



Research article

Geographical differentiation of Cypriot multifloral honeys through specific volatile compounds and the use of DFA

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Abstract: The objective of the present work was to investigate the volatile profile of multifloral honeys collected from different regions in Cyprus, and whether these specific volatiles could serve as true markers of geographical origin. An effort to highlight differences in the volatile amounts of Cypriot honeys according to altitude of harvesting was also carried out. Aqueous solutions of honeys were subjected to headspace solid phase microextraction coupled to gas chromatography/mass spectrometry analysis (HS-SPME/GC-MS). Results showed that 25 volatile compounds of different classes were identified in all samples by 100% frequency rate. Application of multivariate analysis of variance (MANOVA) showed that 3 volatiles [1-(2-furanyl-ethanone), cis-linalool oxide, para-cymenene] recorded significant variations ($p < 0.05$) according to geographical origin. Discriminant function analysis (DFA) classified honeys according to geographical origin providing a total classification rate of 85% using the original and 70% the cross validation method based on the aforementioned volatiles. It is possible then for an analyst to characterize the geographical origin of honey, even in cases when multifloral honeys are subjected to research/analysis. Regarding the effect of altitude on Cypriot honey aroma, results showed that the lower the altitude of harvesting the richer the aroma of honeys.

Keywords: multifloral honey; differentiation; HS-SPME/GC-MS; markers; chemometrics

1. Introduction

The determination of geographical and botanical origin of natural based foods is of great concern to the authorities and organizations that handle food distribution and set specific guidelines and rules for products' identity. Of course, a similar concern on food authentication is exhaustively shown in the recent literature in which numerous articles that cover this topic have been published. A great number of the aforementioned articles deal with the aroma profile of foodstuffs [1–8].

The determination of geographical and botanical origin of natural products has flourished the past decades, as analytical methodologies and chemometric techniques have shown a great evolution. Honey, the sweet product of honeybees (*Apis mellifera*) has earned great attention in terms of its authentication and adulteration control [2]. Hence, the authentication of a natural based product may be defined as the uniqueness in its properties and chemical composition. Analytical methodologies such as solid phase microextraction in combination with gas chromatography-mass spectrometry (SPME/GC-MS), high performance liquid chromatography (HPLC) or high performance liquid chromatography in combination with tandem mass spectrometry (HPLC/MS-MS), nuclear magnetic resonance (^{13}C NMR/ ^1H NMR), etc., may provide actual data on honey composition in terms of aroma [1–8], minerals [9,10], saccharides [11], phenolic compounds or any other fingerprint/chemical marker related to honey authenticity [12].

Honey may be classified into two main categories: blossom or nectar honeys and honeydew honeys. There are substantial differences among these honey types including pollen contribution, physicochemical composition and sensory properties [14].

At the same time, the plant microflora that dominates a region may be termed as 'characteristic of the region'. However, there is a possibility of a honey type to be of mixed flowers since honeybees cannot be restrictedly controlled and kept only in a specific place while collecting nectar from a wider area. If so, we can easily understand the differences in the pollen, physicochemical and sensory properties of such honey types compared to monofloral ones. In addition, parameters such as sunlight, rainfall, humidity, altitude, bee species, etc. may affect honey composition and plant contribution in the final product [10].

Regarding the preference among consumers, monofloral honeys are normally preferred by consumers since these have a well defined composition and characteristic sensory properties. On the other hand, multifloral ones seem to have a lower price in the market and lesser acceptability.

A fast screening of the literature points out that there are lesser studies on the characterization and geographical differentiation of multifloral or less common honey types [15–17]. As a matter of fact, studies on the authentication of Cypriot honeys using volatile compounds analysis are scarce.

Therefore, the objective of the present work was to differentiate multifloral Cypriot honeys according to geographical origin using volatile compounds data in combination with chemometrics. The impact of altitude during honey harvesting was also monitored.

2. Materials and methods

2.1. Honey samples

Twenty multifloral honey samples were collected directly from beekeepers during two consecutive harvesting seasons (2011–2013) from different regions in Cyprus. Respective number (N) of honey samples that were collected from Limassol was N = 5, from Larnaca N = 10, and from Nicosia N = 5. The contribution of plant taxa and harvesting year of honey samples are given in Table 1.

Table 1. Geographical and botanical origin of honey samples along with year of harvesting as provided by beekeepers.

