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Volatile Profile, Pigment Content, and *In Vitro* Radical Scavenging Activity of Flower, Thyme, and Fir Honeys Produced in Hellas

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Abstract

The philosophy of the present work was to investigate the volatile profile, pigment content, and *in vitro* radical scavenging activity of Hellenic honeys, using aqueous honey solutions. Twenty-one honey samples representing flower, thyme and fir honeys were donated from professional beekeepers during two consecutive harvesting years, from three different regions in Hellas.

Volatile profile was estimated using headspace solid phase micro extraction coupled to gas chromatography/mass spectrometry (HSPME-GC/MS). For semi-quantification purposes the internal standard method was used. Pigment content along with the *in vitro* radical scavenging activity was determined using spectrophotometric methods.

Results showed that the twenty-one honeys analyzed exhibited variations in volatile profile, pigment content and antioxidant activity depending on the floral source ($p<0.05$). Honey aroma was found to be the outcome of the combination of different class compounds, namely: carboxylic acids, alcohols, aldehydes, ketones, benzene derivatives, etc. The most pronounced aromas were recorded for flower honey, whereas fir honey possessed the highest pigment content and radical scavenging activity ($p<0.05$). In addition, specific volatiles (2-hydroxy-benzoic acid methyl ester, alpha-pinene, para-cymene, and delta-3-carene) were perfectly correlated (Pearson's correlation coefficient $r = 1$) with radical scavenging activity ($p<0.01$).

The present results enhance the quality control and authenticity of Hellenic honey and may be of great interest to a wider audience including: consumers, honey industry, and honey researchers, at an international level.

Keywords

Honey, Aroma, Pigment content, Antioxidant activity, Authenticity

Abbreviations

HS-SPME: Headspace solid phase micro extraction; GC/MS: Gas chromatography/Mass spectrometry; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; RSA: Radical scavenging activity; VOCs: Volatile organic compounds; 2-(OH)-BAME: 2-hydroxy-benzoic acid methyl ester; DAEE: Decanoic acid ethyl ester; IS: Internal standard

Introduction

"Complex as the universe", could be a few words to describe honey, the product of *Apis mellifera* honeybees. Nectar from different flowers or honeydew secretions

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from conifer trees are all well exploited by honeybees for the production of honey.

Honeybee honey is a highly concentrated solution of a complicated mixture of several sugars. Water represents the second major constituent, whereas there are small amounts of other components, such as: minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes, and numerous volatile organic compounds (VOCs).

VOCs in honey originate from different biosynthetic pathways and contribute to the development of honey distinguishable aroma, which is greatly affected by botanical and geographical origin [1, 2]. Despite their presence in low proportions, VOCs could contribute to biomedical activity of honey due to their natural radical scavenging activity potential [1].

As a multi plant-based foodstuff, honey contains certain bioactive compounds (phenolic acids, flavonoids, carotenoids, enzymes, free amino acids, ascorbic acid, etc), as aforementioned, that may have specific functions within the human body which helps maintain and promote health or prevent the body from several diseases [3, 4].

On the other hand, there is a great tendency for simple, fast, non-expensive, and eco-friendly methodologies to be applied on the quality control of agricultural products [1].

HS-SPME/GC-MS is a fast, simple and well developed technique that may provide useful information on the headspace aroma of untreated or low treated foodstuffs, while the volatile profile of the determined compounds is closely associated with that of sensory perception [1, 5, 6].

DPPH method serves as a good monitor of the *in vitro* radical scavenging activity of antioxidant compounds being present in plant-based food matrices [7]. It may be characterized as a fast, reproducible spectrometric technique that has been used to estimate, among others, the radical scavenging activity of water- soluble antioxidants [8, 9]. It should also be stressed that, the *in vitro* radical scavenging activity of honey, depends greatly on its botanical origin [8, 9].

Based on the afore mentioned, the purpose of the present study was to evaluate volatile profile, pigment content and antioxidant activity of flower, thyme and fir honeys aqueous solutions, using less organic solvents and a low amount of untreated honey sample, by application of simple and fast modern techniques. To the best of our knowledge, there are limited research studies that enhance the potential of water-soluble antioxidants of different honey types produced in the Hellenic zone [9] this constituting a fair novelty.

