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ORIGINAL PAPER

Phenolic profile, colour intensity, and radical scavenging activity of Greek unifloral honeys

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Abstract The present study was designed to investigate the antioxidant properties [selected polyphenol content (SPC), radical scavenging activity] and colour intensity of Greek unifloral honeys. For this purpose, one hundred and seventy honey samples of different botanical origin (thyme, pine, orange blossom, and fir) were collected during the harvesting period 2011 and 2012 from thirteen different regions in Greece. The phenolic profile was evaluated based on the quantification of syringic acid, myricetin, quercetin, kaempferol, and chrysin using high performance liquid chromatography. Radical scavenging activity and colour intensity were estimated using spectrometric assays. Honey samples analysed showed variations in the SPC (3.86 ± 0.67 to 65.79 ± 8.08 mg/kg), colour intensity (164 ± 49 to 517 ± 110 mAU) and % radical scavenging activity (% RSA) (17.24 ± 5.50 to 50.38 ± 19.17), depending on botanical origin. On the basis of results obtained, the tested honey samples may be considered as easily accessible natural sources of antioxidants and valuable supplements to the daily diet.

Keywords Honey · Polyphenols · Radical scavenging activity · Colour intensity · Chemometrics

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Introduction

"Let food be thy medicine and medicine be thy food" was first voiced by Hippocrates, the father of modern medicine, nearly 2500 years ago, indicating that a food of sufficient nutritive value promotes and maintains health. Many plant-based foods contain bioactive compounds that may have specific functions within the human body that maintain health or prevent disease.

Naturally occurring compounds, known as phytochemicals, are thought to be largely responsible for the protective health benefits of these plant-based foods and beverages, beyond those conferred by their vitamin and mineral contents. Such phytochemicals, which are part of a large group of chemical compounds, also are responsible for the colour, flavour, and odour of many plant foods. Approximately ten thousands phytochemicals are already known and researchers speculate that there are likely many more that have not been identified yet in the foods we eat. Even though the broadest groups of phytochemicals such as flavonoids, isoflavones, or anthocyanidins, are often referred to as a homogenous group, it is well documented that individual compounds within each group are metabolized differently by the body and may have different health effects [1].

Honeybee honey is produced by Western or European honey bees (*Apis mellifera*) from carbohydrate-containing exudates produced by plants (blossom or nectar honeys) or from excretions of plant-sucking insects (*Hemiptera*) on the living parts of plants or secretions of living parts of plants (honeydew honeys). Honey is a highly concentrated solution of a complex mixture of sugars. It also contains small amounts of other nutrients such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other phytochemicals. The components which are responsible for the antioxidative activity in honey are flavonoids, phenolic acids, ascorbic acid, catalase, peroxidase, carotenoids,

and products of the Maillard reaction. The quantity of these components varies widely according to the botanical and geographical origin of honey. In addition, processing, handling, and storage of honey may influence its composition [2–5].

Over the past 15 years, a number of studies have reported enhanced antioxidant properties of human serum after honey consumption in vivo. These indicate that consumption of honey alone or in combination with other antioxidant beverages significantly increases the antioxidant capacity of human serum [6, 7]; honey has potential therapeutic value in the treatment of heart disease, cancer, antiatherogenic, cataracts, and several other inflammatory diseases as reported previously [8–11].

The scientific community has recently focused on determination of various honey components such as amino acids, protein content, trace elements, sugars, pollen, volatile compounds [12–15], as well as antioxidants [16–20] in an effort to evaluate product quality and authenticity.

In the EU honey regulation [21], it is stated that both botanical and geographical origin of the product must be declared on the package label. However, it does not mention any specific chemical marker(s) characteristic of the geographical and botanical origin or the antioxidant properties of honey.

More recently, the international honey commission (IHC) in its 2013 annual meeting [22] in Ukraine, set the need for the development of new techniques for characterizing and testing the authenticity of bee products (honey, beeswax, royal jelly).

