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Determination of Unifloral Honey Volatiles from *Centaurea iberica* and *Zizyphus spina-christi* by Solid-Phase Microextraction and Gas Chromatography-Mass Spectrometry

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Summary. The volatiles of two different unifloral Palestinian honeys from botanical species *Centaurea iberica* and *Zizyphus spina-christi* have been investigated for the first time. They were isolated, identified, and quantitatively analyzed using Headspace Solid-Phase Microextraction and Gas Chromatography Mass Spectrometry (HS-SPME-GCMS) methodology. The resulted total ion current (TIC) chromatographic profiles reflected the uniqueness of each type of honey and therefore the proposed procedure can be used to characterize each kind of honey by revealing the absence or presence of certain volatile constituents.

A total of 18 compounds were seen in *Centaurea iberica* honey with phenylacetaldehyde, phenylethylalcohol, 2-ethyl hexanoic acid, 2,4,6-trimethylphenol and nonanoic acid as the principal components, whereas 25 compounds were seen in *Zizyphus spina-christi* honey with benzaldehyde, phenylacetaldehyde, phenylethylalcohol, benzeneacetonitrile, 2-ethyl hexanoic acid, octanoic acid, 2-methoxy-4-(1-propanol)-6-acetate phenol, nonanoic acid, decanoic acid, 1-hydroxy 2,4,6-trimethylbenzene, and 5-hydroxymethyl-2-furaldehyde as the principal constituents.

Ziziphus spina-christi honey was found to have two unequivocal potential markers: phenylacetonitrile and 5-hydroxymethyl-2-furancarboxaldehyde, while *Centaurea Italica* honey has only one representative floral origin marker compound: the 2,4,6-trimethylphenol.

Key Words: honey, volatiles, *Centaurea iberica*, *Zizyphus spina-christi*

Introduction

There is a large number of endogenous plant species in Palestine, of which more than 2600 are documented on such a very small Mediterranean area [1]. The rich ethnobotanical and pharmacological importance of these plants prompted unifloral beekeeping lately. Most of these honeys have only a local importance and are marketed on a limited scale and rarely have been described in the literature. Unifloral honeys possess highly characteristic

aromas, indicating the presence of various specific volatile components. These volatiles originated from the plant species where the nectar had been collected. It is believed that the precursors of the volatiles are responsible for the specific flavor of unifloral honeys which originate from the corresponding plants [2–3].

The determinations of these volatiles by using headspace technology (HS) with solid phase microextraction (SPME) connected in tandem to gas chromatography mass spectrometry (GCMS) allow an objective characterization and classification of these unifloral honeys from the polyfloral ones.

In previous work, we scanned a variety of volatile markers from wild Palestinian herbs of *Thymus capitatus*, *Thymelaea hirsuta*, and *Tolpis virgata* [4]. In the current work, however, we investigate the existence of potential volatile markers that originates from *Centaurea iberica* and *Zizyphus spina-christi* honeys. The two types of unifloral honeys were deliberately selected merely because of their health benefits reputation among Palestinians.

Centaurea iberica is a wild herb that belongs to the family Asteraceae and is predominantly distributed around the Mediterranean area and Western Asia [5]. It is known locally as *Murar* and has been used in traditional medicine to treat many diseases, such as cancer, microbial infections, stimulant, tonic, antidiabetic, diuretic, and antirheumatic [6–15]. Several reports mentioned anti-inflammatory, wound healing, and insulin secretion activities of the plant extract [16, 17]. More recently, new potent anti-platelet constituents were isolated and identified [18].

Although there are reports that focused on the endogenous nonvolatiles of *Centaurea iberica,* nothing is mentioned about its volatile contents. As far as we know, the present study is the first to give a complete profile of the volatile compounds emitted from *Centaurea iberica* honey by using Headspace Solid-Phase Microextraction and Gas Chromatography Mass Spectrometry (HS-SPME-GCMS) methodology.

Zizyphus spina-christi, locally known as *Sidr*, belongs to the botanical family *Rhamnaceae*. Sidr tree, is cultivated mainly for its nutritious fruits and honey production purposes. The flowers are important for the production of wild unifloral bee honey, which is highly demanded by Palestinians for its medicinal qualities in addition to its excellent taste and fragrant smell. The plant was reported to exhibit antibacterial, anticancer, antidiabetic, antinociceptive, antihypertensive, antidiarrhoeal, and CNS effect [19, 20].

