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Determination of potential volatiles markers from *citrus*, *eucalyptus*, *cotton* and *wildflower* Palestinian honeys using SPME followed by GCMS analysis

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Abstract

The volatiles of Palestinian honeys from *citrus* of the orange blossom (*citrus* spp.), *eucalyptus* (*eucalyptus camaldulensis*), *cotton* (*Gossypium hirsutum* L.) and *wildflower* (*poyfloral*) were investigated. They were separated, identified and quantitatively analyzed by using Headspace Solid-Phase Microextraction and Gas Chromatography Mass Spectrometry (HS-SPME-GCMS) technology to estimate the amount of volatiles evolved. Although the investigated honeys have some volatiles in common but still each of them possess specific characteristic volatiles. For example, *citrus* honey was characterized by the presence of three volatile compounds namely, 2-methoxy-4 (1-propanol) phenol, 1-hydroxylinalool, and 2-amino benzoic acid methylester. These compounds are absent from all other honeys. *Eucalyptus* honey was found to have 2-propyl-1-pentanol and pentadecane as potential markers. *Cotton* honey was characterized by the presence of three markers, 2-furanomethanol, eicosane and 2-methyl decanol. The wildflower honey is distinguished from other floral honey by the presence of three volatile marker compounds the hexadecane, heptadecane and 3,4-dimethyl benzaldehyde.

Introduction

Many studies reported the analysis of variety of volatiles from unifloral honeys that are collected from different places all over the world by using Headspace Solid-Phase Microextraction and Gas Chromatography Mass Spectrometry (HS-SPME-GCMS) methodology. Unifloral honeys by default are honeys that are dominated by a single nectar source. The single flower origin usually assures a better quality when it guarantees a specific and well defined flavor and aroma. A large number of organic volatile compounds have been described in different types of honeys from different places (Serra Bovehi *et al*., 1995; Bouseta *et al*., 1996; D'Arcy *et al*., 1997; Pérez *et al*., 2002; Wolski *et al*., 2006; Vázquez *et al*., 2006; Soria *et al*., 2008). The identification and quantification of the isolated volatile compounds from honey is considered to be challenging because of the complexity of the honey resin matrix.

HS-SPME is a technique that has been used mainly to obtain highly specific compositional properties and therefore it offers reliable information related to the floral source of the honey. The method is fast, inexpensive and requires little sample preparation. The characterization of various Palestinian unifloral honeys has been investigated via the existence of key organic volatile markers (Odeh *et al*., 2007; Odeh *et al*., 2013). The scanning comprises unifloral honeys from *thymus capitatus*, *thymelaea hirsuta*, and *tolpis virgata*, *centaurea iberica* and *zizyphus spina-christi* (Odeh *et al*., 2007; Odeh *et al*., 2013). In the current work however, a screening was conducted to explore potential volatile markers from *citrus* of the orange blossom (*citrus* spp.), *eucalyptus* (*eucalyptus camaldulensis*), *cotton* (*Gossypium hirsutum* L.) and *wildflower* honeys.

Palestinian *citrus* honey is an important type of honey, with a distinctive floral citrus aroma and taste which makes it especially appreciated among the monoflorals of Jericho, the oldest city on the world. This kind of honey is usually mild in flavor and has a fresh aroma (Escriche *et al*., 2011). The *eucalyptus* honey has a pungent aroma, strong flavor and unique healthy characteristics (Soria *et al*., 2008). *Cotton* honey is light in color, with a mild aroma with very sweet taste (Alissandrakis *et al*., 2005). *Wildflower* honey is the product of wild growing flowers in the mountains of Hebron, Palestine. It has a pleasant taste and can be characterized by its dark color in comparison to the other kinds of honey. Darker honey usually has more vitamins, minerals, and antioxidant

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nutritional benefits than lighter honey (Marceau *et al*., 2009). Therefore, there is a great demand for this honey because of its diverse health benefits.

The aim of this research is to investigate the presence of potential volatiles that can be utilized as markers in the Palestinian honeys specifically *citrus*, *eucalyptus*, *cotton* and *wildflower*. The amounts of the volatiles present are determined for each type to support quantitatively their marker profiles.

