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Comparison of the Volatile Composition in Thyme Honeys from Several Origins in Greece

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Thyme honey is the most appreciated unifloral Greek honey in Greece as well as around the world. In an effort to investigate the headspace composition of this type of honey, 28 samples were analyzed by means of solid-phase microextraction coupled to a gas chromatography–mass spectrometry system. The botanical origin of the samples was ascertained by pollen analysis, and samples displayed relative frequencies of thyme pollen between 18 and 41%. A total of 62 compounds were isolated, and phenylacetaldehyde was the most abundant (32.9% of the total peak area). Possible botanical markers are 1-phenyl-2,3-butanedione (13.4%), 3-hydroxy-4-phenyl-2-butanone, 3-hydroxy-1-phenyl-2-butanone (14.7%), phenylacetonitrile (4.8%), and carvacrol (0.9%), since these compounds are found only in thyme honey. Additionally, high proportions of phenylacetaldehyde are also characteristic ($F = 12.282$, $p < 0.001$). The average concentrations of seven compounds were significantly different ($p < 0.05$), namely phenylacetaldehyde, acetophenone, octanoic acid, carvacrol, phenylethyl alcohol, nonanal, and hexadecane. Applying principal component analysis to the data, six components were extracted, explaining 85.4% of the total variance. The first component explained 46.2% of the total variance and was positively correlated to phenylacetaldehyde, nonanoic acid, acetophenone, decanoic acid, benzaldehyde, phenylacetonitrile, isophorone, and nonanal. The extracted components were used as variables to the discriminant analysis, which showed good discrimination, especially for samples from Crete. A leave-one-out classification showed 85.7% of cross-validated grouped cases correctly classified. These results are promising to establish a discrimination model for these geographical regions. This is crucial for local beekeeper corporations on their effort to produce honey with geographical origin label.

KEYWORDS: Thyme honey; SPME; volatile compounds; botanical origin; geographical origin; 1-phenyl-2,3-butanedione; 3-hydroxy-4-phenyl-2-butanone; 3-hydroxy-1-phenyl-2-butanone; phenylacetonitrile; carvacrol; phenylacetaldehyde

INTRODUCTION

Honey is considered the most appreciable natural product. It is not only the taste and the aroma but also the various nutritional and medicinal properties that attract consumers worldwide. Consumer preference, and hence the price of the product, depends mainly on its botanical origin. The need for finding reliable marker compounds to discriminate between unifloral honeys is obvious.

During the past years, the analysis of volatile compounds has become a powerful tool to assist the determination of honey origin (*1–5*). Phenolic compounds (*6–10*), norisoprenoids (*6, 11–14*), terpenoids (*3, 15–18*), and aliphatic dicarboxylic acids (*19*) have been proposed as potent markers for honey botanical origin.

Solid-phase microextraction (SPME) is a solvent-free isolation technique, gaining at increased rate the appreciation of food industry as a means to isolate headspace volatile compounds. Lately introduced (*20, 21*), SPME is highly appreciated by the food industry for the analysis of volatile compounds. This method has proven effective for the analysis of honey headspace volatile compounds (*22–31*).

Thyme honey is the most preferable unifloral honey in Greece, with its price ranging from 2 to 3 times higher than any other honey. Highly appreciated worldwide, thyme is the most important honey harvest in Greece, and thus it has been receiving great attention for a long time. It is this importance that requires measures to ensure the best product for the consumer and one of the major aims is to find in adequate ways to determine its floral origin.

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Corydothymus capitatus (L.) Reichenb. [syn. *Thymus capitatus* (L.) Hoffmans & Link] is the most widespread species of thyme in Greece, among the 23 found all over the country. It blossoms at the beginning of summer, and the flowers provide nectar for about 40 days. The climatic conditions at this period make honey production even more difficult. The honey produced is light colored, with a distinct and desirable aroma. More than 10% of the annual production in Greece is unifloral thyme honey.