Honey sample	Geographical origin	Botanical origin	Harvesting Year
1	Limassol (Limnatis region)	<i>Sinapis</i> L., <i>Prunus dulcis</i> L., <i>Malus domestica</i> , <i>Heliotropium europaeum</i> ., <i>Prunus persica</i> L., <i>Citrus</i> <i>× sinensis</i> L., <i>Verbascum thapsus</i> L.	2011–2012
2	Limassol (Mathikoloni region)	<i>Thyme</i> spp.	2011–2012
3	Limassol (Monagroulli region)	<i>Eucalyptus</i> spp., <i>Ceratonia siliqua</i> , <i>Opuntia ficus indica</i> L.	2011–2012
4	Limassol (Monagroulli region)	<i>Eucalyptus</i> spp., <i>Ceratonia siliqua</i> , <i>Opuntia ficus indica</i> L.	2011–2012
5	Limassol (Lofou region)	<i>Rosmarinus officinalis</i> L.	2011–2012
6	Larnaca (Agioi Vavatsinias)	<i>Lavandula stoechas</i> , <i>Cistus creticus</i> <i>Acacia Lefkokefala</i> , <i>Phoenix</i> spp., <i>Albizzia</i> , <i>Rosmarinus officinalis</i> , <i>Eucalyptus</i> spp., <i>Parkinsonia</i>	2011–2012
7	Larnaca (Airport)	<i>aculeata</i> L., <i>Heliotropium europaeum</i>	2011–2012
8	Larnaca (Pirga, Nissou, Alampra regions)	<i>Thyme</i> spp., <i>Eucalyptus</i> spp., <i>Rosmarinus officinalis</i> , <i>Heliotropium europaeum</i>	
9	Larnaca (Lefkara region)	–	2011–2012
10	Larnaca (Kosti region)	<i>Thyme</i> spp., <i>Heliotropium europaeum</i>	2011–2012
11	Larnaca (Kosti region)	<i>Thyme</i> spp., <i>Heliotropium europaeum</i>	2012–2013
12	Larnaca (Kosti region)	<i>Thyme</i> spp., <i>Heliotropium europaeum</i>	2012–2013
13	Larnaca (Athienou region)	Multiflower	2012–2013
14	Larnaca	Multiflower	2012–2013
15	Larnaca (Vavla region)	Multiflower	2012–2013
16	Nicosia (Calopanagiotis-Marathassa regions)	<i>Thyme</i> ssp, <i>Rhus coriaria</i> , wild flowers	2011–2012
17	Nicosia (Agros-Pitsilia regions)	<i>Thyme</i> ssp, <i>Heliotropium europaeum</i>	2011–2012
18	Nicosia (Agios Ioannis Malountas region)	<i>Eucalyptus</i> spp., <i>Heliotropium europaeum</i> , <i>Thyme</i> spp., <i>Phoenix</i> spp., , <i>Parkinsonia aculeata</i> L. <i>Pinus brutia</i> , <i>Quercus alnifolia</i> , <i>Arbutus andrachne</i> ,	2012–2013
19	Nicosia (Troodos, Nicosia side)	<i>Quercus infectoria</i> , <i>Acer obtusifolium</i> , <i>Olea europaea</i> var. <i>Sylvestris</i>	2012–2013
20	Nicosia (Forest of Macheras , Nicosia side)	<i>Pinus brutia</i> , <i>Quercus alnifolia</i> , <i>Arbutus andrachne</i> , <i>Quercus infectoria</i> , <i>Acer obtusifolium</i> , <i>Olea europaea</i> var. <i>Sylvestris</i> , <i>Rhus coriaria</i>	2012–2013

2.2. Headspace solid phase microextraction coupled to gas chromatography/mass spectrometry analysis (HS-SPME/GC-MS)

2.2.1. HS-SPME

Volatile compounds of multifloral honeys were extracted using a divinyl benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber 50/30 μm (Supelco, Bellefonte, PA, USA). The fiber was firstly conditioned for 1h at 270 $^{\circ}\text{C}$ in the injection port of the GC unit. The optimum SPME analysis conditions were as follows [7,15]: 2 g of honey was placed in a 15 mL screw-cap vial equipped with PTFE/silicone septa, with 2 mL of distilled water, and 0.20 g NaCl (Merck, Darmstadt, Germany). Finally, 20 μL of internal standard (benzophenone, 100 $\mu\text{g}/\text{mL}$, Sigma-Aldrich), were placed in the aqueous honey solution obtained. The vials were then sealed and maintained at 45 $^{\circ}\text{C}$ in a water bath under continuous stirring at a speed regulation of 600 rpm during the headspace extraction. A magnetic stirrer (cross shaped PTFE-coated magnetic stirrer, diameter 10 mm, Semadeni, Ostermundigen–Bern, Switzerland) was placed inside the vials to assist in the extraction of volatiles. The fiber was removed from the vial at the end of extraction, and was inserted into the injection port of GC for 15 min. Honey samples were prepared daily prior to HS-SPME/GC-MS analysis and the fiber was cleaned exhaustively prior analysis, using the clean program method (22.5 min). Each sample was run in duplicate and the results were averaged [16].

2.2.2. GC-MS analysis

The GC-MS analysis was performed on an Agilent 7890A gas chromatograph coupled to an Agilent 5975 mass detector with an electron ionization source. The chromatographic separation of volatile compounds was accomplished using a DB-5MS (cross linked 5% PH ME siloxane) capillary column (60 m \times 320 μm i.d., \times 1 μm film thickness). The GC oven temperature program followed the sequence : i) held at 40 $^{\circ}\text{C}$ for 3 min, ii) then was increased to 260 $^{\circ}\text{C}$ at 8 $^{\circ}\text{C}/\text{min}$ (6 min hold). The injector and MS-transfer line were maintained at 250 $^{\circ}\text{C}$ and 270 $^{\circ}\text{C}$, respectively. The mass spectra were recorded from m/z 50 to 550 at ionization energy of 70 eV. Finally, helium was used as the carrier gas (purity 99.999%), at a flow rate of 1.5 mL/min.