Materials and Methods

Reagents

Helium was purchased from a local supplier with high grade purity (99.999%). All the standards were purchased from Sigma-Aldrich. These including: 2-ethyl hexanoic acid, benzylalcohol, 2-furanomethanol, decanal, decanoic acid, nonanoic acid, 2-furaldehyde-5-methyl, octanoic acid, nonanal, 2-furancarboxaldehyde, benzaldehyde, phenylacetaldehyde, phenylethyl alcohol, 5-hydroxymethyl -2-furanaldehyde and Linalool.

Honey collection

All the honey samples were collected by Palestinian beekeepers. The four honey samples were obtained from four different sites in Palestine. Three samples of *eucalyptus* and *cotton* were obtained from Jenin, located in the northern part of Palestine, while *citrus* (orange blossom) samples were from Jericho, the oldest city in the world which is located near to Jordan River in the West Bank of Palestine. The *wildflower* was collected from Hebron Mountains, which is located in the southern part of Palestine. Each sample was collected fresh and directly analyzed in replicates. The collected samples were stored in a refrigerator at 4^{o}C in the dark until they were analyzed.

HS-SPME-GCMS

A Shimadzu GC-17A connected to an MS-QP5050A was utilized for the honey sample analysis. The GCMS was operated in the electron impact ionization mode (EI) at 70 eV. An Omegawax 250 capillary column (30 m x 0.25 mm x 0.25 µm film thickness) was obtained from (Supelco, USA). A SPME microextraction syringe with a 65 µm carbowax/divinylbenzene (CW/DVB) fiber was used for collecting the polar and nonpolar volatiles and semi volatiles from the headspace of honey samples. A Shimadzu autosampler AOC-20I was used with 2 ml vials sealed with 8 mm double-faced rubber septa and a screw cap with 12 mm hole. (5.00 g) of honey was put in a 27 ml vial and the vial 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 disrobed in the injection port for five minutes. The carrier gas flow was 1.6 ml He/min., column pressure was 100 Kpa. The injector and detector temperatures were 220^{o}C and 250^{o}C respectively. The column temperature was held at 60^{o}C for 1 minute, then raised from 60^{o}C to 200^{o}C at 100C/min and held there for 5 minutes and from 200^{o}C to 240^{o}C at 10^{o}C /min and held there for 6 minutes. The program was run in the splitless mode with a mass range of 50 to 400u, and the scan interval was 0.5 seconds. Detector voltage was set at 1.5 KV. By this program we have a good separation and short time.

Qualitative and quantitative SPME-GCMS analysis

As a rule, before use the fiber was put in the injector port for one hour at 220^{o}C, and every two runs the fiber was conditioned again for 10 min. The loaded fiber of the SPME was desorbed in the injection port for 5 min at 220^{o}C. An ethanol solution of the volatile standard samples (1 ml) was placed in a 2 ml vial equipped with a septum cap and one µl was injected into the GCMS. The injected standards concentrations were 20, 50, 100, 500, and 1000 ngmL^{-1}. The identification of the eluted unknown compounds was based mainly on their retention times in comparison with those of authentic standards, and on comparison of their MS spectra to those of the MS-NIST library.

Results and Discussion

HS-SPME method conditioning on citrus honey

The HS-SPME analysis of the volatiles of four Palestinian honey samples from different floral origin (citrus, eucalyptus, cotton and wildflower) was optimized. Several parameters affect the sensitivity and selectivity of the SPME technique particularly incubation temperature, SPME fiber exposure time, fiber polarity, and honey matrix weight were investigated. Different incubation temperatures of 40, 50, 60, 70, and 80^{o}C were tested at fixed time and triplicate injections were made at each temperature. Temperature influenced the vapor pressure of the volatiles and thus their extraction rates. It was found that the concentrations of nonanoic acid and phenylacetaldehyde, a principal components present in citrus honey, were dramatically increased upon increasing the temperature from 60 to 70^{o}C. However, extensive pressure built up in the sample vial and leakage apparently occurred when the temperature was raised up to 80^{o}C, followed by a dramatic fall dawn in their concentration.

Another parameter was the duration of incubation and its effect on the equilibration of volatiles in the SPME fiber. Different incubation times of 40, 50, 60, 70, and 80 minutes was tested, at a fixed temperature of 70°C. The result showed that a 50 minute incubation time has a significant effect on the concentration of *citrus* honey volatiles. Therefore, an incubation time of 50 minutes at 70°C was chosen as an optimized condition for extracting the volatiles in *citrus* honey. The same conditions used in *citrus* were adopted for the other types of honeys under investigation. It was noticed that the optimized method extracted more polar compounds such as alcohols and acids in comparison to other methods, probably due to the relatively high temperature and incubation period along with the polarity of the column and the selected fiber, which generate greater affinity to these polar compounds (Serra Bovehi *et al*., 1995; Pérez *et al*., 2002; Wolski *et al*., 2006; Vázquez *et al.*, 2006).