Based on the above, the aim of the present work was to evaluate the phenolic profile, the antioxidant activity of Greek honeys, and pigment content, in an effort to highlight the prospective health benefits of unifloral honey through regular consumption. This was carried out by setting up a relatively simple procedure for: (a) the determination of radical scavenging activity of an aqueous honey solution and (b) the estimation of pigment content, along with the selected phenolic compounds determined in the ethyl acetate extract of honey.

To our knowledge, this is the first study in Greece using such a large number of honey samples, to highlight the potential of water-soluble antioxidants of Greek honeys as a source of food antioxidants in relation to honey botanical origin.

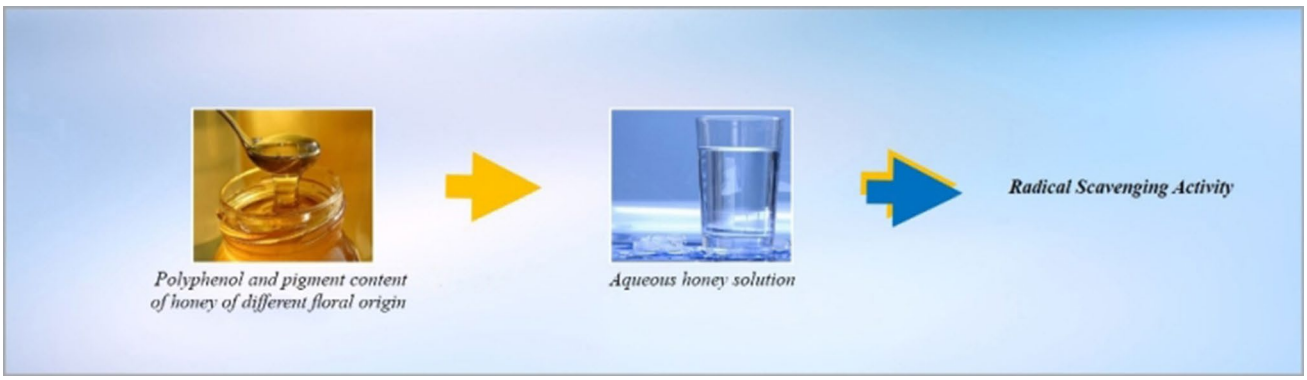
Experimental

Honey samples

One hundred and seventy unifloral honey samples were collected; 83 thyme [*Thymus capitatus* (L.)], 13 orange blossom [*Citrus sinensis* (L.)], 47 pine (*Pinus* spp.), and 27 fir (*Abies cephalonica* Loudon, *Pinaceae* or Greek fir) from ATTIKI Honey S.A. and local beekeepers' associations, during the harvesting periods 2011 and 2012, from 13 different regions in Greece (Irakleio, Hania, Kefalonia, Symi, Kos, Lakonia, Halkidiki, Thassos, Samos, Arta, Messinia, Arkadia, Karditsa) known to produce good quality honey, according to the standards set by the 2001/110/EC regulation [21]. All honey samples were stored in glass containers, shipped to the laboratory and maintained at 4 °C until analysis.

Reagents and solutions

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) ≥95 %, myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) ≥95 %, kaempferol (3,5,7,4′-tetrahydroxyflavone) ≥97 %, chrysin (5,7-dihydroxyflavanone) 97 %, syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid) ≥95 %, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and ethyl acetate anhydrous 99.8 % were purchased from Sigma-Aldrich (Germany). Methanol and acetate buffer ($CH_3COONa*3H_2O$) were purchased from Merck (KGaA, 64271, Darmstadt, Germany).



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

Preparation of DPPH free radical standard solution

A standard solution of DPPH 1.12×10^{-4} mol/L (M) was prepared by dissolving 0.0044 g of the radical DPPH in 100 mL methanol. The volumetric flask was wrapped in foil and stirred in a vortex apparatus. The solution obtained (pH 7.02) had a deep purple colour and was left in the refrigerator for 2 h in order to stabilize.