Materials and Methods

Honey samples

Twenty-one honey samples were collected; 7 flower honeys from Samos Island, 12 thyme (*Thymus capitatus* (L.)) from Kos Island, and 2 fir honeydew honeys from Aitolokarnania region, during the harvesting years 2011 and 2012, from professional beekeepers. All honey samples were stored in glass containers and maintained at 4 ± 1 °C before analysis.

Reagents and solutions

Benzophenone was purchased from Sigma-Aldrich (Germany). Sodium chloride (NaCl), methanol, and acetate buffer (CH$_3$COONa*3H$_2$O) were purchased from Merck (KGaA, 64271, Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl [DPPH·], was purchased from Sigma-Aldrich (Germany). All aqueous honey solutions were prepared using distilled water.

HS-SPME-GC/MS analysis

Headspace volatile compounds were extracted from honey, using a divinyl benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber 50/30 µm (Supelco, Bellefonte, PA, USA). The fiber was conditioned by following the manufacturer's recommendations, prior analysis. Honey samples of 2 g dissolved in 2 mL of distilled water containing 0.20 g NaCl and 20 µL of internal standard (benzophenone, 100 µg/mL), were placed in 15 mL screw-cap vials equipped with PTFE/silicone septa. Each vial was maintained at 45 °C in a water bath under stirring at 600 rpm for the headspace extraction. A cross-shaped PTFE-coated magnetic stirrer (diameter 10 mm) (Semadeni, Ostermundigen–Bern, Switzerland) was placed inside the vials. The sample preparation conditions were: 15 min equilibration time, 30 min sampling time, 4 mL sample volume and 45 °C water bath temperature. The temperature of 45 °C was chosen in order to avoid enzymatic activity and keep at minimum the formation of thermal artifacts like furan derivatives, furfural, etc.

Honey samples analyzed, were prepared daily prior to HS-SPME-GC/MS analysis. Blank runs were carried out before sample analysis to make sure that there was no contamination that could cause memory effects, and thus, the collection of false results. Each sample was run in duplicate (n = 2).

Identification and semi-quantification of volatile compounds

GC/MS instrumentation and conditions

A GC unit (Agilent 7890 A) coupled to a MS detector (Agilent 5975) was used to analyze the prepared honey solutions. A DB-5MS (cross-linked 5% PH ME siloxane) capillary column, having dimensions of 60 m x 320 µm i.d., x 1 µm film thickness, was used, with helium as the carrier gas (purity 99.999%), at 1.5 mL/min flow rate. The temperature for the injector and MS-transfer line were maintained constant at 250 °C and 270 °C, respectively. The oven temperature was held at 40 °C for 3 min, and was further increased to 260 °C having a rate of 8 °C/min, for 6 min. Electron impact mass spectra were recorded at the mass range of 50–550. The ionization energy of the electron ionization system was 70 eV [2].

Mass spectral data processing

The identification of compounds was achieved using the Wiley 7, NIST 2005 mass spectral library. For the determination of linear retention indices a mixture of n-alkanes (C$_8$–C$_{20}$) dissolved in n-hexane, was employed. The standard mixture was purchased from Supelco (Bellefonte,

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PA, USA). Calculation of retention time indices was carried out for components eluting between n-octane and n-eicosane. Benzophenone (m/z = 182) was chosen as an internal standard since it did not naturally occur in any of the isolated compounds [10]. Volatile compounds having ≥ 85% similarity with the Wiley mass spectral library were tentatively identified using GC-MS spectra. For semi-quantification, compound content was expressed as µg/kg based on the ratio:

$$Volatiles\ (\mu g\,/\,kg) = \left(\frac{peak\ area\ of\ analyte}{peak\ area\ of\ internal\ standard} \right) \text{--------Eq. (1)}$$

In vitro estimation of radical scavenging activity (%RSA) of Hellenic honeys

Preparation of [DPPH·] standard solution

The free radical [DPPH·] standard solution was prepared according to Karabagias et al. [9].

Preparation of [DPPH·] calibration curve

This step is mandatory, in order to estimate the %decrease in [DPPH·] free radical absorbance (%RSA), % decrease in [DPPH·] free radical concentration, %[DPPH·] remaining of the mixture obtained by the addition of honey aqueous solution, when the reaction between free radical and honey antioxidant components reaches plateau (steady state). A calibration curve of different [DPPH·] concentrations, covering the range of 0-40 mg/L, versus the respective absorbance, was prepared according to Karabagias et al. [9].

