.2 | 0.3 | -   | 0.3 | -   | -   |
| 25  | Benzoic acid 4 | 1181 | 1178 | 4.4 | 3.1 | 2.0 | 2.2 | 1.2 | 0.3 |
| 26  | Terpendiol I | 1191 | 1191 | 0.7 | 0.5 | 0.7 | 0.3 | -   | -   |
| 27  | 5-Hydroxymethylfurural 1 | 1230 | 1226 | 0.8 | 2.6 | -   | 1.1 | 13.3 | 0.8 |
| 28  | 4-Methoxybenzaldehyde 4 | 1259 | 1258 | 0.1 | 0.2 | 0.7 | -   | 0.3 | -   |
| 29  | Phenylacetic acid 4 | 1269 | 1270 | 0.8 | 0.8 | -   | 0.6 | 0.1 | -   |
| 30  | Benzoic acid 4 | 1273 | 1276 | 0.1 | 0.2 | 0.7 | -   | -   | -   |
| 31  | 1,4-Benzenediol 4 (Hydroquinone) | 1328 | /    | 25.8 | 2.4 | -   | 36.8 | 27.7 | 0.7 |
| 32  | 3,4,5-Trimethylphenol ** | 1336 | 1331 | 0.3 | 0.5 | -   | 0.3 | -   | -   |
| 33  | 2-Phenylethanol 4 | 1354 | 1348 | -   | -   | 2.8 | -   | -   | -   |
| 34  | Phenylpropionic acid 4 | 1359 | 1361 | 1.8 | 1.6 | -   | 0.4 | -   | -   |
| 35  | 1-Hydroxinalool ** | 1365 | /    | 0.3 | 0.3 | -   | -   | 0.1 | -   |
| 36  | 4-Hydroxybenzaldehyde 4 | 1393 | /    | 10.3 | 9.9 | -   | 5.3 | 2.5 | 1.2 |
| 37  | 4-Hydroxy-3-methoxy-benzaldehyde (Vanillin) 4 | 1412 | 1394 | 0.3 | 0.7 | -   | -   | -   | -   |
| 38  | 3,5,5-Trimethyl-4-(3-oxo-1-butenyl)cyclohex-2-en-1-one (3-Oxo-α-ionone) 4 | 1452 | 1451 | 3.7 | 4.2 | -   | 2.5 | 0.7 | 0.4 |
| 39  | (E)-3-Phenylprop-2-enolic acid (trans-Cinnamic acid) 4 | 1455 | 1457 | 0.9 | 0.7 | -   | 0.4 | 0.1 | 0.1 |
| 40  | Methyl 4-hydroxybenzoate 4 | 1482 | /    | 0.2 | 0.3 | -   | -   | -   | -   |
| 41  | 4-Hydroxy-phenylacetone 4 | 1502 | /    | 1.0 | 1.3 | -   | 0.8 | 0.3 | 0.3 |
| 42  | Methyl 4-hydroxy-3-methoxybenzoate 4 | 1530 | 1527 | 0.2 | 0.2 | -   | -   | -   | -   |
| 43  | 4-Hydroxybenzoic acid 4 | 1558 | 1558 | 16.4 | 0.2 | -   | 16.6 | 11.6 | -   |
| 44  | 3,5,5-Trimethyl-4-(3-oxo-1-butenyl)cyclohex-2-en-1-one (3-Oxo-α-ionone) 4 | 1665 | 1661 | 1.8 | 1.5 | 2.4 | -   | 0.3 | -   |
| 45  | Syringaldehyde 4 | 1668 | 1667 | 0.7 | -   | -   | 0.1 | -   | -   |
| 46  | 3,5,5-Trimethyl-4-(3-oxo-1-butenyl)cyclohex-2-en-1-one (3-Oxo-α-ionone) 4 | 1682 | 1681 | 0.5 | 0.1 | 0.7 | 0.3 | 0.3 | -   |
| 47  | Heptadecene 4 | 1700 | 1700 | 0.2 | -   | 0.9 | 0.1 | -   | -   |
| 48  | Methyl syringate 4 | 1744 | 1744 | 9.8 | 12.2 | 26.2 | 3.0 | 6.6 | 1.0 |
| 49  | 4-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)cyclohex-2-en-1-one (Vomifoliol) 4 | 1802 | 1796 | 1.1 | 2.6 | -   | 1.1 | 0.9 | -   |
| 50  | Hexadecane 4 | 1882 | 1883 | 0.1 | 0.2 | 1.2 | 0.2 | 0.4 | 1.5 |
| 51  | (E)-4-(t1 ’,t2 ’,e4 ’,t6 ’,e4 ’,t6 ’,trimethylcyclohexyl)-but-3-en-2-one | 1960 | /    | 3.1 | 18.3 | -   | 6.2 | 3.3 | 49.1 |
| 52  | Hexadecanoic acid 4 | 1970 | 1977 | 1.1 | 4.1 | 1.8 | 0.7 | 0.1 | 0.1 |
| 53  | (Z)-Octadec-9-en-1-ol 4 | 2060 | 2060 | 0.8 | 6.2 | 3.1 | 2.0 | 0.1 | 8.9 |
| 54  | Octadecan-1-ol 4 | 2084 | 2081 | 0.1 | 1.5 | 0.8 | 0.2 | 2.2 | 2.8 |
| 55  | (Z)-Octadec-9-enoic acid 4 | 2142 | 2140 | 1.5 | 2.4 | 2.8 | 1.7 | 0.1 | 0.1 |
| 56  | Docosane 4 | 2200 | 2200 | 0.1 | 1.0 | 23.0 | 0.2 | 14.0 | 25.9 |
| 57  | Tricosane 4 | 2300 | 2300 | 0.7 | 1.0 | 4.3 | 0.7 | 0.1 | 0.1 |