2.3. Identification and semi-quantification of volatile compounds

The identification of volatile compounds was achieved using the Wiley 7, NIST 2005 mass spectral library. In addition, a mixture of n-alkanes ($\text{C}_8\text{--C}_{20}$) dissolved in n-hexane was employed in order to calculate the linear retention time indices (Kovats indices). The mixture was supplied by Supelco (Bellefonte, PA, USA). The calculation was carried out for volatiles eluting between n-octane and n-eicosane. Volatile compounds having $\geq 90\%$ similarity with Wiley library were tentatively identified using the GC-MS spectra. Data were then expressed as concentration (C_{analyte}, $\mu\text{g}/\text{kg}$) according to the eq. 1:

$$C_{\text{analyte}} (\mu\text{g}/\text{kg}) = \left(\frac{E_{\text{analyte}}}{E_{\text{benzophenone}}} \right) \times C_{\text{IS}} \quad (1)$$

where E_{analyte} and $E_{\text{benzophenone}}$ are the peak areas of the isolated volatile compounds and internal standard; whereas C_{IS} is the final concentration of internal standard inside the aqueous honey solution, assuming a response factor equal to 1 for all the volatile compounds [16]. Benzophenone (m/z = 182) proved to be an ideal internal standard since it did not cause any co-elution

problems. It should also be marked that this compound did not comprise a 'natural' honey volatile compound. The reproducibility, that is the closeness of the agreement between the results of consecutive measurements of the same measurand carried out with same methodology described in work, of the metabolite semi-quantification with respect to sample preparation and analysis was $\geq 90\%$.

2.4. Statistical analysis

Semi-quantitative data of volatile compounds were subjected to multivariate analysis of variance (MANOVA) in order to investigate which are the significant ones ($p < 0.05$) that could differentiate Cypriot multifloral honeys according to geographical origin. MANOVA may be termed as a generalized form of univariate analysis of variance (ANOVA) although, unlike univariate ANOVA, it uses the covariance between outcome variables in testing the statistical significance of the average differences [18]. Volatile compounds were taken as the dependent variable, while geographical origin was taken as the independent variable. Wilk's Lambda and Pillai's Trace indices were computed to determine/or not a significant effect of volatile compounds on geographical origin of multifloral honey samples. Discriminant function analysis (DFA) was then applied only to the significant volatile compounds to explore the effectiveness, in terms of prediction rates, of differentiating multifloral honey samples according to geographical origin. The original and cross validation methods for the prediction ability were both considered. More specifically, in the original method the correct prediction rate arises from the contribution of all cases (significant parameters) in the discriminant functions. In cross validation, each case is classified by the functions derived from all cases other than that case [7].

3. Results and discussion

3.1. Volatile compounds of Cypriot multifloral honeys

In Table 2 are listed the 25 volatile compounds that were tentatively identified and semi-quantified. The volatiles that dominated the aroma of multifloral Cypriot honeys were acids, alcohols, aldehydes, esters, hydrocarbons, ketones, and terpenoids. A typical gas chromatogram of multifloral honey sample from Limassol is given in Figure 1.

At this point it should be stressed that in Table 2 some values for the amount of certain components comprise of standard deviations higher than the average. It is owed to the sample variability regarding the specific volatile compound and not the analytical method variability. More specifically, a wide range in the amounts of specific volatile compounds was observed during the analysis of samples (Table 2). Since honey is a natural product, hence, variations in the volatile composition among different samples of the same geographical origin may also occur. This is also confirmed further by Table 1 in which the contribution of plant species among honey samples of the same geographical origin was different.

Numerous of these compounds have been previously identified in blossom and honeydew honeys harvested in different parts of the world [3–6, 15–17].

Acetic acid has been reported previously aiding to the volatile fraction of Spanish and Greek thyme honeys, respectively [7,8]. On the other hand, Spanish lavender honey proved to have a higher amount ($\mu\text{g}/\text{kg}$) of acetic acid compared to that of Spanish [8].

Octanoic, nonanoic, decanoic, and dodecanoic acid ethyl esters showed diverse amounts ($\mu\text{g}/\text{kg}$) with respect to multifloral honey geographical origin. Spanik et al. [5] reported the presence of numerous acid methyl esters in rape, sunflower, acacia, lime, raspberry, and phacelia honeys from Slovakia. Acetic acid ethyl ester was also identified in Spanish lavender and thyme honeys [8].

The same trend, in terms of diversity, showed linear aldehydes such as octanal, nonanal, and decanal. Such aldehydes are typical honey aroma compounds since these have been identified in numerous honey types [3–9]. For instance, the amounts of octanal and decanal were higher in Larnaca region.

Phenolic aldehydes such as benzaldehyde and phenylacetaldehyde were identified in higher amounts in multifloral honeys from Limassol region. Phenolic volatiles may be characterized as a major class of volatile compounds included in the typical volatile compounds of honey. Variations in their amounts have been reported in relation to botanical and geographical origin of honey [3,7,16].

The ketones, 1-hydroxy-2-propanone and 1-(2-furanyl)-ethanone have been reported previously to enhance the aroma of monofloral thyme honey from Greece and Spain [7,8]. 1-hydroxy-2-Propanone recorded higher amounts ($\mu\text{g}/\text{kg}$) in multifloral honeys from Limassol region. On the other hand, 1-(2-furanyl)-ethanone recorded higher amounts ($\mu\text{g}/\text{kg}$) in Nicosia region.