Calculation of %RSA of Hellenic honeys

The *in vitro* radical scavenging activity of aqueous honey solutions was estimated using the [DPPH·] assay according to the method of Karabagias et al. [9]. The reaction was completed in 4 h (plateau). The absorbance at the plateau for each sample was measured at 517 nm using a UV/VIS Spectrometer (PerkinElmer, Lambda 25, USA).

Radical scavenging activity was calculated using the following equation:

$$\%RSA = \left(\frac{A_o - A_t}{A_o} \right) \times 100 \quad \text{----------------- Eq. (2),}$$

Taking into account that: A_0 is the initial absorbance of the [DPPH·] free radical standard solution and A_t is the absorbance of the remaining [DPPH·] free radical after the reaction with aqueous honey solution antioxidants, at steady state. Each sample was run in triplicate (n = 3). Methanol and acetate buffer (2:1, v/v) served as the blank [9].

Determination of pigment content of Hellenic honeys: Net absorbance ABS$_{450-720}$

Honey color is the outcome of a combination of numerous pigments with antioxidant properties [8]. Such pigments (carotenoids, flavonoids, etc.) are associated with honey floral origin. Pigment content, may be defined as the difference between spectrometric absorbance at 450 and 720 nm (ABS$_{450-720}$) and was used to evaluate the contribution of honey colored phytochemical to the total antioxidant capacity of honey aqueous solution. Aqueous honey solutions were prepared according to Karabagias et al. [9]. Results were expressed as absorbance units (mAU).

Statistical analysis

Mean values of the investigated parameters were subjected to T-test in order to test the existence of significant variations with respect to honey botanical origin ($p<0.05$). Pearson's correlation coefficient (r), was computed to estimate any kind of correlations between the investigated parameters at the confidence level $p<0.05$. Statistical treatment of data was performed using the SPSS v.22.0 statistics software [9].

Results and Discussion

Volatile profile of Hellenic honeys

The aroma of Hellenic honey is owed to the combination of numerous compounds including: alcohols, aldehydes, benzene derivatives, esters, hydrocarbons, ketones, norisoprenoids, etc. Volatile fraction of Hellenic honeys proved to be dependent on botanical origin (Tables 1-3). In particular, flower honeys possessed a richer volatile pattern compared to thyme or fir honeys. Respective total volatile content (µg/kg), calculated from the average of each compound presented in Tables 1-3, followed the order: flower honey (55.19 µg/kg) > thyme honey (31.24 µg/kg) > fir honey (24.11 µg/kg). A typical gas chromatogram of fir honey from the region of Aitolokarnania is shown in Figure 1.
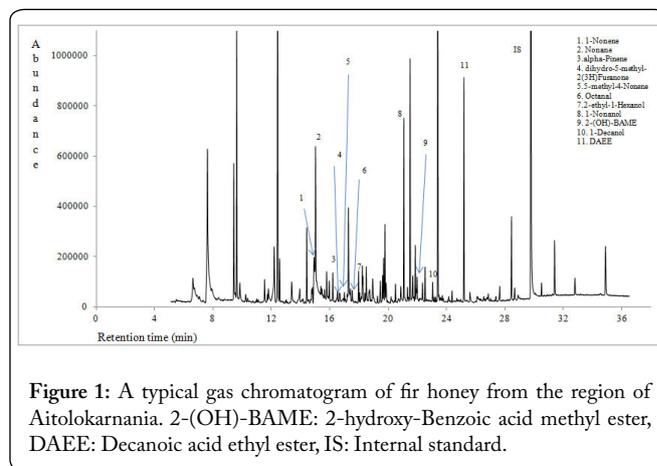
Figure 1: A typical gas chromatogram of fir honey from the region of Aitolokarnania. 2-(OH)-BAME: 2-hydroxy-Benzoic acid methyl ester, DAEE: Decanoic acid ethyl ester, IS: Internal standard.