In the literature, many works have been published on the volatile compounds isolated from thyme honey from different geographical origins (*23, 24, 30–33*), and several compounds are referred to as characteristic such as ethenyl phenylacetate and α-hydroxybenzenepropanoic acid (*24*), 1,3-diphenyl-2-propanone, (3-methylbutyl)benzene, 3,4,5-trimethoxybenzaldehyde, 3,4-dimethoxybenzaldehyde, vanilline, and thymol (*30*), 3,4,5-trimethoxybenzaldehyde (*31*), and phenylacetonitrile and 1-phenyl-2,3-butanedione combined with increased concentrations of phenylacetaldehyde (*33*).

The scope of this work is to evaluate SPME as a method to isolate and identify potential botanical markers for unifloral Greek honey and to discriminate thyme honeys from different geographical regions of Greece.

MATERIALS AND METHODS

Honey Sample. Thyme honeys (28 samples) were from different locations all over Greece and were obtained from local beekeepers. They were stored at −18 °C until used. All samples met with Greek legislation requirement for unifloral thyme honey regarding relative frequency of pollen, which has to exceed the value of 18%. Pollen content varied between 18 and 41% among the samples. Also, citrus (*Citrus* spp., 33 samples), cotton (*Gossypium hirsutum*, 7 samples), heather (*Erica manipuliflora*, 4 samples), chestnut (*Castanea sativa*, 3 samples), eucalyptus (*Eucalyptus* spp., 3 samples), pine (*Pinus* spp., 5 samples), and fir (*Abies* spp., 6 samples) unifloral honeys were analyzed.

Reagents. Benzaldehyde and α-terpineol were purchased from Merck (Darmstadt, Germany), octanoic acid from Riedel-de Haën (Steinheim, Germany), and phenylacetaldehyde from Aldrich (Steinheim, Germany). Octane, octanal, *p*-cymene, acetophenone, *trans*-furanoid linaloxide, *cis*-furanoid linaloxide, undecane, linalool, nonanal, phenylethyl alcohol, isophorone, methyl octanoate, 1-nonanol, terpinen-4-ol, benzoic acid, methyl salicylate, decanal, methyl nonanoate, *p*-anisaldehyde, nonanoic acid, thymol, undecanal, carvacrol, methyl decanoate, eugenol, 4-methoxyphenethyl alcohol, decanoic acid, (*E*)-β-damascenone, dodecanal, methyl dodecanoate, hexadecane, heptadecane, and benzophenone were purchased from Fluka Chemika (Buchs, Switzerland).

Isolation of Volatile Compounds. The isolation of the aroma compounds was performed using the SPME procedure. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber was used to extract headspace volatiles from honey. This type of fiber provides the best sorption capacity and the broadest range of volatiles extracted from the headspace of a mixed honey sample (*34*). The samples (6 mL of water solution of 3 g honey mL^{-1}) were placed in 15 mL screw-top vials with PTFE/silicone septa. Benzophenone was used as internal standard, and a portion of 20 μL (10 μg/mL in methanol) was added prior to extraction. The vials were maintained in a water bath at 60 °C under stirring during the whole procedure. Equilibration time was set at 30 min, followed by 60 min sampling time. The standard deviation of the isolated compounds ranged between 2.51% for 1-nonanol to 11.19% for octanal.

Analysis of the Isolated Compounds. The analysis of the extracts was performed using a Hewlett-Packard 5890 II GC, equipped with a Hewlett-Packard 5972 MS detector. The column used was an HP-5MS (cross-linked 5% PH ME siloxane) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness), and the gas carrier was helium, at 1 mL/min rate. The injector and MS-transfer line temperatures were maintained at 220 and 290 °C, respectively. Oven temperature was held at 40 °C for 3 min and raised to 160 °C at 3 °C/min and then to 200 °C at 10 °C/min.