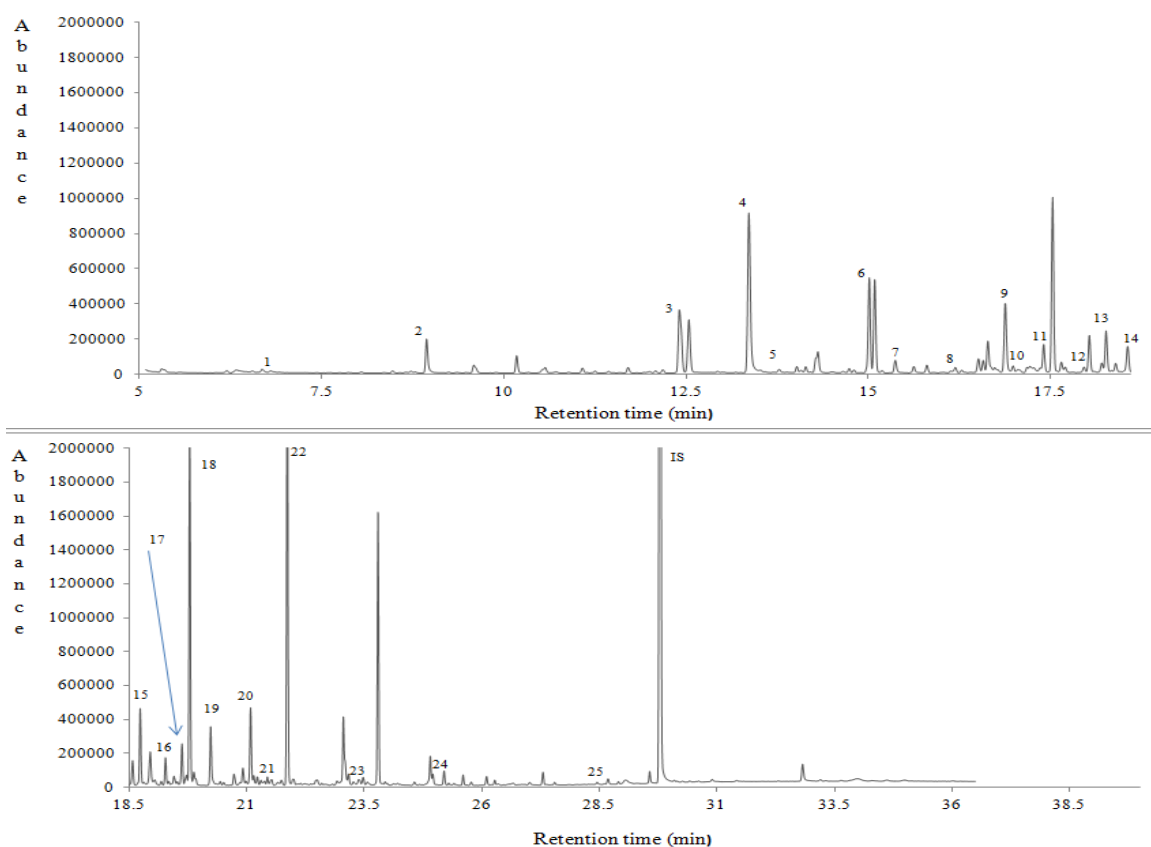


Figure 1. A typical gas chromatogram of multifloral honey from Limassol. Volatile compounds are numbered according to retention time given in Table 1. IS: internal standard.

Table 2. Volatile compounds tentatively identified in Cypriot multifloral honeys according to geographical origin: Semi-quantitative data assuming a response factor equal to 1 for all compounds.

Volatile compounds	RT (min)	RI _{exp}	RI _{lit}	Limassol average (\pm SD) (μ g/kg)	Range (μ g/kg)	Larnaca average (\pm SD) (μ g/kg)	Range (μ g/kg)	Nicosia average (\pm SD) (μ g/kg)	Range (μ g/kg)	F
Acetic acid	6.84	<800	<800	200 \pm 300	ni–600	100 \pm 200	ni–600	ni	ni	1.491ns
1-hydroxy-2-Propanone	8.91	<800	<800	100 \pm 100	ni–200	30 \pm 100	ni–200	ni	ni	1.903ns
Octane	12.42	800	800	50 \pm 40	20–100	40 \pm 20	10–100	100 \pm 120	10–300	0.700ns
Furfural	13.25	852	835	90 \pm 60	40–200	70 \pm 50	ni–200	50 \pm 20	30–70	0.970ns
2-Furanmethanol	13.83	888	864	30 \pm 40	ni–110	10 \pm 30	ni–110	ni	ni	1.158ns
Nonane	15.03	900	900	20 \pm 20	ni–40	20 \pm 20	ni–50	30 \pm 10	10–40	0.215ns
1-(2-furanyl)-Ethanone	15.40	916	914	ni	ni	ni	ni	2.0 \pm 3.0	ni–5.0	4.250*
alpha-Pinene	16.21	950	936	3.0 \pm 3.0	ni–5.0	2.0 \pm 4.0	ni–10	6.0 \pm 4.0	ni–10	1.735ns
Benzaldehyde	16.89	979	970	80 \pm 70	10–200	60 \pm 30	20–120	40 \pm 20	20–60	0.883ns
5-methyl-4-Nonene	17.01	984	–	ni	ni	1.0 \pm 3.0	ni–10	ni	ni	0.472ns
Octanal	17.45	1002	1003	20 \pm 7.0	10–30	30 \pm 30	10–100	30 \pm 20	5.0–50	0.199ns
delta-3-Carene	17.98	1026	1020	ni	ni	4.0 \pm 5.0	ni–10	4.0 \pm 5.0	ni–10	1.417ns
ortho-Cymene	18.27	1039	1039	40 \pm 20	10–70	40 \pm 30	ni–110	60 \pm 40	ni–100	0.624ns
1,8-Cineole	18.56	1052	1044	ni	ni	ni	ni	6.0 \pm 10	ni–30	2.783ns
Benzeneacetaldehyde	18.73	1060	1048	80 \pm 60	10–180	220 \pm 320	50–980	60 \pm 30	30–90	1.043ns
cis-Linalool oxide	19.28	1085	1091	ni	ni	10 \pm 10	ni–30	20 \pm 10	ni–30	4.247*
para-Cymenene	19.71	1105	1094	ni	ni	10 \pm 10	ni–30	30 \pm 30	ni–60	5.061*
Nonanal	19.78	1108	1106	80 \pm 60	ni–170	80 \pm 60	ni–210	80 \pm 50	20–120	0.011ns
Benzeneethanol	20.23	1130	1114	70 \pm 80	10–200	90 \pm 110	ni–310	40 \pm 30	20 \pm 80	0.582ns
1-Nonanol	21.08	1170	1172	ni	ni	2.0 \pm 4.0	ni–10	ni	ni	1.063ns
Octanoic acid ethyl ester	21.50	1192	1198	10 \pm 7.0	10–20	20 \pm 10	ni–40	30 \pm 30	ni–80	1.647ns