Numerous volatile compounds identified in the present study such as: carboxylic acids (acetic acid, 2-methyl-propanoic acid, pentanoic acid, decanoic acid), alcohols (2-ethyl-1-hexanol, 1-nonanol, 1-decanol, etc.), linear aldehydes (heptanal, octanal, nonanal, decanal, etc.), benzene derivatives (methyl-benzene, 1,3-dimethyl-benzene, 1-methyl-4-(1-methylethyl)-benzene, 2,2-diethoxyethyl-benzene, benzeneacetonitrile, benzenemethanol, benzeneethanol, etc.), esters (formic acid ethyl ester, hexanoic acid ethyl ester, octanoic acid ethyl ester, nonanoic acid ethyl ester, decanoic acid ethyl ester, etc.), hydrocarbons (octane, 1-nonene, nonane, 5-methyl-4-nonene, undecane), ketones (1-2-furanyl-ethanone), norisoprenoids (2,6,6-trimethyl-2-cyclohexene-1,4-dione), terpenoids (alpha-pinene, 2-beta-pinene, dL-limonene, etc.), and furan derivatives (2,5-diethyltetrahydro-furan, 3-phenyl-furan2,5-dimethyl-3-ethyl-4-acetyl-furan), have been reported

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previously to enhance the volatile profile of several honey types produced in different countries [2, 5, 6, 10, 11-15].

In particular, acetic, 2-methyl-propanoic, pentanoic, and decanoic acids were identified only in thyme honey samples

Table 1: Volatile compounds (VOCs, μg/kg) identified in flower honeys from Samos Island.

RT	KI	VOCs	Average	± SD	Method of identification
12.17	<800	1-Octene	4.67	3.47	MS
12.40	800	Octane	95.89	81.32	MS/KI
13.37	837	2-Furancarboxaldehyde	16.63	23.99	MS/KI
14.48	879	1,3-dimethyl- Benzene	3.51	5.40	MS/KI
14.79	892	1-Nonene	2.84	1.81	MS/KI
15.00	900	Nonane	25.56	18.71	MS/KI
15.10	904	para-Xylene	6.72	6.67	MS/KI
16.18	949	alpha-Pinene	39.09	25.74	MS/KI
16.86	978	Benzaldehyde	11.86	11.20	MS/KI
17.25	994	Hexanoic acid ethyl ester	12.17	7.82	MS/KI
17.33	997	2-beta-Pinene	9.31	7.53	MS/KI
17.51	1005	Octanal	33.14	16.06	MS/KI
17.94	1025	delta-3-Carene	17.34	9.40	MS/KI
18.01	1028	2-ethyl-1-Hexanol	1.41	1.37	MS/KI
18.25	1038	1-methyl-4-(1-methylethyl)- Benzene	25.19	11.09	MS/KI
18.37	1044	dL-Limonene	3.47	2.50	MS/KI
18.70	1059	Benzeneacetaldehyde	14.06	13.30	MS/KI
19.75	1107	Nonanal	186.19	85.87	MS/KI
20.82	1160	2,6,6-trimethyl-2-Cyclohexene-1,4-dione	1.44	1.42	MS/KI
21.04	1170	1-Nonanol	18.60	15.94	MS/KI
21.47	1191	Octanoic acid, ethyl ester	46.98	16.87	MS/KI
21.82	1209	Decanal	135.96	75.31	MS/KI
21.98	1216	2-hydroxy- Benzoic acid methyl ester	6.49	3.75	MS/KI
23.37	1290	Nonanoic acid ethyl ester	99.28	35.97	MS/KI
23.76	1311	Undecanal	4.23	3.02	MS/KI
25.15	1389	Decanoic acid ethyl ester	63.14	17.73	MS/KI
25.56	1413	Dodecanal	4.33	2.06	MS/KI
26.83	1489	Undecanoic acid ethyl ester	1.78	2.04	MS/KI
26.98	1498	Pentadecane	2.18	1.00	MS/KI
28.41	1588	Dodecanoic acid ethyl ester	17.40	8.01	MS/KI
34.87	1983	Tridecanoic acid ethyl ester	7.41	7.13	MS/KI

RT: Retention time (min), **KI**: Experimental Kovats index values using hydrocarbons being naturally present in honey. Volatile compounds are given with respect to retention time. Average±standard deviation values of seven (N = 7) floral honey samples expressed as μg/κg of honey. Method of identification: MS, identification by comparison with MS data in Wiley 7 NIST 2005 mass spectral library. **KI**: identification by comparison of Kovats index with the literature cited or included in the Wiley library. Comparison of values using T-test at the confidence level *p<0.05*.