1 RI—retention indices on HP-5MS column relative to C9–C25 alkanes; 2 RI from the literature (NIST Chemistry WebBook, NIST Standard Reference Database Number 69, http://webbook.nist.gov/chemistry/); 3 Area percentages (%); 4 identification confirmed with standard compound; **—tentatively identified; **—correct isomer is not identified; - indicates that compound is not identified. A—USE with pentane:diethyl ether (1:2, v/v); B—USE with dichloromethane; C—sequence I/II: USE with pentane; D—sequence I: USE with diethyl ether after C (pentane extraction), E—sequence II: USE with the mixture pentane:diethyl ether (1:2, v/v) after C (pentane extraction); F—sequence II: USE with dichloromethane after E (the extraction with the mixture pentane:diethyl ether (1:2, v/v) and C (pentane extraction).
1,4-Dihydroxybenzene was proposed as a floral marker compound for vipers bugloss (*A. ursinum*), though found abundant by HPLC in buckwheat (*Fagopyrum esculentum*), which could be useful for its isolation from the honey matrix. Such a result was also expected and it is dominated by hydroquinone (36.8%), and 4-hydroxymethylfurfural (13.3%), 4-hydroxybenzoic acid (11.6%), methyl syringate, and 4-hydroxybenzaldehyde with 5.3%. Other C₁₃-norisoprenoids were found with minor abundance, such as 3-oxo-α-ionone, 3-oxo-7,8-dihydro-α-ionone, and vomifoliol. Only trans-linalool oxide (furan type) was found. It can be seen that this extract was purified from less polar compounds by previous extraction with pentane (sequence I). The extract with pentane:diethyl ether (1:2, v/v) applied in sequence II (E) contained as major compounds 1,4-benzenediol (27.7%), docosane (14.0%), 5-hydroxymethylfurfural (13.3%), 4-hydroxybenzoic acid (11.6%), methyl syringate (6.6%), and (E)-4-(r-1′,t-2′,c-4′-trihydroxy-3′,6′,6′-trimethylcyclohexyl)-but-3-en-2-one (3.3%). Other C₁₃-norisoprenoids (3-oxo-α-ionone, 3-oxo-7,8-dihydro-α-ionone, and vomifoliol) were present. Lot of similarities were noted among diethyl ether extract in sequence I (D) and the extract with the mixture of pentane:diethyl ether (1:2, v/v) in sequence II (E) regarding the distribution of hydroquinone, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, and C₁₃-norisoprenoids. The major difference was the abundance of docosane in the extract E (sequence II). Since dichloromethane extract in sequence II (F) was applied after pentane extraction and after the extraction with pentane:diethyl ether (1:2, v/v), it was expected to contain the least compounds of all the extracts (Table 2). However, this extract was dominated by (E)-4-(r-1′,t-2′,c-4′-trihydroxy-3′,6′,6′-trimethylcyclohexyl)-but-3-en-2-one (49.1%), which could be useful for its isolation from the honey matrix. Such a result was also expected and it is in accordance with the data obtained from the direct extraction with dichloromethane. It is interesting to note that other C₁₃-norisoprenoids were extracted with pentane:diethyl ether (1:2, v/v) previously applied in sequence II (E), and they were not present in dichloromethane extract (F). Docosane was the second major compound (25.9%) in this extract, followed by (Z)-octadec-9-en-1-ol (8.9%) and octadecan-1-ol (2.8%).