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Volatile compounds	RT (min)	RI _{exp}	RI _{lit}	Limassol average (\pm SD) (μ g/kg)	Range (μ g/kg)	Larnaca average (\pm SD) (μ g/kg)	Range (μ g/kg)	Nicosia average (\pm SD) (μ g/kg)	Range (μ g/kg)	F
Decanal	21.85	1210	1206	50 \pm 50	10–110	90 \pm 80	20–250	90 \pm 90	10–200	0.413ns
Nonanoic acid ethyl ester	23.40	1292	1295	50 \pm 90	10–220	25 \pm 20	ni–50	30 \pm 25	ni–60	0.641ns
Decanoic acid ethyl ester	25.19	1391	1393	30 \pm 20	5.0–60	20 \pm 20	ni–50	20 \pm 15	ni–30	0.520ns
Dodecanoic acid ethyl ester	28.46	1591	1593	10 \pm 10	ni–10	10 \pm 6.0	ni–20	10 \pm 4.0	ni–10	0.038ns
TVSQD				1000 \pm 500		970 \pm 600		700 \pm 400		

Results reported are the average \pm standard deviations values of two independent replicates ($n = 2$). MANOVA results (F values and significant differences); ns: not significant; $p < 0.05^*$. RT: retention time, RI_{exp}: experimental retention indices values based on the calculations using the standard mixture of alkanes. RI_{lit}: Retention indices of the identified compounds according to literature data cited in Wiley 7 NIST MS library. ni: not identified; these values were treated as zeros for chemometrics, not as missing values. Range: minimum to maximum value of each volatile with respect to geographical origin. TVSQD: total volatile semi-quantitative data (μ g/kg).

Linear hydrocarbons such as octane and nonane are typical volatile markers of honey. Octane recorded higher amounts (μ g/kg) in multifloral honeys from Nicosia, whereas nonane recorded higher amounts in samples from Limassol. In a previous study involving Italian *Citrus* spp. honeys decane was the dominant hydrocarbon that was identified. [4].

Terpenoids are among the major class of volatiles found in numerous natural based products, and enhance the aroma of honey by a distinctive note. Volatiles such as alpha-pinene, ortho-cymene and para-cymenene, cis-linalool oxide, delta-3-carene and 1,8-cineole dominated the volatile fraction of multifloral honeys from Cyprus. Terpenoids have been also identified in Italian, Slovak, Greek Spanish and Irish floral honeys [4–6,8,19].

Furan or pyran derivatives are quite common volatiles that are found in honey. These compounds may originate from the thermal processing of honey during the headspace extraction [16].

3.2. Differentiation of multifloral Cypriot honeys according to geographical origin based on specific volatile compounds' semi-quantitative data

Data of volatile compounds (semi-quantitative results) originating from all the analyzed multifloral honey samples were subjected to MANOVA analysis to determine which volatile compounds could effectively contribute to geographical differentiation ($p < 0.05$). Dependent variables included the 25 volatile compounds while geographical origin was taken as the independent variable. Wilks' Lambda and Pillai's Trace indices showed that there was a multi-significant effect of volatiles on honey geographical origin. Three of the 25 volatile compounds (Table 2) were found to be significant ($p < 0.05$) for the geographical discrimination of multifloral Cypriot honeys (Wilks' Lambda = 0.327, $F = 3.738$, $df = 6$, $p = 0.007$; Pillai's Trace = 0.733, $F = 3.083$, $df = 6$, $p = 0.017$). Afterwards, these 3 volatiles were subjected to DFA.

Discriminant function analysis results showed that two discriminant functions were formed. However, only one was significant and could be used for the differentiation of multifloral honeys according to geographical origin: Wilks' Lambda = 0.327, $X^2 = 17.864$, $df = 6$, $p = 0.0007$ for the first function and Wilks' Lambda = 0.906, $X^2 = 1.581$, $df = 2$, $p = 0.454$, for the second. The first discriminant function recorded the higher eigenvalue (1.767) and canonical correlation of 0.799, accounting for 94.4% of total variance. The second discriminant function recorded a much lower eigenvalue (0.104) and canonical correlation of 0.307, accounting for 5.6%.

In Figure 2 it is shown that the geographical regions are satisfactorily separated. The overall correct classification rate was 85% using the original and 70% using the cross validation method, considered a satisfactory rate for this method of "modeling" (Table 4). The unstandardized canonical discriminant functions evaluated at group means (functions at group centroids) are also shown in Figure 2 and were (-1.183, -0.427) for Limassol; (-0.436, 0.278) for Larnaca; and (2.056, -0.128) for Nicosia. The best classification results (100%) were obtained for multifloral honey samples from Limassol, followed by those of Nicosia (80%) and Larnaca (50%). Discriminant function coefficients are given in Table 3. In that sense the canonical discriminant functions based on the 3 volatiles may be written as follows:

$$DF1 = -1.183 + 588.688[1-(2-furanyl)-ethanone] + 28.915[\text{cis-linalool oxide}] + 47.131[\text{para-cymenene}] \quad (2)$$

$$DF2 = -0.427 - 454.847[1-(2-furanyl)-ethanone] + 80.687[\text{cis-linalool oxide}] - 12.305[\text{para-cymenene}] \quad (3)$$

The structures of the marker compounds are given in Figure 3. Classification result showed that geographical differentiation of Cypriot multifloral honeys based on specific volatile compounds was in general satisfactory. We should not forget that the discrimination of multifloral honeys according to geographical origin may comprise a more difficult approach compared to research carried out on monofloral honeys. In addition, botanical origin differentiation of honey is normally a simpler procedure and may reach classification rates of ca. 100% [6].