Preparation of DPPH free radical calibration curve

A calibration curve of concentration versus absorbance of DPPH was prepared as follows: The 1.12×10^{-4} M solution of DPPH was diluted with the addition of methanol. The resulting solutions were vortexed, left in the dark (until measurements were made), and their absorbance was measured in a UV/VIS Spectrometer (PerkinElmer, Lambda 25, USA) at λ_{max} of 517 nm. The calibration curve of absorbance (y) versus concentration (x) of [DPPH·] was expressed by the following equation:

$$y = 0.0243x - 0.0004;\ R^2 = 0.9985 \quad (1)$$

Parameters such as the % decrease in [DPPH·] free radical absorbance (% RSA), % decrease in [DPPH·] free radical concentration, % [DPPH·] remaining after the addition of the honey solution to the [DPPH·] radical at the point where the reaction reached plateau, were estimated using the above calibration curve.

Determination of radical scavenging activity of Greek unifloral honeys

The radical scavenging activity of honey samples was calculated in vitro using the DPPH assay according to the method of Beretta et al. [17] with some modifications. All honeys were dissolved in distilled water to obtain a concentration of 0.12 g/mL (solution simulating the daily consumption of 30 g of honey), equal to a soup spoon of honey dissolved in a glass of water (250 mL) (mother solution, (w/v)). Additionally, 1:2, 1:4, 1:10 dilutions were prepared from the mother solution in order to estimate concentrations of the honey solution causing 50 % inhibition of the DPPH free radical (EC_{50}). Volumes of 1.9 mL of methanol solution of [DPPH·] (0.044 mg/mL, 1.12×10^{-4} mol/L) and 1 mL of acetate buffer 100 mM (pH 7.10) were placed in a cuvette, and the absorbance of the [DPPH·] radical was measured at $t = 0$ (A_0).

Subsequently, 0.1 mL of each of the tested honey solutions was added to the above medium (final DPPH· concentration of 70.9 μmol/L) and the absorbance was measured every 30 min (regular time periods) until the absorbance value reached a plateau (steady state, A_t). The reaction was completed in 4 h. The absorbance of the reaction mixture was measured at 517 nm.

The [DPPH·] radical scavenging activity was calculated using the following equation:

$$\%\,\mathrm{RSA} = [A_0 - A_t/A_0] \times 100 \quad (2)$$

where A_0 is the initial absorbance of the DPPH free radical standard solution and A_t is the absorbance of remaining [DPPH·] free radical after reaction with honey antioxidants, at steady state (t, plateau). Each analysis was run in triplicate. For this antioxidant test, methanol and acetate buffer (2:1, v/v) were used as the blank. EC_{50} (concentration of the honey solution required to decrease the [DPPH·] concentration by 50 %) was estimated from graphs of the % [DPPH·] remaining free radical concentration = $[DPPH\cdot]_t/[DPPH\cdot]_0 \times 100$ (Fig. 1) versus concentrations of honey prepared dilutions 1:1, 1:2, 1:4, 1:10 (w/v).

At this point it should be mentioned that a model honey simulant containing the predominant sugars in honey: fructose and glucose did not exhibit any antioxidant activity [2, 17, 23].

Determination of selected polyphenol content (SPC) of Greek unifloral honeys

Syringic acid, myricetin, quercetin, kaempferol and chrysin were determined according to the validated method of Karabagias et al. [20].

Determination of colour intensity of Greek honeys: ABS_{450}

The colour of honey usually reflects the content of pigments with antioxidant properties (carotenoids, flavonoids, etc.). Such pigments are related to its botanical origin