In comparison with HS-SPME (Tables 1 and 2), only a few compounds were similar, while linalool and its derivates were found with significantly lower abundance in the extracts than in the headspace. Epoxidation of linalool gives 6,7-epoxylinalool, which undergoes further reactions to form linalool oxides, while hotrienol is derived from hydroxylated linalool derivates [11]. Higher abundance of linalool, cis-, and trans-linalool oxide were found in the headspace of *Coriandrum sativum* L. [14] and *Citrus* spp. [13,15,16] honey types. Regarding the extract chemical profiles, no major similarity was found among the profiles of other honey types. A combination of predominant compounds (E)-4-(r-1′,t-2′,c-4′-trihydroxy-3′,6′,6′-trimethylcyclohexyl)-but-3-en-2-one, hydroquinone, methyl syringate, and 4-hydroxybenzoic acid is unique to *A. ursinum* honey. 1,4-Dihydroxybenzene was proposed as a floral marker compound for vipers bugloss (*Echium vulgare* L.) honey [17]. High proportions of benzoic acid and its derivates were found in *Salix* spp. honeydew extratives [18], but with a minor percentage of 4-hydroxybenzoic acid. The latter was found abundant by HPLC in buckwheat (*Fagopyrum esculentum* L.) honey [19]. (E)-4-(r-1′, t-2′,c-4′-trihydroxy-2′,6′,6′-trimethylcyclohexyl)but-3-en-2-one contains a megastigmane structure. Structurally, megastigmanes are C₁₃-carbon skeleton compounds, which are commonly classified as C₁₃-norisoprenoids, also assumed to be apocarotenoids. Megastigmanes possess a unique basic skeleton with a six-membered ring with a double bond within the ring system, followed by methyl and
dimethyl substitutions and an attached four membered chain with a double bond in the trans-mode [20]. The biosynthesis of this compound can be envisaged as proceeding via the alkene with a double bond within the ring system and via one or both of the epoxides [20]. Although a wide variety of degraded carotenoid-like substances have been identified from different honey types [13], this appears to be a rare situation where a trihydroxy ketone has been found. In fact, it was previously isolated and characterized by X-ray crystallographic analysis as a dominant substance from the ether extracts of New Zealand thyme honey [21]. Its recorded MS spectra were \( m/z \) 224 (6%), 141 (9), 140 (8), 125 (55), 124 (12), 123 (18), 109 (8), 99 (7), 97 (23), 95 (6), 83 (9), 71 (13), 69 (8), 55 (17), 43 (96) and the reported data [21] on MS of (E)-4-(\( \alpha \)-1,\( \beta \)-2,\( \gamma \)-4'-trihydroxy-3',6',6'-trimethylcyclohexyl)-but-3-en-2-one were \( m/z \) 224 (6%), 141 (9), 140 (8), 125 (43), 124 (10), 123 (17), 109 (8), 99 (7), 97 (23), 95 (6), 83 (9), 71 (13), 69 (8), 55 (17), 43 (100). This compound exerted significant apoptotic activity in PC-3 prostate cancer cells at 100 \( \mu \)M, while it inhibited NF-\( \kappa \)B phosphorylation and IL-6 secretion at a concentration range of \( 10^{-6} \)–\( 10^{-4} \) M [22].