Stanimirova et al. [3] showed that 26 volatile compounds such as benzaldehyde, octanal, nonanal, decanal, para-cymene, nonanoic acid ethyl ester, decanoic acid ethyl ester, etc., were the marker volatiles for the classification of Corsican and non Corsican honeys by application of DFA. The classification rate was 86.4%.

Karabagias et al. [7] classified *Thymus capitatus* L. honeys according to geographical origin based on volatile compounds such as formic acid, acetic acid, 1-hydroxy-2-propanone, octane, decanal, etc. and reported a classification rate of 64.3% using DFA and the cross-validation method.

Escriche et al. [8] classified Spanish lavender and thyme honeys according to botanical origin, based on volatile compounds such as hexanal, 1-hexanol, hotrienol, acetic acid, etc. achieved a correct prediction rate of 85.7% using discriminant function analysis.

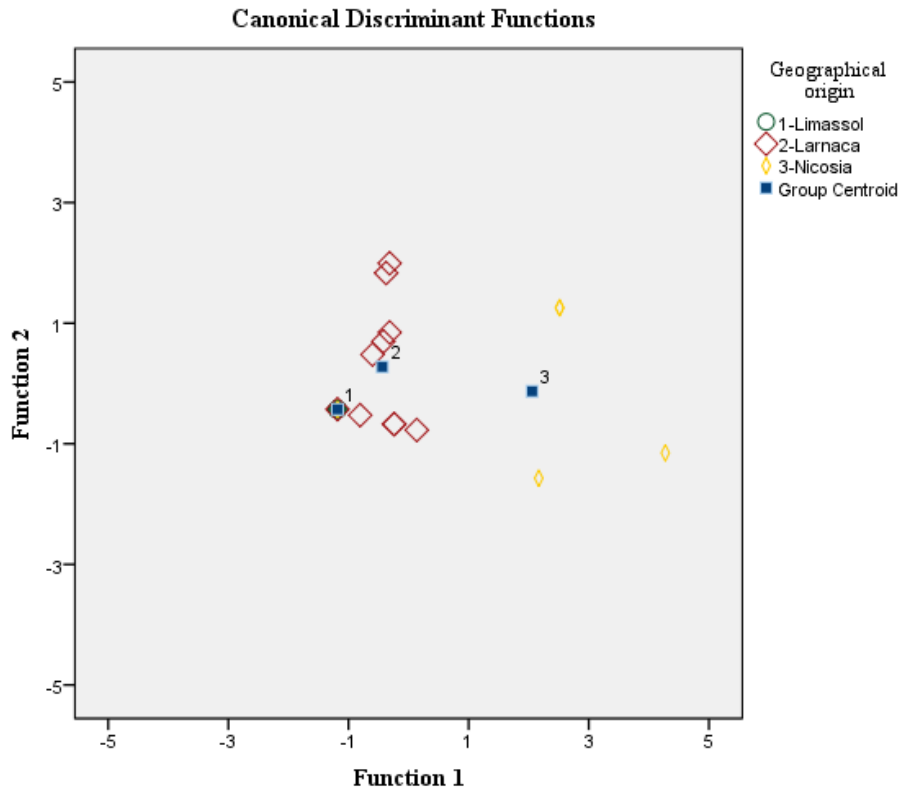


Figure 2. Differentiation of Cypriot multifloral honeys according to geographical origin using 3 volatile compounds and DFA.

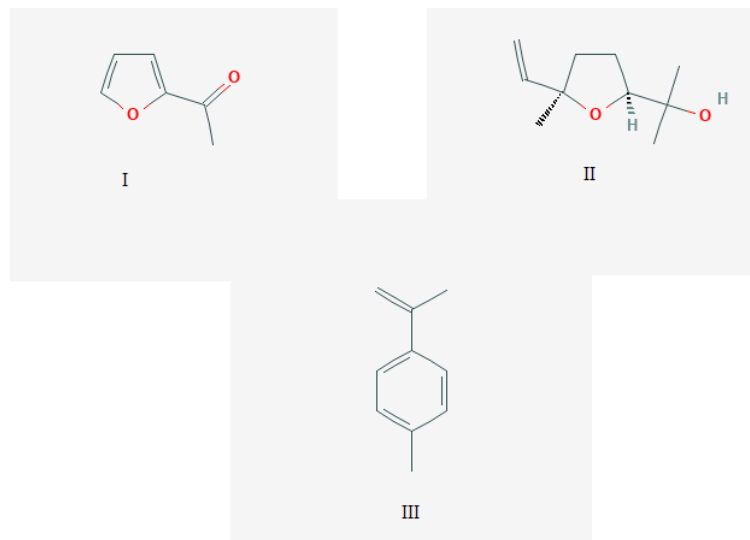


Figure 3. Structures of marker compounds. I:1-(2-furanyl)-Ethanone, II:cis-Linalool oxide, III: para-Cymenene (PubChem, Open Chemistry, Database).