Table 2: Volatile compounds (VOCs, μg/kg) identified in thyme honeys from Kos Island.

RT	KI	VOCs	Average	± SD	Method of identification
5.20	<800	Formic acid, ethyl ester	2.50	1.72	MS
7.03	<800	Acetic acid	17.50	13.42	MS
7.58	<800	2-methyl- Propanenitrile	4.16	3.27	MS
8.47	<800	3-methyl- Butanal	1.57	1.13	MS
10.81	<800	2-methyl- Propanoic acid	7.16	3.38	MS
11.70	<800	methyl- Benzene	14.00	8.75	MS
12.40	800	Octane	21.57	19.92	MS/KI
13.02	823	Pentanoic acid	12.14	8.09	MS/KI
13.26	833	2-methyl- Butanoic acid	2.04	2.90	MS/KI
13.37	836	2-Furancarboxaldehyde	46.05	11.82	MS/KI
15.01	852	2,5-diethyltetrahydro-Furan	21.80	22.19	MS/KI
15.08	903	Heptanal	2.11	3.93	MS/KI
15.37	914	1-(2-furanyl)-Ethanone	1.37	1.55	MS/KI
16.18	948	alpha-Pinene	3.03	4.42	MS/KI
16.87	977	Benzaldehyde	148.23	33.08	MS/KI
17.39	1000	Decane	3.20	3.15	MS/KI
17.51	1005	Octanal	16.48	5.56	MS/KI
17.94	1025	delta-3-Carene	5.32	5.42	MS/KI
18.01	1027	2-ethyl-1-Hexanol	2.21	2.41	MS/KI
18.24	1038	1-methyl-4-(1-methylethyl)- Benzene	18.45	7.09	MS/KI
18.39	1044	Benzenemethanol	2.67	2.74	MS/KI
18.71	1059	Benzeneacetaldehyde	749.68	398.87	MS/KI
19.59	1100	Undecane	3.89	4.56	MS/KI
19.75	1107	Nonanal	36.83	21.55	MS/KI
20.21	1129	Benzeneethanol	93.45	33.85	MS/KI
20.71	1154	Benzeneacetonitrile	63.45	31.86	MS/KI
21.47	1191	Octanoic acid ethyl ester	7.63	5.63	MS/KI
21.82	1209	Decanal	28.31	8.88	MS/KI
22.56	1248	3-phenyl- Furan	5.28	7.74	MS/KI
22.65	1253	Benzeneacetic acid ethyl ester	9.02	8.65	MS/KI
23.35	1288	alpha,-ethylidene- Benzeneacetaldehyde	6.86	10.05	MS/KI
23.68	1306	2-methyl-5-(1-methylethyl)- Phenol	4.76	5.25	MS/KI
24.06	1328	2,2-diethoxyethyl- Benzene	17.92	12.43	MS/KI
24.43	1348	Decanoic acid	8.73	5.07	MS/KI
25.37	1401	beta-Damascenone	4.48	4.03	MS/KI
26.61	1475	Propanedioic acid, -ethoxymethylene- diethyl ester	1.14	1.35	MS/KI
27.98	1561	2,5-dimethyl-3-ethyl-4-acetyl-Furan	2.45	3.64	MS/KI

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28.42	1588	Dodecanoic acid ethyl ester	1.38	2.38	MS/KI

RT: Retention time (min), **KI**: Experimental Kovats index values using hydrocarbons being naturally present in honey. Volatile compounds are given with respect to retention time. Average ± standard deviation values of seven (N = 15) thyme honey samples expressed as μg/κg of honey. Method of identification: MS, identification by comparison with MS data in Wiley 7 NIST 2005 mass spectral library/ KI, identification by comparison of Kovats index with the literature cited or included in the Wiley library. Comparison of values using T-test at the confidence level $p<0.05$.

from Kos Island. This finding is in conformity with previous works on Italian, Spanish and Hellenic thyme honeys, respectively [2, 5, 6, 11].