3. Materials and Methods

A rare and representative Allium ursinum L. honey sample was collected from a professional beekeeper in Croatia (more unifloral samples were not available). The sample was stored in a hermetically closed glass bottle at 4 °C until the volatiles were isolated. Melissopalynological analysis was performed according to the International Commission for Bee Botany [23]. Microscopical examination was carried out on a Hund H 500 light microscope (Helmut Hund GmbH, Wetzlar, Germany) attached to a digital camera (Motic m 1000, Motic Deutschland GmbH, Wetzlar, Germany) and coupled to an image analysis system (Motic Images Plus software, Motic Deutschland GmbH) for the morphometry of pollen grains.

3.1. Headspace Solid-Phase Microextraction (HS-SPME)

The headspace solid-phase extraction (HS-SPME) was performed using a manual SPME holder using polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber that was conditioned prior to the usage according to Supelco (Bellefonte, PA, USA) instructions. The honey/saturated water solution (5 mL, 1:1 (v/v); saturated with NaCl) was placed in a 15-mL glass vial and hermetically sealed with polytetrafluorethylene (PTFE)/silicone septa. The vial was maintained in a water bath at 60 °C during equilibration (15 min) and HS-SPME (45 min) under constant stirring (1000 rpm) with a magnetic stirrer, and the sample was kept below the water level of the water bath. After sampling, the SPME fiber was withdrawn into the needle, removed from the vial, and inserted into the injector (250 °C) of the GC-FID and GC-MS for 6 min, where the extracted volatiles were thermally desorbed directly to the GC column. The experiment was performed in triplicate.

3.2. Ultrasonic Solvent Extraction (USE)

Ultrasonic-assisted solvent microextraction (USE) was performed in an ultrasound cleaning bath (Clean 01, MRC Scientific Instruments, London, UK) by the indirect sonication mode at a frequency of 37 kHz at 25 ± 3 °C. The advantage of using USE is the isolation of volatile and semi-volatile as well as water-soluble organic compounds without the application of heat. Different solvents were used for USE: a mixture of pentane/diethyl ether, 1:2 (v/v), dichloromethane, pentane, and diethyl ether. The mixture and dichloromethane were separately used for the extractions. A previously developed USE method was modified with the solvent sequences that were applied for the honey extraction. Sequence I consisted of the extraction with pentane followed by the extraction with diethyl ether (pentane → diethyl ether). Sequence II consisted of pentane extraction followed by the extraction with pentane:diethyl ether 1:2 (v/v) and afterwards with dichloromethane (pentane → pentane:diethyl ether 1:2 (v/v) → dichloromethane). For each extraction, 40 grams of the honey was dissolved in distilled water (22 mL) in a 100-mL flask. Magnesium sulfate (1.5 g) was added and vortexed (10 min). The solvent volume was 20 mL and the sonication was applied for 30 min. After the sonication, the
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Organic layer was separated by centrifugation and filtered over anhydrous MgSO₄. The aqueous layer was returned to the flask and another batch of the same extraction solvent was added and extracted for 30 min. The organic layer was separated in the same way as the previous layer and filtered over anhydrous MgSO₄, and the aqueous layer was sonicated a third time for 30 min with another batch of the extraction solvent. Combined organic extracts were concentrated to 0.2 mL by distillation with a Vigreaux column, and 1 µL was used for GC-FID/GC-MS analyses. The experiments were performed in triplicate.

3.3. GC-FID and GC-MS Analyses

The GC-FID analyses were conducted with an Agilent Technologies (Palo Alto, CA, USA) gas chromatograph model 7890A equipped with a flame ionization detector (FID) and a HP-5MS capillary column (5% phenyl-methylpolysiloxane, Agilent J and W, Santa Clara, CA, USA). The GC conditions were described previously [13,18]. In brief, the oven temperature was programmed isothermal at 70 °C for 2 min, increasing from 70–200 °C at 3 °C·min⁻¹, and held isothermally at 200 °C for 15 min; the carrier gas was He (1.0 mL·min⁻¹); and the total run time was 65 min.