Table 3. Canonical discriminant function coefficients ^{uc} based on the three significant volatile compounds identified in Cypriot multifloral honeys according to geographical origin.

Volatile markers of the provenience of Cypriot multifloral honeys	Discriminant function	
	1	2
1-(2-furanyl)-Ethanone	588.688	-454.847
cis-Linalool oxide	28.915	80.687
para-Cymenene	47.131	-12.305
(Constant)	-1.183	-0.427

^{uc}: Unstandardized coefficients.

Table 4. Classification results based on semi-quantitative data of volatile compounds identified in Cypriot honeys.

Chemometric technique	Prediction rates	Geographical origin	Predicted Group Membership			Number of honey samples
			Limassol	Larnaca	Nicosia	
DFA	Percentage (%)					
	Count	Limassol	5	0	0	5
		Larnaca	2	8	0	10
Original method ^a	%	Nicosia	1	0	4	5
		Limassol	100.0	0.0	0.0	100.0
		Larnaca	20.0	80.0	0.0	100.0
	Count	Nicosia	20.0	0.0	80.0	100.0
		Limassol	5	0	0	5
		Larnaca	5	5	0	10
Cross-validation method ^b	%	Nicosia	1	0	4	5
		Limassol	100.0	0.0	0.0	100.0
		Larnaca	50.0	50.0	0.0	100.0
		Nicosia	20.0	0.0	80.0	100.0

^a 85.0% of original grouped cases correctly classified.

^b Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

^c 70.0% of cross-validated grouped cases correctly classified.

On the other hand, environmental factors should be also taken into account. Indeed, the altitude of honey harvesting was also an important parameter that showed fluctuations with respect to the total volatile content or individual volatile content (semi-quantitative data) of Cypriot multifloral honeys. The non significant differences ($p < 0.05$), hence, may be probably due to the limited honey samples grouped into the three zones or the multifloral nature of honey samples analyzed.

It is very important then for a researcher that works in the field of honey authentication to test the classification models by using in the analysis, among other varieties, mixed floral honey types. In addition, when data regarding environmental factors are available, this should be also considered in the final report. In that sense, more rigorous honey authentication approaches may be planned. The present study addresses also this questioning.

3.3. The impact of altitude on total volatile semi-quantitative data of Cypriot multifloral honeys

In order to test whether the altitude could affect volatile compounds' data (semi-quantitative data) honey samples originating, hence, from different regions were classified into three groups: Group A: honey samples harvested at 0–300 m (N = 8); Group B: honey samples harvested at 300–600 m (N = 6); and Group C: honey samples harvested at 600–1000 m (N = 6). Before going any further it is important to stress that this procedure may imply a useful strategy for the evaluation of the impact of altitude on the volatile profile of honey, since grouped honey samples originated from the 3 different regions in Cyprus. Thus, present data may be well generalized in a larger population of samples. For instance, this study could be extended, i.e. to the entire Mediterranean area to a larger number/population of samples, from more wide-spread sampling in order the obtained results to be more accurate.

Therefore, the collected data may be characterized as preliminary in nature. However, results showed that the lower the altitude, the richer the aroma of honeys (Figure 4). This finding shows that the contribution of aromatic plants is favored in regions of lower altitude, compared to those with higher one. This is extremely important information for beekeepers or any other authorities that are involved in honey production; that is the production of a richer in aroma honey. Therefore, the altitude of honey harvesting may comprise an additional criterion for the quality of honey. As we mentioned above, future research using a larger number of honey samples harvested at different altitudes will enhance further present results.

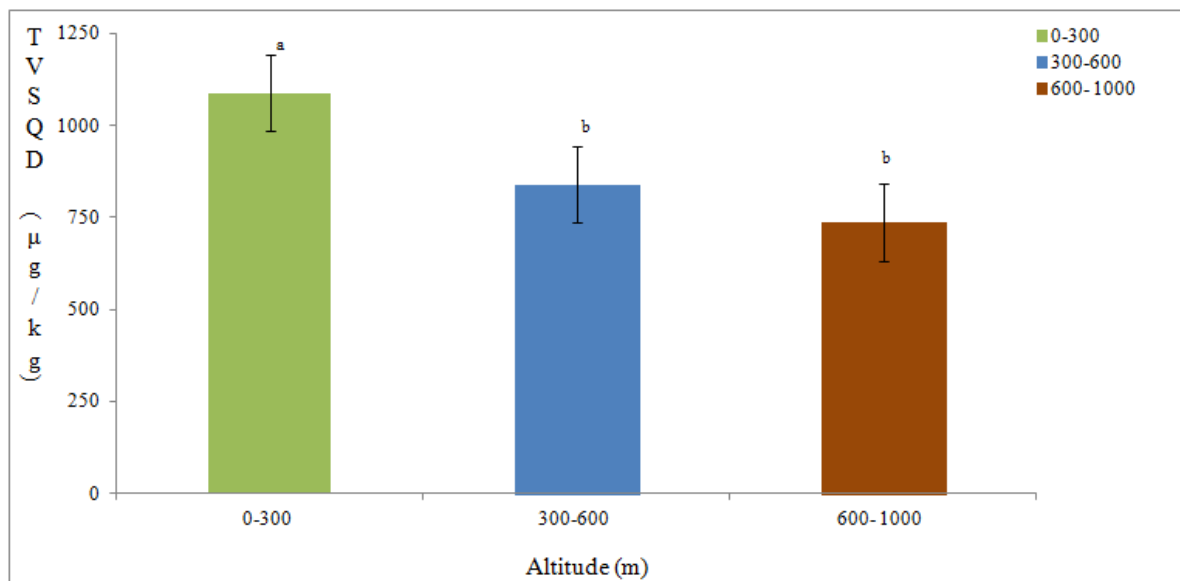


Figure 4. The effect of altitude on the total volatile semi-quantitative data (TVSQD) ($\mu\text{g}/\text{kg}$) of Cypriot multifloral honeys. Different letters in each bar indicate statistically significant differences at the confidence level $p < 0.05$.