Electron impact mass spectra recorded at the 40–500 mass range. An electron ionization system was used with ionization energy of 70 eV. The identification of the isolated compounds was achieved by comparing retention times and mass spectra with those of authentic samples. For tentative identification, the Nist98 and Wiley275 mass spectral libraries were employed as well as spectral data and Kovats index provided by Adams (*35*) or published in the literature cited.

Concentrations of the isolated compounds are expressed as ratios of the response of each compound against the response of the internal standard (benzophenone) according to the equation $C_{analyte} = (E_{analyte}/E_{benzophenone}) \times (1000/3)$, where $C_{analyte}$ is the concentration of the target compound, $E_{analyte}$ and $E_{benzophenone}$ are the peak areas of the target compound and the internal standard, respectively, and 1000/3 is the correction number to express concentrations in ng/kg honey. Statistical evaluation of the data was performed with SPSS v. 11.0 software.

RESULTS AND DISCUSSION

Headspace Composition of Thyme Honey. In total, 62 volatile components were isolated (**Table 1**). Phenolic compounds were the most abundant. Peaks with high intensity at the GC trace (**Figure 1**) are phenylacetaldehyde (32.9% of the total peak area), 1-phenyl-2,3-butanedione (13.4%), the acyloins 3-hydroxy-4-phenyl-2-butanone and 3-hydroxy-1-phenyl-2-butanone (14.7%), benzaldehyde (5.8%), phenylethyl alcohol (4.7%), phenylacetonitrile (benzyl cyanide, 4.8%), nonanal (3.6%), 3-hydroxy-4-phenyl-3-buten-2-one (3%), and decanal (2.1%). Minor components with significance are 2-phenyl-2-butenal (α-ethylidene phenylacetaldehyde), 2-methylbutyrophenone, and carvacrol.

Phenylacetaldehyde is a common honey constituent (*22, 23, 25, 30, 31, 36, 37*), with a pleasant honey-like, floral odor. It was found in all honeys analyzed (**Table 2**), yet thyme honey contained significantly higher proportions than other unifloral honeys ($F = 12.282$, $p < 0.001$). Our results are in agreement with earlier findings on Greek thyme honey (*33*).

1-Phenyl-2,3-butanedione was first reported in honey as a component of Australian blue gum and yellow box (*12*). 3-Hydroxy-4-phenyl-2-butanone has been isolated from honey before (*1, 12, 32, 38*), including unifloral Greek thyme (*33*). It has a low odor threshold (75–100 ng) and an intense floral-sweet odor (*39*). 3-Hydroxy-1-phenyl-2-butanone was isolated as a food component for the first time from dry fino sherry (*39*). These two components seem to occur in foodstuff together, as in the case of dry fino sherry, and the absence of 3-hydroxy-1-phenyl-2-butanone where 3-hydroxy-4-phenyl-2-butanone was identified was probably due to small proportions or coelution matters. 3-Hydroxy-4-phenyl-3-buten-2-one is a possible precursor of 3-hydroxy-4-phenyl-2-butanone. Phenylacetonitrile has been reported in various honeys, including thyme (*24, 31, 33*). It has been claimed to characterize Greek thyme together with 1-phenyl-2,3-butanedione and increased concentrations of phenylacetaldehyde (*33*). **Table 2** lists the potent botanical marker compounds of thyme honey and their concentrations in other unifloral Greek honeys. The presence of low proportions of most of the impact thyme components in cotton and pine honeys is due to the simultaneous blossoming of cotton and thyme flowers and the secretions of pine honeydew, which leads—in some cases—to the contribution of thyme nectar to the production of cotton and pine honeys.

Referring to other compounds of interest, phenylethyl alcohol is a common honey compound and increased proportions are found in thyme honey (*24*). Carvacrol is a primary essential oil component of thyme as well as of other Lamiaceae species (*40*).