The GC-MS analyses were conducted with an Agilent Technologies (Palo Alto, CA, USA) gas chromatograph model 7820A equipped with a mass selective detector (MSD) model 5977E (Agilent Technologies) and a HP-5MS capillary column, under the same conditions as those described for the GC-FID analysis. The MSD (EI mode) was operated at 70 eV, and the mass range was 30–300 amu, as previously reported [13].

The identification was based on the comparison of VOC retention indices (RI), determined relative to the retention times of a homologous series of n-alkanes (C₉–C₂₅), with those reported in the literature and their mass spectra with authentic compounds available in our laboratories or those listed in Wiley 9 (Wiley, New York, NY, USA) and NIST 14 (D-Gaithersburg) mass spectral libraries. The percentage composition of the samples was computed from the GC peak areas using the normalization method (without correction factors). The average component percentages in Tables 1 and 2 were calculated from duplicate GC-FID and GC-MS analyses.

4. Conclusions

The unusual chemical profile of A. ursinum honey was investigated and described for the first time. The headspace was dominated by linalool and its derivatives, which is not specific. The extracts showed remarkable variabilities according to the solvents applied, which is important to point out since the use of only one solvent could lead to incomplete results for A. ursinum honey. Namely, the extracts obtained with diethyl ether as the solvent contained 1,4-benzenediol and 4-hydroxybenzoic acid as the major compounds, while (E)-4-(r-1′,t-2′,c-4′-tri hydroxy-2′,6′,6′-trimethylcyclohexyl)but-3-en-2-one predominated in the dichloromethane extracts. The applied sequence of solvents enabled the fractionation of the compounds according to polarity, and sequence II was useful for the concentration and possible isolation of (E)-4-(r-1′,t-2′,c-4′-tri hydroxy-2′,6′,6′- trimethylcyclohexyl)but-3-en-2-one. More samples should be investigated to confirm these compounds as characteristic of this honey type.

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References

1. Sobolewska, D.; Podolak, I.; Makowska-Waś, M. Allium ursinum: Botanical, phytochemical and pharmacological overview. Phytochem. Rev. 2015, 14, 81–97. [CrossRef] [PubMed]

2. Kubec, R.; Svolodova, M.; Velisek, J. Distribution of S-alk(en)lycysteine sulfoxides in some Allium species. Identification of a new flavour precursor: S-ethylcysteine sulfoxide (ethiin). J. Agric. Food Chem. 2000, 48, 428–433. [CrossRef] [PubMed]

3. Godевac, D.; Vujišić, L.; Mojišević, M.; Ignjatović, A.; Spasojević, I.; Vajs, V. Evaluation of antioxidant capacity of Allium ursinum L. volatile oil and its effect on membrane fluidity. Food Chem. 2008, 107, 1692–1700. [CrossRef]

4. Blazewicz-Woźniak, M.; Michowska, A. The growth, flowering and chemical composition of leaves of three ecotypes of Allium ursinum L. Acta Agrobot. 2011, 64, 171–180. [CrossRef]

5. Górecki, L.; Dinic, R.; Parnavel, R. The influence of extraction method on the apparent content of bioactive compounds in Romanian Allium spp. leaves. Not. Bot. Horti Agrobot. Cluj Napoca 2012, 40, 93–97.

6. Sobolewska, D.; Janeczko, Z.; Kisiel, W.; Podolak, I.; Galanty, A.; Trojanowska, D. Steroidal glycosides from the underground parts of Allium ursinum L. and their cytostatic and antimicrobial activity. Acta Pol. Pharm. Drug Res. 2006, 63, 219–223.

7. Sendl, A. Allium sativum and Allium ursinum: Part I. Chemistry, analysis, history, botany. Phytomedicine 1995, 1, 323–329. [CrossRef]

8. Farkas, Á.; Zajacsz, E. Nectar production for the Hungarian honey industry. Eur. J. Plant Sci. Biotechnol. 2007, 1, 121–151.