2-ethyl-1-hexanol was found in higher amounts (μg/kg) in fir honeys from Aitolokarnania compared to thyme honeys from Kos Island or flower honeys from Samos Island. The same holds for nonanol which was found at a content (μg/kg) ca. 3 times fold higher in fir honeys, compared to flower honeys (Table 1 and 3). Finally, decanol was identified only in fir honeys from Aitolokarnania and may be indicative of this particular honey type [2].

Table 3: Volatile compounds (VOCs, μg/kg) identified in fir honeys from Aitolokarnania.

RT	KI	VOCs	Average	± SD	Method of identification
14.80	892	1-Nonene	7.57	0.61	MS/KI
15.01	900	Nonane	79.19	10.16	MS/KI
16.19	949	alpha-Pinene	16.97	8.44	MS/KI
16.41	959	dihydro-5-methyl-2(3H)-Furanone	3.36	3.33	MS/KI
16.99	982	5-methyl-4-Nonene, (Z)	19.99	24.02	MS/KI
17.53	1005	Octanal	30.54	37.54	MS/KI
18.02	1028	2 -ethyl-1-Hexanol	8.04	7.12	MS/KI
21.07	1170	1-Nonanol	53.58	13.31	MS/KI
21.98	1216	2-hydroxy- Benzoic acid methyl ester	10.16	4.02	MS/KI
23.05	1273	1-Decanol	10.00	2.83	MS/KI
25.19	1389	Decanoic acid ethyl ester	47.80	41.30	MS/KI

RT: Retention time (min), **KI**: Experimental Kovats index values using hydrocarbons being naturally present in honey. Volatile compounds are given with respect to retention time. Average ± standard deviation values of two (N =2) fir honey samples expressed as μg/κg of honey. Method of identification: MS, identification by comparison with MS data in Wiley 7 NIST 2005 mass spectral library **KI**: identification by comparison of Kovats index with the literature cited or included in the Wiley library. Comparison of values using T-test at the confidence level $p<0.05$.

Octanal, nonanal, and decanal were identified in higher amounts (μg/kg) in flower honeys from Samos Island compared to thyme honeys from Kos Island, or fir honeys from Aitolokarnania. The aforementioned aldehydes have been reported previously to enhance the volatile fraction of Spanish [2, 5, 6, 10], and Hellenic thyme honey [2], or even in Turkish pine honey [16] The lower content of octanal, nonanal

and decanal in fir honeys (dark colored honeys) as compared to flower or thyme honeys, is in agreement with the results reported by Petretto et al. [17] for Moroccan dark colored honeys. It should also be mentioned that, undecanal and dodecanal were detected only in flower honeys from Samos Island.

Phenolic volatiles with sweet or honey like aroma such as benzaldehyde and benzeneacetaldehyde were identified in much higher proportions (μg/kg) in thyme honeys from Kos Island compared to flower honeys from Samos Island, whereas such compounds were absent in fir honeys from Aitolokarnania region. This observation may be indicative of both: botanical and geographical origin aspects. Benzaldehyde and benzeneacetaldehyde have been reported previously to enhance the volatile fraction of Italian [11], Spanish [5, 6], Hellenic [2, 10], and Turkish thyme honeys [15].

Para-cymene (1-methyl-4-(1-methylethyl)-benzene) was identified at higher amounts (μg/g) in flower honey from Samos Island compared to thyme honey from Kos Island, whereas it was not detected in fir honey from Aitolokarnania. This compound has been reported previously to enhance the volatile pattern of Hellenic thyme honeys [2], whereas it was not identified in Spanish thyme or blossom honeys [6].

Benzenemethanol and benzeneethanol (or 2-phenylethanol) were identified only in thyme honeys from Kos Island. These compounds have been reported previously to enhance the volatile fraction of Spanish thyme honeys [6].

Hexanoic, octanoic, nonanoic, decanoic, dodecanoic, and tridecanoic acid ethyl esters were detected in high amounts (μg/kg) in flower honeys from Samos Island compared to thyme or fir honeys (Tables 1-3). However, formic acid ethyl ester, ethoxymethylene-propanedioic acid diethyl ester and benzene acetic acid ethyl ester (ethyl phenyl acetate) were detected only in thyme honeys from Kos Island. These ethyl ester derivatives have not been reported previously, to enhance the volatile pattern of Spanish thyme honeys [5, 6].