Table 1. Volatile Compounds Isolated from the Headspace of Thyme Honey by Means of SPME

no.	compound	KI[a]	ID[b]	avg[c]	min	max	%[d]	% S[e]
1	octane	800	MS, RT, KI	10	3	57	0.1	100
2	furfural	848	MS, KI	125	17	607	1.0	100
3	isovaleric acid	875	MS, KI	45	6	359	0.3	100
4	2-methylbutanoic acid	894	MS, KI	14	0	255	0.1	32
5	benzaldehyde	966	MS, RT, KI	670	126	2673	5.8	100
6	6-methyl-5-hepten-2-one	991	MS, KI	2	0	23	0.1	32
7	octanal	1005	MS, RT, KI	78	25	217	0.6	100
8	*p*-cymene	1027	MS, RT, KI	18	2	90	0.1	100
9	phenylacetaldehyde	1049	MS, RT, KI	4546	659	16515	32.9	100
10	acetophenone	1068	MS, RT, KI	66	8	667	0.5	100
11	*trans*-furanoid linaloxide	1076	MS, RT, KI	10	0	231	0.1	14
12	*cis*-furanoid linaloxide	1091	MS, RT, KI	2	0	41	0.1	7
13	cymenene	1092	MS, KI	29	0	437	0.3	89
14	undecane	1100	MS, RT, KI	8	2	25	0.1	100
15	linalool	1103	MS, RT, KI	6	0	38	0.1	32
16	nonanal	1105	MS, RT, KI	389	38	2249	3.6	100
17	hotrienol	1109	MS	32	0	607	0.1	29
18	phenylethyl alcohol	1116	MS, RT, KI	517	21	2965	4.7	100
19	isophorone	1124	MS, RT, KI	46	0	1114	0.5	25
20	methyl octanoate	1128	MS, RT, KI	58	13	284	0.5	100
21	phenylacetonitrile	1143	MS	508	19	3797	4.8	100
22	lilacaldehyde isomer	1146	MS	8	0	224	0.1	4
23	lilacaldehyde isomer	1154	MS	21	0	456	0.2	14
24	2-phenylpropenal	1161	MS	17	0	61	0.1	54
25	lilacaldehyde isomer	1169	MS	10	0	235	0.1	7
26	1-nonanol	1186	MS, RT, KI	59	5	582	0.5	100
27	methyl phenylacetate	1187	MS	2	0	15	0.1	21
28	terpinen-4-ol	1187	MS, RT, KI	3	0	28	0.1	21
29	octanoic acid	1191	MS, RT, KI	41	7	239	0.3	100
30	benzoic acid	1193	MS, RT, KI	3	0	91	0.1	4
31	α-terpineol	1195	MS, RT, KI	17	0	52	0.1	71
32	methyl salicylate	1197	MS, RT, KI	6	0	59	0.1	25
33	decanal	1208	MS, RT, KI	260	70	644	2.1	100
34	1-phenylbutane-2, 3-dione	1211	MS	1280	58	5095	13.5	100
35	methyl nonanoate	1227	MS, RT, KI	155	34	956	1.3	100
36	ethyl phenylacetate	1252	MS	13	0	99	0.1	32
37	*p*-anisaldehyde	1258	MS, RT, KI	5	0	75	0.1	11
38	2-decenal	1266	MS, KI	9	0	33	0.1	32
39	2-phenyl-2-butenal	1281	MS	69	37	1044	0.3	100
40	2-methylbutyrophenone	1283	MS	85	66	383	0.9	100
41	nonanoic acid	1297	MS, RT, KI	103	30	344	0.9	100
42	thymol	1306	MS, RT, KI	10	0	83	0.1	46
43	1-nitro-2-phenylethane	1307	MS	7	0	62	0.1	21
44	undecanal	1310	MS, RT, KI	10	0	48	0.1	32
45	carvacrol	1312	MS, RT, KI	114	16	427	0.9	100
46	methyl decanoate	1328	MS, RT, KI	67	25	181	0.5	100
47	3,4,5-trimethylphenol	1331	MS	17	0	396	0.2	11
48	3-hydroxy-4-phenyl-2-butanone	1348	MS, KI	1370[f]	129	6067	14.7	100
49	3-hydroxy-1-phenyl-2-butanone	1351	MS	1370[f]	129	6067	14.7	100
50	1,2-dihydro-1,1,6-trimethylnaphthalene	1355	MS	158	0	4330	1.8	14
51	eugenol	1359	MS, RT, KI	14	0	68	0.1	43
52	4-methoxyphenethyl alcohol	1374	MS, RT, KI	17	0	196	0.2	32
53	decanoic acid	1387	MS, RT, KI	27	11	116	0.2	100
54	(*E*)-β-damascenone	1388	MS, RT, KI	26	4	57	0.2	100
55	dodecanal	1412	MS, RT, KI	11	7	40	0.1	100
56	3-hydroxy-4-phenyl-3-buten-2-one	1433	MS	273	77	1125	3.0	100
57	geranyl acetone	1458	MS	22	13	74	0.2	100
58	5-methyl-2-phenyl-2-hexenal	1483	MS	34	0	544	0.3	29
59	methyl dodecanoate	1527	MS, RT, KI	21	8	49	0.2	100
60	hexadecane	1600	MS, RT, KI	7	2	19	0.1	100
61	3,4,5-trimethoxybenzaldehyde	1608	MS	37	17	142	0.2	100
62	internal standard (benzophenone)	1664						
63	heptadecane	1700	MS, RT, KI	11	5	17	0.1	100
	total			13198	4952	30350	99.7	