Quantitative determination of the amount of volatiles in the Palestinian honey

The HS-SPME method was partially validated and applied to determine the amount of volatiles in the unifloral honey samples. In order to address the optimized method reproducibility, three successive injections of the same honey type were made using identical experimental conditions (incubation time and temperature, fiber type) as shown in Table 1.

Table 1. Reproducibility of average retention time and average peaks area of *citrus* honey volatile constituents using the optimized HS-SPME-GCMS conditions and carbowax/divinylbenzene (CW/DVB) fiber coating, (*n*=3)

Volatile compound	average t_R (mins)	average peak area	RSD of t_R *(n=3)*	RSD of Area *(n=3)*
Nonanal	7.342	260645.1	0.31	4.351
2-Furancarboxaldehyde	8.460	223161.5	0.39	3.584
Benzaldehyde	9.201	696163.6	0.09	13.20
Linalool	9.425	214158.8	0.15	2.537
Benzeneacetaldehyde	10.89	6654729	0.05	6.638
Phenylethyl alcohol	14.12	464113.7	0.03	15.99
2-Ethylhexanoic acid	14.63	2034598	0.06	7.466
Pantolactone	15.58	172539.5	0.07	9.284
Octanoic acid	16.33	415795.5	0.02	22.80
Nonanoic acid	17.70	7255635	0.09	1.766
2-Amino benzoic acid methylester	18.60	1152364	0.09	6.325
1-Hydroxylinalool	19.64	4745624	0.12	5.324
2-methoxy,4-(1-propanol)phenol	20.39	3806550	0.25	6.325
5-hydroxymethyl2-furancarboxaldehyde	23.29	3919349	0.37	21.91

Relative standard deviation RSD % values of retention time and peak areas showed that the method is fairly reproducible. Table 1 demonstrate *citrus* retention time reproducibility as reflected by RSD values (0.02- 0.39%) with fairly acceptable peak area (1.76-22.8%). As to the other types of honey, injection of triplicates of *eucalyptus* honey gave RSD of (0.053-0.329%) in retention time and (1.53-19.01%) in peak area. *Cotton* honey revealed RSD of (0.015-0.27%) and (2.1-24.74%) in retention time and signal area respectively. *Wildflower* honey shows an RSD values of (0.013-0.49%) and (1.47-24.43%) in retention time and signal area respectively. The above results indicate that the retention time was always almost constant but the peak area shows variation but within the accepted limits.

The method was applied to evaluate the concentration of the unifloral honey volatiles that desorbed from the SPME carbowax/divinylbenzene (CW/DVB) fiber to the GC carbowax column. It was determined by comparing their average peak areas with those of known standard. Standard calibration curves were prepared for 14 volatile compounds as shown in Table 2. The concentrations of each volatile were calculated and the regression coefficients (r) values were always > 0.99, for all the tested compounds. The HS-SPME/GCMS method proved to be sensitive as reflected from the limits of detection (LOD) and limits of quantitation (LOQ).

Table 2. Quantitation parameters of standards injections used to calculate the amounts of volatiles from 5 g of *citrus*, *eucalyptus*, *cotton* and *wildflower* honeys using the optimized HS-SPME-GCMS conditions and carbowax/divinylbenzene (CW/DVB) fiber coating, (*n*=3)

		Amount (ng/5 g)					
Target Volatile	**(r)**	***LOD***	***LOQ***	***Citrus***	***Eucalyptus***	***Cotton***	***Wildflower***
Nonanal	0.9954	7.777	31.08	37.33	35.84	34.12	180.9
Benzeneacetaldehyde	0.9944	32.60	130.4	280.1	693.3	363.8	848.1
2-Ethyl hexanoic acid	0.9917	28.88	115.3	487	152.9	252.7	256.7
Octanoic acid	0.9929	37.50	150.3	157.6	232.6	537.9	271.5
Nonanoic acid	0.999	41.66	166.6	972.2	601.1	931.3	961.3
5-Hydroxymethyl 2-furancarboxaldehyde	0.9989	16.21	64.86	289.1	230.5	777.7	absent
Decanoic acid	0.9998	31.25	132.5	absent	absent	243.8	887.4
2-Furancarboxaldehyde	0.9974	9.615	38.46	99.96	absent	40.88	absent
Phenylethyl alcohol	0.9903	3.386	13.54	52.42	absent	146.4	absent
Pantolactone	0.9921	5.244	20.97	27.55	absent	90.35	absent
2-furanomethanol	0.9953	9.740	38.96	absent	absent	60.08	absent
Decanal	0.9952	3.865	15.46	20.01	absent	absent	42.47
Linalool	0.9979	3.482	13.95	17.55	absent	absent	21.66
Benzaldehyde	0.9974	2.021	8.086	28.05	absent	absent	absent