Beta-damascenone, a cyclic carotenoid derivative, was identified only in thyme honeys which conform to previous work involving Hellenic thyme honeys [2]. Furthermore, beta-damascenone has been reported previously to enhance the volatile fraction of Spanish artisanal and eucalyptus honeys, respectively [14, 18]. It is characterized by a fruity, spicy and typical honey aroma [14].

With respect to furan derivatives, 2,5-diethyltetrahydro-furan, 1-(2-furanyl)-ethanone, 3-phenyl-furan, and 2,5-dimethyl-3-ethyl-4-acetyl-furan were identified in the volatile fraction of thyme honeys from Kos Island, whereas dihydro-5-methyl-2(3H)-furanone was detected only in the volatile fraction of fir honeys from Aitolokarnania region.

Soria et al. [5] reported that dihydro-5-methyl-2(3H)-furanone enhanced the volatile fraction of Spanish eucalyptus honey. Furan related compounds usually serve as indicators of heat treatment and storage conditions [11]. Similar compounds have been reported previously to contribute to the volatile profile of Italian [11] and Spanish thyme honeys [5, 6].

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Isophorone related volatiles (i.e. 2,6,6-trimethyl-2-cyclohexene-1,4-dione) were found in low amounts (1.44 μg/kg) in flower honeys from Samos Island. These compounds have been reported previously to enhance the volatile fraction of Spanish [5, 6] and Hellenic thyme honeys [2].

Finally, carvacrol [2-methyl-5-(1-methylethyl) phenol], was detected only in the volatile fraction of thyme honeys from Kos Island. Carvacrol may not be considered as an undisputed volatile marker of thyme honey since it has been previously identified only in traces in the volatile fraction of Spanish thyme honeys [6], or it was absent in the volatile fraction of Italian thyme honeys [11]. However, this volatile compound may be indicative of thyme honey production area.

VOCs in honey serving as a biomonitor of environmental pollution

Honey could serve as a biomonitor of environmental pollution due to anthropogenic activities. Indeed, the use of SPME-GC/MS on the headspace aroma of honey may provide information regarding specific volatiles that are pollutants [12]. The average ± SD values (μg/kg) of such compounds (data not shown) were: chloroform (5.86 ± 1.52), cyclohexane (5.96 ± 6.87), benzyl chloride (5.60 ± 5.89), and 1,1-dichloro-ethene (1.20 ± 0.95) for thyme honey from Kos Island; chloroform (6.59 ± 4.41), cyclohexane (2.91 ± 2.10) and 1,1-dichloro-ethene (3.76 ± 2.46) for flower thyme from Samos Island. Finally, in fir honeys from Aitolokarnania 1-bromo-napthalene was determined at 2.17 ± 3.06 μg/kg. Probably, the use of aerosol containing these compounds, could led to its deposition on flowers around the plant [12]. It should be noted that such pollutants have not yet been regulated by the official authorities worldwide.

Pigment content of Hellenic honeys

Pigment content (mAU) of honey samples analyzed followed the order: fir > flower > thyme (Figure 2 and Table 4). As it can be observed, dark colored honeys such as fir, possess a higher pigment content compared to light colored or nectar honeys (flower and thyme honeys). This is in conformity with the results of Beretta et al. [8], Bertoncelj et al. [19] and Karabagias et al. [9]. It should also be noted, that these differences were statistically significant ($p<0.05$), highlighting thus, the impact of floral source on pigment content of honey.
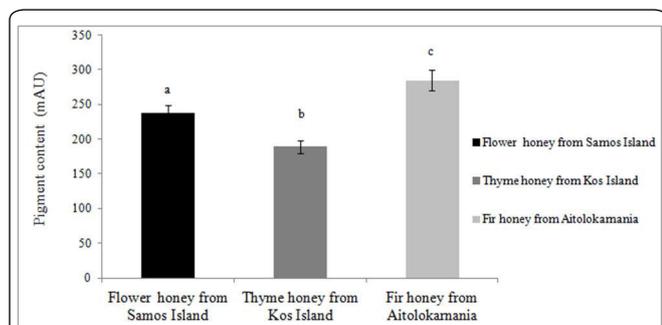
Figure 2: Pigment content (mAU) of Hellenic honeys according to botanical origin. Different lower case letters indicate statistically significant differences ($p<0.05$).