There are few papers that report about volatiles from *Zizyphus spina-christi* fruits, oil leaves, and flowers [21]; however, their volatiles from the unifloral honey has not been yet investigated.

The aim of this work is to separate and characterize the volatile markers from the Palestinian unifloral honeys of *Centaurea iberica* and *Zizyphus spina-christ.* Quantitative analysis of some potential volatile markers was de-

termined for each selected type of honey using HS-SPME-GCMS technology.

Experimental

Chemicals and Materials

All the authentic standards were purchased from Sigma-Aldrich. The standards are 2-ethyl hexanoic acid, 2,4,6-trimethyl phenol, decanoic acid, nonanoic acid, octanoic acid, benzaldehyde, benzeneacetaldehyde, dodecanal, phenylethyl alcohol, and 5-hydroxymethyl-2-furancarboxaldehyde.
The two different unifloral honey samples were collected by Palestinian beekeepers. Three separate samples of *Ziziphus spina-christi* were obtained from Tulkarem, while the three samples of *Centaurea iberica* were from Nablus, both districts are located in the northern part of Palestine. Each sample was collected fresh and directly analyzed in replicates. The collected samples were stored directly in a refrigerator at 4 °C in the dark until they were analyzed.

Apparatus and Chromatographic Analysis

The unifloral honey samples were analyzed using a Shimadzu GC-17A connected to an MS-QP5050A. A Shimadzu autosampler AOC-20I was used with 2 mL vials sealed with 8 mm double-faced rubber septa and a screw cap with a 12-mm hole. The GCMS was operated under electron impact (EI) ionization mode at 70 eV.

Omegawax 250 capillary column (30 mm × 0.25 mm × 0.25 μm film thickness) was obtained from Supelco, USA. The honey contains relatively polar constituents, such as acids, and therefore, an SPME microextraction syringe with a 65-μm carbowax/divinylbenzene (CW/DVB) fiber was used for collecting the volatiles and semi-volatiles from the headspace of honey samples.

Five grams of honey was put in a 27-mL vial which was sealed with a rubber septum secured with an aluminum cap. The vial was heated to constant temperature in a water path. The SPME fiber was introduced into the vial then removed and desorbed in the injection port for 7 min.

The carrier gas flow was 1.6 mL He/min, column pressure was 100 kPa. The injector and detector temperatures were 220 and 250°C, respectively. The column temperature was started at 50°C and held for 1 min, then raised from 50 to 200°C at 10°C/min and held there for 5 min, and from 200 to 240°C at 10°C /min and held there for 5 min. The program was run in the

splitless mode with a mass range of 50 to 400 μ, and the scan interval was 0.5 s. Detector voltage was set at 1.5 kV.

Before use, the fiber was put in the injector port for 1 h at 220°C, and every two runs, the fiber was conditioned again for 15 min. The loaded fiber of the SPME was desorbed in the injection port for 5 min at 220°C. Sample temperature equilibration was optimized to 80°C, and the sampling equilibration time of the SPME fiber was determined to be 50 min.

Qualitative and Quantitative Determination of Honey Volatiles

Qualitative analysis was based on the comparison of the obtained spectra with those of the NIST (US National Institute of Standards and Technology) mass spectral electronic library and was confirmed by injecting authentic reference standards.

Quantitative data were calculated from the total ion current (TIC) peak areas and by relating it to the calibration curve using 20, 50, 100, 500, and 1000 ng mL^{-1} standard concentrations.

Results and Discussion

The volatiles constituents of two different unifloral Palestinian honeys from *Centaurea iberica* and *Zizyphus spina-christi* have been investigated for the first time.

Various volatile compounds present in the *Centaurea iberica* and *Zizyphus spina-christi* unifloral honeys have been separated, identified, and quantified using HS-SPME-GCMS analysis. Carbowax/divinylbenzene (CW/DVB) fiber was used for collecting the volatiles and semi-volatiles from the headspace of honey samples. *Fig. 1* and *Fig. 2* represent the TIC generated for the two types of honeys. Among the volatiles detected were compounds that belong to aldehyde, phenol, carboxylic acid, and unexpected nitrile families. A total of 18 signals appeared in the *Centaurea iberica* honey, of which seven were positively identified by comparing their retention time with authentic standards and by comparing their mass spectrum with MS-NIST library *(Fig. 1)*. Phenylacetaldehyde, phenylethanol, 2-ethyl hexanoic acid, 2,4,6-trimethyl phenol, and nonanoic acid appeared as the principal components according to their calculated average peak area parentages of three samples of the same honey.