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

Figures 1-3 revealed the total ion chromatogram (TIC) profiles of three types of honey investigated using HS-SPME-GCMS and carbowax/divinylbenzene (CW/DVB) fiber coating using the optimized conditions. Generally among the volatiles detected were compounds that belong to the phenol, ketone, ester, acid and aldehyde families.

A total of 36 compounds were observed in *citrus*

honey with phenylacetaldehyde, phenylethylalcohol, 2-ethyl hexanoic acid, nonanoic acid, benzoic acid and 1-hydroxylinalool as the principal components. Fourteen of them were positively identified as shown in Table 2 by comparing their retention with authentic standards. In general they are of low molecular weight, linear and branched aldehydes, phenols and acids (Figure 1).

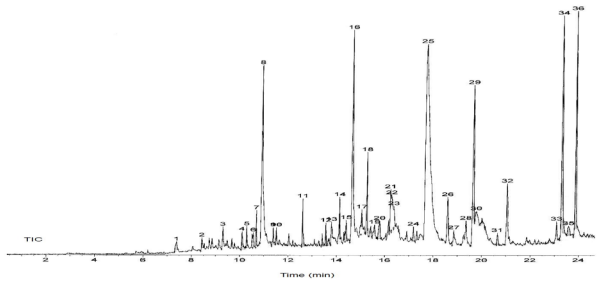

Figure 1. TIC of citrus honey using HS-SPME-GCMS and carbowax/divinylbenzene (CW/DVB) fiber coating. Major peaks identities: 1: nonanal; 2: 2-furancarboxaldehyde; 3: benzaldehyde; 4: 3,7-dimethyl 1,6-Octadien-3-ol; 8: benzeneacetaldehyde; 14: phenylethyl alcohol; 16: 2-ethyl hexanoic acid; 19: pantolactone; 23: octanoic acid; 24: 5-hydroxymethyl 2-furancarboxaldehyde; 25: nonanoic acid; 26: benzoic acid 2-aminomethylester; 29: 1-hydroxylinalool; 31: 2-methoxy-4-(1-propanol) phenol. All compounds except (26, 29, 31) were identified by injecting standards and by comparing their MS with NIST library.

Eucalyptus SPME however released 28 compounds with phenylacetaldehyde, tetradecane, 2-ethyl hexanoic acid, pentadecane, nonanoic acid, octadecane, and 5-hydroxymethyl 2-furancarboxaldehyde as the principal constituents (Figure 2). About 31 volatiles were observed in *cotton* honey with phenylacetaldehyde, phenylethyl alcohol, 2-ethyl hexanoic acid, nonanoic acid and 5-hydroxymethyl 2-furancarboxaldehyde as the major constituents (Figure 3). *Wildflower* revealed 25 peaks of which 3,7-dimethyl 1,6-octadien-3-ol, phenylacetaldehyde, 3,4,-dimethyl benzaldehyde, 2-ethyl hexanoic acid, nonanoic acid, and nonadecane are the principal constituents.

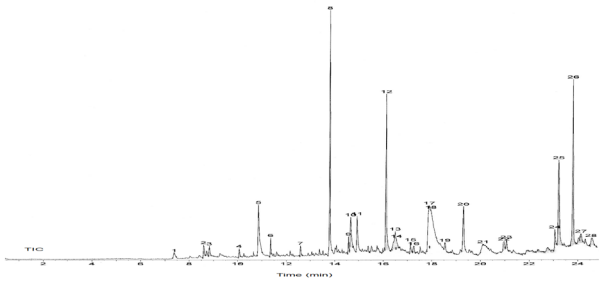

Figure 2. TIC of eucalyptus honey using HS-SPME-GCMS and carbowax/divinylbenzene (CW/DVB) fiber coating. Major peaks identities: 1: nonanal; 2: 2-propyl, 1-pentanol; 3: decanal; 5: benzeneacetaldehyde; 8: tetradecane; 10: 2-ethyl hexanoic acid; 12: pentadecane; 13: octanoic acid; 18: nonanoic acid; 20: octadecane; 24: nonadecane; 25: 5-hydroxymethyl 2-furancarboxaldehyde. All compounds except (2, 8, 12, 20, 24) were identified by injecting standards and by comparing their MS with NIST library.