Radical scavenging activity (%RSA) of Hellenic honeys

%RSA values of honey samples analyzed followed the order: fir > flower > thyme (Table 4). The capacity of an aqueous honey solution to reduce the activity of [DPPH·] free radical is shown in Figure 3. As in the case of pigment content, fir honeys recorded higher %RSA values compared to flower or thyme honeys. This is also in agreement with previous work in the literature [8, 9, 20, 21].
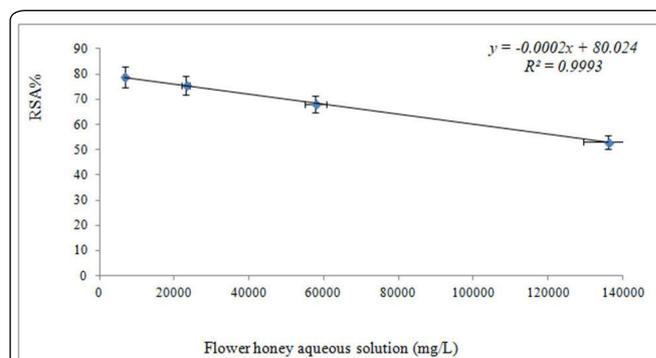
Figure 3: Radical scavenging activity (%RSA) of flower honey aqueous solution from Samos Island.

Table 4: Pigment content and *in vitro* radical scavenging activity of flower, thyme, and fir honeys from Hellas.

Geographical origin	Botanical origin	Pigment content (mAU)	%RSA
Samos Island	Flower honey (N=7)	237 ± 31	40.96 ± 5.40
Kos Island	Thyme honey (N=12)	189 ± 28	25.30 ± 9.47
Aitolokarnania	Fir honey (N=2)	285 ± 52	56.52 ± 9.22

Average ± standard deviation values of three measurements (n = 3). N: number of honey samples. Comparison of values using T-test at the confidence level *p<0.05*.

An excellent Pearson's correlation ($r = 1$) was obtained between pigment content and %RSA values of honey samples with respect to botanical origin ($p<0.01$).

Correlations between VOCs and %RSA

Pearson's correlation is defined by the coefficient *r*, which is an indicator of the degree of the linear relationship between two different parameters. A positive and high (close to 1) value of Pearson's correlation coefficient, adequately represents a linear relationship between the examined parameters. In that sense, the mean values of specific volatiles determined in flower vs. thyme, or thyme vs. fir honeys, were subjected to Pearson's correlation with their respective %RSA values. These compounds were: alpha-pinene, p-cymene, delta-3-carene and 2-OH-BAME.

Perfect Pearson's correlation ($r = 1$) was obtained for the sets: a) alpha-pinene-%RSA involving thyme and fir honeys (Tables 2-4), b) para-cymene-%RSA involving flower and thyme honeys (Table 1, 2, and 4), c) delta-3-carene-%RSA involving flower and thyme honeys (Tables 1, 2, and 4), and d) 2-OH-BAME-%RSA involving thyme and fir honeys (Tables 2-4).

To the best of our knowledge, this is the first report in the literature about such correlation.

Volatile Profile, Pigment Content, and In Vitro Radical Scavenging Activity of Flower, Thyme, and Fir Honeys Produced in Hellas

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Conclusion

Honey produced from different floral sources, may have definitely different aroma and taste, owed to differences in the volatile composition. VOCs not only contribute to the aroma of honey, but significantly influence the efforts on honey botanical and geographical origin identification. Information on the VOCs profile of honey, could aid in standardizing the quality of honey and contribute to its authenticity.

However, on the aforementioned efforts, specific geographical aspects including: weather conditions, soil fertilization and environmental purity, beekeepers practicing, honey extraction methods, etc., should be also considered.

In addition, the present study clearly demonstrates a strong impact of honey botanical origin on pigment content and radical scavenging activity ($p<0.05$). To the best of our knowledge, this is the first report in the literature associating honey volatiles namely: alpha-pinene, delta-3-carene, p-cymene, and 2-hydroxy-benzoic acid methyl ester with antioxidant properties ($p<0.01$).

Finally, this research was carried out in aqueous honey solutions and by using limited toxic solvents, providing thus, an eco-friendly scientific character.

Conflict of Interest

The authors declare that the research was conducted in the absence of any interests.

Acknowledgments

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