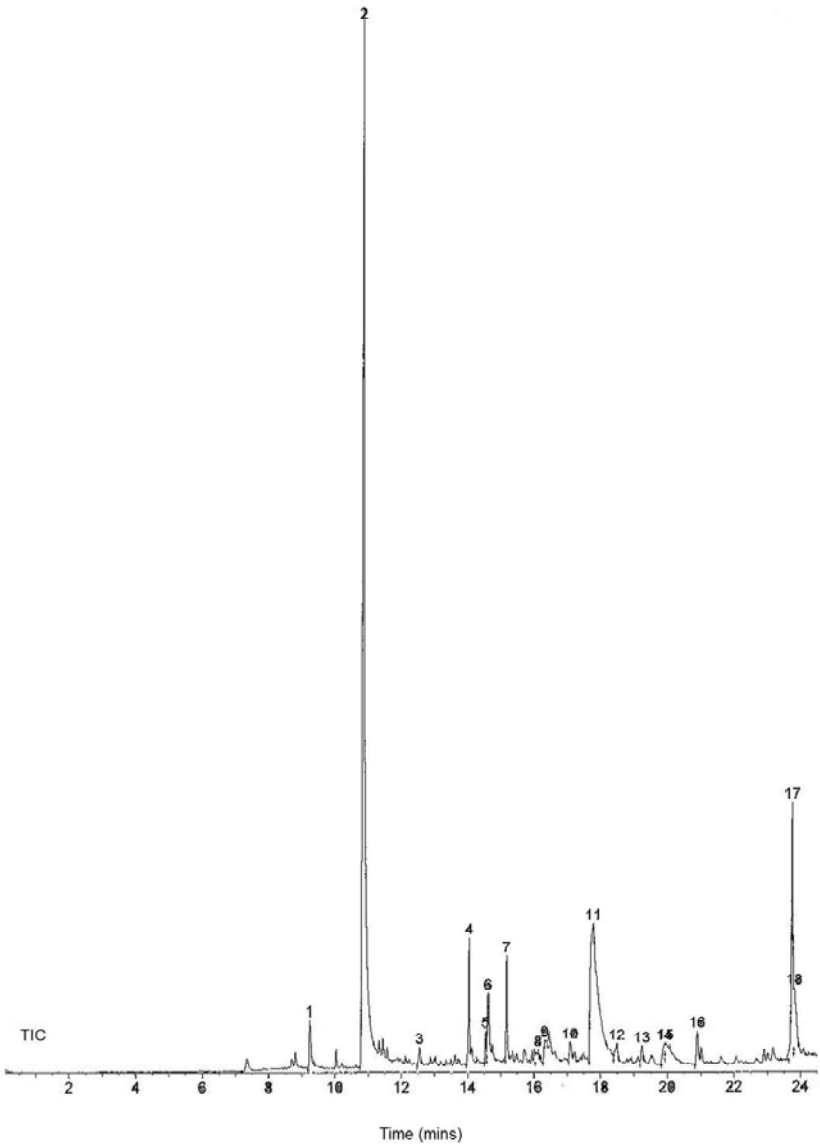

Fig. 1. Total ion chromatogram of *Centaurea iberica* honey using HS-SPME-GCMS and carbowax/divinylbenzene (CW/DVB) fiber coating. Major peaks identities: 1: benzaldehyde; 2: phenylacetaldehyde; 4: phenylethylalcohol; 6: 2-ethyl hexanoic acid; 7: 2,4,6-trimethyl phenol; 11: nonanoic acid;14: decanoic acid. All compounds were identified by injecting standards and by comparing their MS with NIST library

A total of 25 signals appeared in *Zizyphus spina-christi* honey, of which eleven were identified *(Fig. 2)*. Benzaldehyde, phenylacetaldehyde, phenylethylalcohol, benzeneacetonitrile, 2-ethyl hexanoic acid, octanoic acid, 2-