[a] KI values were calculated using the hydrocarbons naturally present in honey. [b] Method of identification: MS, identification by comparison with stored MS data in NIST98 & Wiley275 MS libraries; RT, identification by comparison of retention tomes with those of reference compounds; KI, identification by comparison of Kovats index with the literature. [c] The min, max, and avg values (ng/kg honey) refer to the quantification against the internal standard. [d] % refers to the percentage against the total peak area. [e] % of samples the compound was found. [f] These two compounds were quantified together due to coelution matters.

It has been isolated from various honeys before (*3, 38, 41*) and cited as characteristic of lime tree honey (*24*). 3,4,5-Trimethoxybenzaldehyde has been traced in honey before (*22, 24, 32*) and is considered as characteristic of thyme honey (*30, 31*).

In an attempt to establish possible connections between the primary components of thyme honey, very good positive correlation was found between the concentrations of 3-hydroxy-4-phenyl-2-buten-3-one and the isomers 3-hydroxy-4-phenyl-

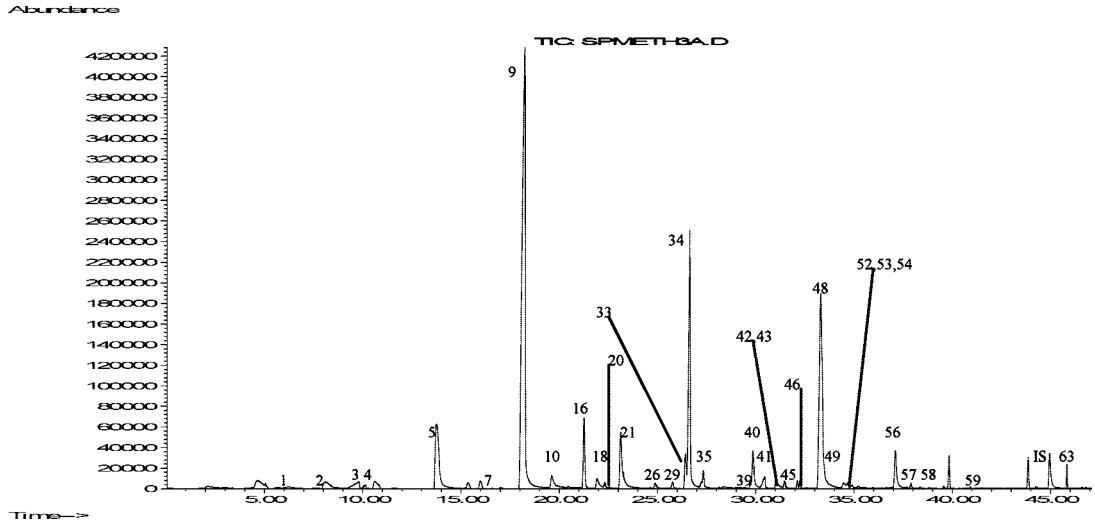

Figure 1. Representative total ion chromatogram of an aroma isolate from thyme honey.

Table 2. Concentrations of the Characteristic Compounds of Thyme Honey in Comparison with Concentrations in Other Unifloral Greek Honeys[a]

no.	compound	Th ($n = 28$)	Or ($n = 33$)	Ct ($n = 7$)	Ht ($n = 4$)	Ch ($n = 3$)	Eu ($n = 3$)	Pn($n = 5$)	Fr ($n = 6$)
9	phenylacetaldehyde	4565	344	350	166	77	25	710	126
21	phenylacetonitrile	508		7				7	
34	1-phenyl-2,3-butanedione	1280		50				37	
39	2-phenyl-2-butenal	69							
40	2-methylbutyrophenone	85							
45	carvacrol	114						4	
48	3-hydroxy-4-phenyl-2-butanone	1370		2				36	
49	3-hydroxy-1-phenyl-2-butanone	1370		2				36	
56	3-hydroxy-4-phenyl-2-buten-3-one	273		5				2	

[a] In ng/kg honey.