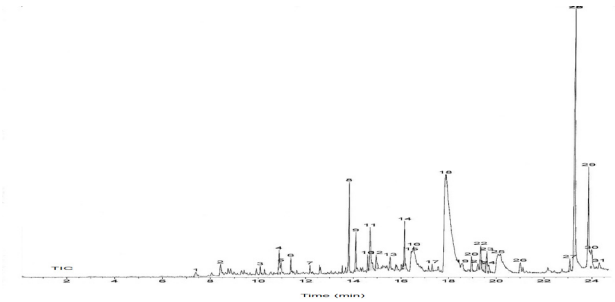

Figure 3. TIC of cotton honey using HS-SPME-GCMS and carbowax/divinylbenzene (CW/DVB) fiber coating. Major peaks identities: 1: nonanal; 2: 2-furancarboxaldehyde; 3: 2-methyldecanol; 4: benzeneacetaldehyde; 5: 2-furanomethanol; 8: phenylethyl alcohol; 11: 2-ethyl hexanoic acid; 13: pantolactone; 16: octanoic acid; 18: nonanoic acid; 22: octadecane, 25: decanoic acid; 27: eicosane, 28: 5-hydroxymethyl 2-furancarboxaldehyde. All compounds except (22, 27) were identified by injecting standards and by comparing their MS with NIST library.

Depending on the results shown in table 2, many observations can be highlighted. All the honey samples analyzed contain three carboxylic acids of molecular weight ranging between 120 and 200 g/mole. The concentration of these acids is higher than 150 ng/5 g in each honey sample. Nonanoic acid concentration was above 600 ng/5 g in all honey types analyzed. These acids appear in the chromatograms as broad peaks as expected. All honey types contain phenylacetaldehyde with concentrations ranging between 280 ng/5 g and 848 ng/5 g. In addition, all honey samples contain small amount of nonanal but in different concentrations. In the *wildflower* honey, the concentration is noticeably high (180.9 ng/5 g), compared with other types of honey. This aliphatic aldehyde, contributes partly to the fruity odor of the four honey types investigated.

Another example is the 2-ethyl hexanoic acid. It is present in all the honey types with a higher extent in *citrus* honey (487 ng/5 g). 5-hydroxymethyl 2-furancarboxaldehyde is present in all except *wildflower* and it is more pronounced in *cotton* (777.7 ng/5 g). 2-furancarboxaldehyde, phenylethyl alcohol and pantolactone are present solely in *citrus* and *cotton* and absent in both *eucalyptus* and *wildflower*. Benzaldehyde however, is absent in nearly all of the samples, but found in *citrus* honey. 2-furanomethanol is only found in *cotton* honey while decanal and linalool are only present in *citrus* and *wildflower* honeys. According to Figure 1, *citrus* honey can be characterized by the presence of phenol, 2-methoxy-4 (1-propanol), 1-hydroxylinalool, and 2-amino benzoic acid methylester. In addition, these compounds are absent from all other honeys.

Figure 2 shows that *eucalyptus* honey possess 2-propyl-1-pentanol and pentadecane as potential markers which are absent in others. As to the *cotton* type, 2-furanomethanol, eicosane and 2-methyl

decanol are important markers that are absent in the other types of honey samples (Figure 3).

Finally the TIC of *wildflower* honey revealed that it contains hexadecane, heptadecane and 3,4-dimethyl benzaldehyde volatiles and absent in others.

Conclusion

The HS-SPME GCMS technology had been optimized and validated as an analytical methodology to characterize the origin of various Palestinian honeys from *citrus* (orange blossom), *eucalyptus*, *cotton* and *wildflower*. In all honey samples the most abundant compounds belonged to aldehydes, acids, phenols and alcohols families. These compounds are found in different percentages and concentrations due to the floral origin of the honey. This result was used to assess certain markers for the types of honey selected.

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