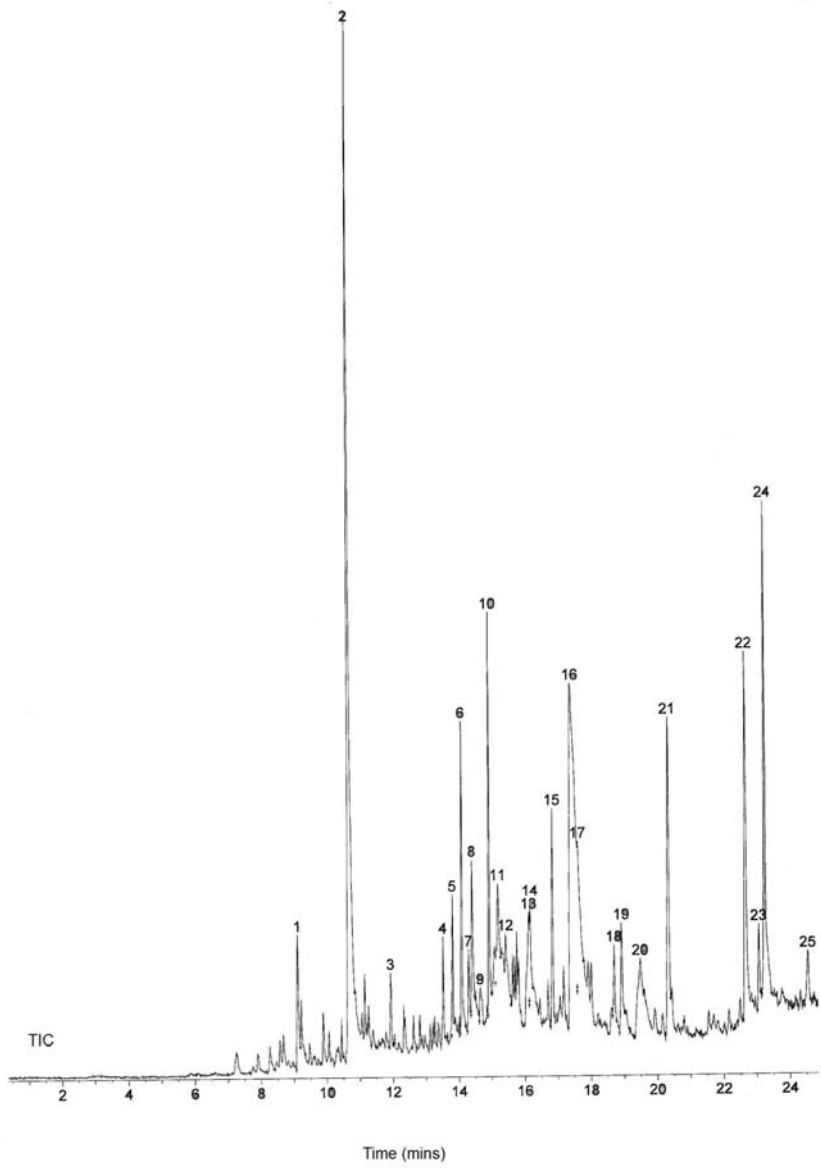

Fig. 2. Total ion chromatogram of *Ziziphus spina-christi* honey using HS-SPME-GCMS and carbowax/divinylbenzene (CW/DVB) fiber coating. Major peaks identities: 1: benzaldehyde; 2: phenylacetaldehyde; 5: benzeneacetonitrile; 6: phenylethylalcohol; 7: dodecanal; 8: 2-ethyl hexanoic acid; 12: 3-methoxy phenol; 14: octanoic acid; 15: 2-methoxy-4-(1-propanol)-6-acetate phenol; 17: nonanoic acid; 20: decanoic acid; 21: 1-hydroxy-2,4,6-trimethylbenzene; 24: 5-hydroxymethyl-2-furancarboxaldehyde. All compounds except (5, 12, 15, and 21) were identified by injecting standards and by comparing their MS with NIST library

methoxy-4-(1-propanol)-6-acetate phenol, nonanoic acid, decanoic acid, 1-hydroxy- 2,4,6-trimethylbenzene, and 5-hydroxymethyl-2-furancarboxaldehyde emerged as the principal components.