4. Conclusions

The volatile profile of Cypriot multifloral honeys was investigated for the first time. Results showed that volatile compounds of Cypriot honeys were affected by geographical origin, involving at the same time the plant contribution in honey. The application of HS-SPME/GC-MS proved to be

an assistive tool for the differentiation of Cypriot honeys according to geographical origin using DFA. Three marker compounds were found: 1-(2-furanyl-ethanone), cis-linalool oxide and p-cymenene. Eventhough, the classification rate obtained using the cross-validation method was lower compared to previous works [3,6], the present study contributes to the state of the art by showing that geographical origin differentiation of multifloral honeys is a more difficult procedure than that of monofloral honeys [3]. In addition, some promising preliminary data regarding the effect of altitude on honey aroma were also collected.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Manyi-Loh CE, Ndip RN, Clarke AM (2011) Volatile compounds in honey: A review on their involvement in aroma, botanical origin determination and potential biomedical activities. *Int J Mol Sci* 12: 9514–9532.
2. Wu L, Dua B, Heyden YV, et al. (2017) Recent advancements in detecting sugar-based adulterants in honey—A challenge. *TrAC Trends Anal Chem* 86: 25–38.
3. Stanimirova I, Ustun B, Cajka T, et al. (2010) Tracing the geographical origin of honeys based on volatile compounds profiles assessment using pattern recognition techniques. *Food Chem* 118: 171–176.
4. Papotti G, Bertelli D, Plessi M (2012) Use of HS-SPME-GC-MS for the classification of Italian lemon, orange and citrus spp. honeys. *Int J Food Sci Technol* 47: 2352–2358.
5. Spanik I, Janacova A, Susterova Z, et al. (2013) Characterisation of VOC composition of Slovak monofloral honeys by GC×GC–TOF-MS. *Chem Pap* 67: 127–134.
6. Castro-Vazquez L, Leon-Ruiz V, Alanon M.E, et al. (2014) Floral origin markers for authenticating Lavandin honey (*Lavandula angustifolia* x *latifolia*). Discrimination from Lavender honey (*Lavandula latifolia*). *Food Control* 37: 362–370.
7. Karabagias IK, Badeka A, Kontakos S, et al. (2014) Characterization and classification of *Thymus capitatus* (L.) honey according to geographical origin based on volatile compounds, physicochemical parameters and chemometrics. *Food Res Int* 55: 363–372.
8. Escriche I, Sobrino-Gregorio L, Conchado A, et al. (2017) Volatile profile in the accurate labelling of monofloral honey. The case of lavender and thyme honey. *Food Chem* 226: 61–68.
9. Petretto GL, Urgeghe PP, Mascia I, et al. (2017) Stir bar sorptive extraction coupled with GC/MS applied to honey: optimization of method and comparative study with headspace extraction techniques. *Eur Food Res Technol* 243: 735–741.

10. Czipa N, Alexa L, Phillips CJC, et al. (2018) Macro-element ratios provide improved identification of the botanical origin of mono-floral honeys. *Eur Food Res Technol* 244: 1439–1445.
11. Karabagias IK, Louppis A, Kontakos S, et al. (2018) Characterization and botanical differentiation of monofloral and multifloral honeys produced in Cyprus, Greece and Egypt using physicochemical parameter analysis and mineral content, in conjunction with supervised statistical techniques. *J Anal Meth Chem* 2018: 7698251.
12. Cotte JF, Casabianca H, Lheritier J, et al. (2007) Study and validity of ¹³C stable carbon isotopic ratio analysis by mass spectrometry and ²H site-specific natural isotopic fractionation by nuclear magnetic resonance isotopic measurements to characterize and control the authenticity of honey. *Anal Chim Acta* 582: 125–136.
13. Ruckriemen J, Henle T (2018) Pilot study on the discrimination of commercial *Leptospermum* honeys from New Zealand and Australia by HPLC–MS/MS analysis. *Eur Food Res Technol* 244: 1203–1209.
14. Pita-Calvo C, Vazquez M (2017) Differences between honeydew and blossom honeys: A review. *Trends Food Sci Technol* 59: 79–87.
15. Zappi A, Melucci D, Scaramagli S, et al. (2018) (In Press) Botanical traceability of unifloral honeys by chemometrics based on head-space gas chromatography. *Eur Food Res Technol* 244: 2149–2157.
16. Karabagias IK, Nikolaou C, Karabagias VK (2018) (In Press) Volatile fingerprints of common and rare honeys produced in Greece: In Search of PHVMs with implementation of the honey code. *Eur Food Res Technol* 245: 23–49.
17. Dekebo A, Kwon SY, Kim DH, et al. (2018) Volatiles analysis of honey by gas chromatography-mass spectrometry (GC-MS): Comparison of SPME volatiles extraction methods. *J Apic* 33: 117–128.
18. Warne RT (2014) A primer on multivariate analysis of variance (MANOVA) for behavioral scientists. *Pract Assess Res Eval* 19: 1–10.
19. Makowicz E, Kafarski P, Jasicka-Misiak I (2018) (In Press) Chromatographic fingerprint of the volatile fraction of rare *Hedera helix* honey and biomarkers identification. *Eur Food Res Technol* 244: 2169–2179.



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