9. Farkas, Á.; Molnár, R.; Morschhauser, T.; Hahn, I. Variation in nectar volume and sugar concentration of Allium ursinum L.ssp. ucrainicum in three habitats. Sci. World J. 2012, 2012, 138579. [CrossRef]

10. Jerković, I. Volatile benzened derivatives as honey biomarkers. Synllet 2013, 24, 2331–2334. [CrossRef]

11. Jerković, I.; Kuš, P.M. Terpenes in honey: Occurrence, origin and their role as chemical biomarkers. RSC Adv. 2014, 4, 31710–31728. [CrossRef]

12. Jerković, I.; Kranjac, M.; Marijanović, Z.; Zekić, M.; Radonić, A.; Tuberoso, C.I.G. Screening of Satureja subspicata Vis. honey by HPLC-DAD, GC-FID/MS and UV/VIS: Prephenate derivatives as biomarkers. Molecules 2016, 21, 377. [CrossRef] [PubMed]

13. Jerković, I.; Pribun, S.; Marijanović, Z.; Zekić, M.; Bubalo, D.; Svečnjak, L.; Tuberoso, C.I.G. Traceability of Satsuma mandarin (Citrus unshiu Marc.) honey through nectar/honey-sac/honey pathways of the headspace, volatiles, and semi-volatiles: Chemical markers. Molecules 2016, 21, 1302. [CrossRef] [PubMed]

14. Jerković, I.; Obradović, M.; Kuš, P.M.; Sarolić, M. Bioorganic diversity of rare Coriandrum sativum L. honey: Unusual chromatographic profiles containing derivatives of linalool/oxygenated methoxybenzene. Chem. Biodivers. 2013, 10, 1549–1558. [CrossRef] [PubMed]

15. Aliassandakis, E.; Tarantilis, P.A.; Harizanis, P.C.; Polissiou, M. Evaluation of four isolation techniques for honey aroma compounds. J. Sci. Food Agric. 2005, 85, 91–97. [CrossRef]

16. Aliassandakis, E.; Tarantilis, P.A.; Harizanis, P.C.; Polissiou, M. Aroma investigation of unifloral Greek citrus honey using solid-phase microextraction coupled to gas chromatographic-mass spectrometric analysis. Food Chem. 2007, 100, 396–404. [CrossRef]

17. Wilkins, A.L.; Tan, S.-T.; Molan, P.C. Extractable organic substances from New Zealand unifloral vipers bugloss (Echium vulgare) honey. J. Apicult. Res. 1995, 34, 73–78. [CrossRef]

18. Jerković, I.; Marijanović, Z.; Tuberoso, C.I.G.; Bubalo, D.; Kezić, N. Molecular diversity of volatile compounds in rare willow (Salix spp.) honeydew honey: Identification of chemical biomarkers. Mol. Divers 2010, 14, 237–248. [CrossRef] [PubMed]

19. Jasicka-Misiak, I.; Poliwooda, A.; Dereni, M.; Kafarski, P. Phenolic compounds and abscisic acid as potential markers for the floral origin of two Polish unifloral honeys. Food Chem. 2012, 131, 1149–1156. [CrossRef]

20. Rao, A.S. Isolation, absolute configuration and bioactivities of megastigmanes or C13 isonorterpinoides. Chem. Int. 2017, 3, 69–91.

21. Tan, S.T.; Wilkins, A.L.; Holland, P.T. Isolation and X-ray crystal structure of (E)-4-(r-l-t-2′,6′,6′-trihydroxy-2′,6′,6′-trimethylcyclohexyl)but-3-en-2-one, a constituent of New Zealand thyme honey. Aust. J. Chem. 1989, 42, 1799–1804. [CrossRef]
22. Kassi, E.; Chinou, I.; Spilioti, E.; Tsiapara, A.; Graikou, K.; Karabournioti, S.; Manoussakis, M.; Moutsatsou, P. A monoterpene, unique component of thyme honeys, induces apoptosis in prostate cancer cells via inhibition of NF-κB activity and IL-6 secretion. *Phytomedicine* **2014**, *21*, 1483–1489. [CrossRef] [PubMed]

23. Louveaux, J.; Maurizio, A.; Vorwohl, G. Methods of melissopalynology. *Bee World* **1978**, *59*, 139–153. [CrossRef]

**Sample Availability:** The honey sample is available from the authors for limited time.