2-butanone and 3-hydroxy-1-phenyl-2-butanone ($R^2 = 0.849$), 3-hydroxy-4-phenyl-2-buten-3-one and 1-phenyl-2,3-butanedione ($R^2 = 0.910$), and the isomers and 1-phenyl-2,3-butanedione ($R^2 = 0.872$). These four components possibly share the same precursor. It is of interest to point out that none of these substances have been traced in the essential oil of thyme species. Moreover, 3-hydroxy-4-phenyl-2-butanone was found in ripe and unripe leatherwood honey, but not in the floral nectar. This component is formed from phenylpyruvic acid, in the presence of pyruvate decarboxylase (*39, 42*). It is possible that these four components are formed from phenylalanine, the most abundant amino acid found in honey, along with proline (*43*).

Geographical Discrimination. As mentioned before, in Greece 23 different thyme species are found, *Corydothymus capitatus* being the most widespread. Moreover, within a certain species of thyme different chemotypes exist. For example, seven chemotypes have been described for *Thymus vulgaris* in France (*44*). Additionally, significant variability within *C. capitatus* essential oil was found in Crete, Greece, attributed to the microclimate of the area the plants were collected from (*45*). These facts provide a satisfactory explanation for the variation of the concentrations observed in **Table 1**.

The thyme honey samples were from four different regions of Greece: Crete, Leros, Kalumnos, and Kos. Analysis of variance showed statistically significant differences ($p < 0.05$) for the average concentrations of seven compounds. Phenylacetaldehyde ($p = 0.009$), acetophenone ($p = 0.001$), and octanoic acid ($p = 0.006$) were found at higher proportions in samples from Crete, while carvacrol was more abundant in samples from Kalumnos ($p = 0.004$). Honeys from Kos contained more phenylethyl alcohol than those from Crete and Leros ($p = 0.019$), those from Leros more nonanal than samples from Kalumnos and Kos ($p = 0.037$), and, finally, honeys from Crete had lower concentrations of hexadecane than those from Leros and Kalumnos ($p = 0.041$).