Quantitation of Some Volatile Components in Honeys

The HS-SPME-GCMS method was partially validated and applied to determine the amount of volatiles in the unifloral honey samples. Among the chromatographic parameters tested was the linearity of the calibration curve of the volatile standards, limits of detection (LOD) and quantitation (LOQ), and reproducibility as reflected via relative standard deviation (RSD %) values of successive triplicate injections. The average RSD % of signal retention time was between 0.02–0.39% for all the compounds tested and 1.7–6.63% for signal peak areas. These results accredited the method to be reproducible. The amount of each volatile compounds emerged from the SPME in ng/g was determined as shown in *Table I*.

Table I. Quantitation parameters of standards injections used to calculate the amounts of volatiles of *Centaurea iberica* and *Ziziphus spina-christi* unifloral honeys using HS-SPME-GCMS and carbowax/divinylbenzene (CW/DVB) fiber coating, (n=3)

Target volatile	*(r)*	LOD (ng)	LOQ (ng)	Amount (ng/g) *C. iberica*	Amount (ng/g) *Z. Christi*
Benzaldehyde	0.9974	2.021	8.084	7.42	9.65
Benzeneacetaldehyde	0.9944	32.60	130.4	149.8	160.9
Phenylethyl alcohol	0.9903	3.386	13.54	19.20	30.08
2-Ethyl hexanoic acid	0.9918	28.88	115.5	32.16	45.56
Nonanoic acid	0.9990	41.66	166.6	157.0	158.8
Decanoic acid	0.9998	31.25	125.0	178.0	143.6
2,4,6-Trimethyl phenol	0.9959	3.441	13.76	37.14	absent
Dodecanal	0.9936	8.253	33.01	absent	12.07
Octanoic acid	0.9929	37.50	150.1	absent	48.24
5-hydroxymethyl-2-furancarboxaldehyde	0.9989	16.21	64.86	absent	160.9

r: regression coefficient, LOD: limits of detection at signal-to-ratio of 3, LOQ: limits of quantitation at signal-to-ratio of 12. The calibration plots were obtained in the standards concentrations range of 20–1000 ng mL^{-1}.

As shown in *Table I*, the regression coefficients (*r*) values were always greater than 0.99 for all the detected compounds. The HS-SPME/GCMS method proved to be sensitive as reflected from the LOD and LOQ values. Most of the components identified are present in the two honey samples but in different amounts. The ratio between some components of different honey could be used to distinguish their different floral origin. For example, the same three carboxylic acids, namely, 2-ethyl hexanoic acid, nonanoic acid, and decanoic acid, were observed in the two honeys. The concentration of these acids is higher than 0.36 μg/g in both honey samples. Octanoic acid, however, only appeared in *Ziziphus spina-christi* honey in a relatively high concentration (48.24 ng/g). These acids possess polar functionality and therefore appear in the chromatograms as a broad signal with tailed rear profile representing typical Langmuir adsorption isotherm because of their strong interaction with the capillary long column.

Moreover, benzaldehyde, benzeneacetaldehyde, and phenylethyl alcohol were also detected in both honeys almost at the same level, but phenylethyl alcohol was more pronounced in *Ziziphus spina-christi* honey. In general, all these compounds are not reliable markers due to their presence in both honeys of different origins.

Surprisingly, phenylacetonitrile has been found in *Ziziphus spina-christi* honey exclusively but in subtle quantity. The fruit flowers of Kiwi also have been reported to contain some phenylacetonitrile [22]. 2,4,6-trimethyl phenol was present solely in *Centaurea Italica* at 37.14 ng/g concentration. The heterocyclic furan, 5-hydroxymethyl-2-furancarboxaldehyde was seen only in *Ziziphus spina-christi* honey at a high concentration of 160.9 ng/g. *Table I* shows the presence and absence of the potential markers in both honeys.

In summary, *Ziziphus spina-christi* honey was found to have two unequivocal potential markers: phenylacetonitrile and 5-hydroxymethyl-2-furancarboxaldehyde, while *Centaurea Italica* honey has only one representative floral origin marker compound: the 2,4,6-trimethyl phenol.

Conclusion

HS-SME-GCMS is a simple, rapid, and reliable method in screening *Centaurea Italica* and *Ziziphus spina-christi* honeys for their potential volatile components that characterize their unifloral authenticity. The method can be easily adapted to investigate other types of unifloral honey from their TIC chromatographic peak profiles.

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