Statistical analysis was performed using the concentrations of those compounds isolated from all the samples of at least one region. A total of 45 variables were employed. At first, principal component analysis was performed to the data matrix with concentrations and six components were extracted, explaining 85.4% of the total variance. The first component explained 46.2% of the total variance and was positively correlated to phenylacetaldehyde, nonanoic acid, acetophenone, decanoic acid, benzaldehyde, phenylacetonitrile, isophorone, and nonanal. The second component (13.6%) was positively correlated to the concentrations of the aliphatic compounds nonanal, dodecanal, hexadecane, and heptadecane. The third component (10.8%) is characterized by the high positive loadings of carvacrol, while the fourth (7.6%) and fifth (7.3%) components are negatively correlated with phenylethyl alcohol and 5-methyl-2-phenyl-2-hexenal, respectively.

The principal components were then used as variables to the discriminant analysis. The first three canonical functions were used in the analysis, two of which was statistically significant. The first function explained 62.6% and the second 21.9% of the total variance (total variance explained 84.3%). **Table 3** shows the discriminant function values at the group centroids. The first function discriminates the samples of Crete from those of the other locations, especially from Kalumnos and Kos. The second function is discriminant for the samples of Leros, especially from those from Crete and Kalumnos.

The graphic representation of discriminant analysis is demonstrated in **Figure 2**. A leave-one-out classification test was performed on the data and 85.71% of cross-validated grouped

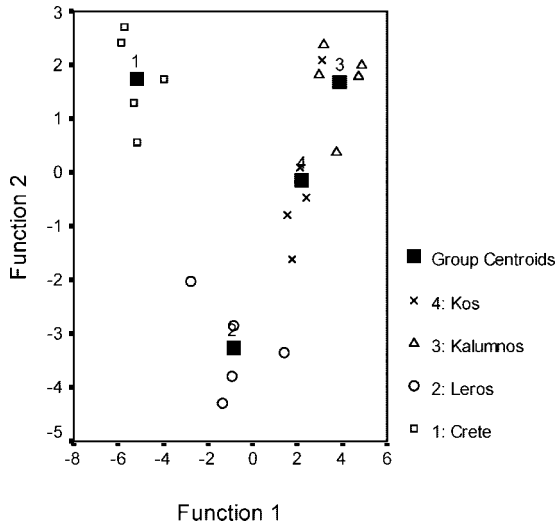

Figure 2. Plot of the canonical discriminant functions. Area codes: (1) Crete, (2) Leros, (3) Kalumnos, (4) Kos.

Table 3. Discriminant Function Values at Group Centroids

	function	
regions	1	2
Crete	−5.200	1.744
Leros	−0.888	−3.266
Kalumnos	3.891	0.668
Kos	2.197	−0.146

Table 4. Classification Results of Leave-On-Out Classification Test

	predicted group				
	Crete	Leros	Kalumnos	Kos	% correct
Crete	8	0	0	0	100
Leros	0	5	1	0	83.33
Kalumnos	0	1	5	1	71.43
Kos	0	0	1	6	85.71
total	8	6	7	7	85.71

cases were correctly classified (**Table 4**). The misclassified samples were from Leros, Kalumnos, and Kos, and this can be attributed to the very close geographical position of these three regions. Samples from Crete were all correctly grouped. These results are promising and more samples should be analyzed to establish a discrimination model for these geographical regions.

SPME-GC-MS is a suitable technique for the isolation of headspace volatile compounds from unifloral Greek thyme honey. Some compounds are possible botanical markers, mainly 1-phenyl-2,3-butanedione, along with the acyloins 3-hydroxy-4-phenyl-2-butanone and 3-hydroxy-1-phenyl-2-butanone, phenylacetonitrile, carvacrol, and high proportions of phenylacetaldehyde. Even though the number of samples is small, the results show good potential for the geographical discrimination of honeys from different locations in Greece. This is crucial for local beekeeper corporations on their effort to produce honey with geographical origin label.

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