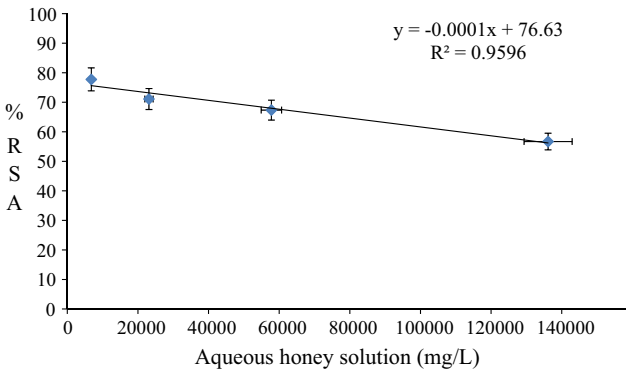


Fig. 1 Radical scavenging activity (% RSA) of thyme honey aqueous solution from Kos island

[17]. The colour intensity (ABS_{450}) was used to evaluate the contribution of coloured phytochemicals (carotenoids, flavonoids) to the overall antioxidant capacity of honey. Honey was diluted to a 50 % (w/v) with warm water (fixed temperature 45°C) (AREX Heating Magnetic Stirrer, VTF Digital Thermoregulator, VELP Scientifica, Italy) sonicated (*Elma*, *Elmasonic* model S 10H, Germany) for 5 min and filtered using Whatman filters (CAT. No. 6780-2504, UK) with a pore size of 0.45 μm, to remove any solid particles. The net absorbance was defined as the difference between spectrometric absorbance at 450 and 720 nm. Results were expressed as mAU [17].

Statistical analysis

In order to test any differences in parameter values determined in relation to differences in honey botanical origin, one-way analysis of variance (ANOVA) was conducted, for each one of the seven dependent variables, used in the analysis (syringic acid, myricetin, quercetin, kaempferol, chrysin, colour intensity, radical scavenging activity). The botanical origin was taken as the independent variable, with 4 levels (treatments) (thyme, orange blossom, pine and fir). The adjusted model used was of the form:

$$Y_{ij} = \mu + a_i + \varepsilon_{ij}, \quad i = 1,2,3,4, \quad j = 1,\ldots j_i$$

where Y_{ij} is the j value (from J_i) of the dependent variables, which arises from i botanical origin, a_i is the effect of the i botanical origin on the value of the dependent variable, μ is a true mean value common to all treatments, and ε_{ij} is the random error. The homogeneity of variances was tested by the Levene's test, where all the ANOVAs seem to satisfy this requirement ($p > 0.05$). In case, where differences among mean values were observed, post hoc tests were used, since no specific hypotheses exist, for the testing of different botanical origin. From these tests, Tukey's multiple comparison tests were used, since they are reliable in case of homogeneity of variances. All the statistical treatment of data was performed using the SPSS v.22.0 Statistics software [24].

Results and discussion

Polyphenol content of Greek unifloral honeys

In the present study variations in the phenolic content of thyme, orange blossom, pine and fir honey were observed, among the 170 honey samples tested. Mean values of each

Table 1 Polyphenol content (mg/kg), colour intensity (mAU) and % radical scavenging activity (% RSA) of Greek unifloral honeys

Botanical origin	Data	Syringic acid	Myricetin	Quercetin	Kaempferol	Chrysin	Colour intensity	(% RSA)
Thyme	Mean	20.25	19.60	11.17	14.44	0.35	209	25.84
$N = 83$	SD	38.26	45.00	20.14	23.79	1.00	71	11.24
	Min	0.06	0.74	0.04	0.10	0.01	10	7.07
	Max	195.04	244.67	129.55	61.38	5.60	449	67.14
	Range	194.98	243.93	129.51	61.28	5.59	348	60.06
Pine	Mean	0.94	1.87	0.47	0.32	0.13	405	50.38
$N = 47$	SD	1.54	5.54	1.69	1.25	0.33	135	19.17
	Min	0.12	1.54	0.21	5.04	0.73	196	13.77
	Max	9.10	26.64	11.36	5.07	1.27	494	79.59
	Range	8.98	10.22	11.15	0.03	0.54	690	65.82
Orange blossom	Mean	1.57	0.57	1.71	2.82	0.37	164	17.24
$N = 13$	SD	2.93	0.96	1.50	2.73	0.92	49	5.50
	Min	0.01	0.11	0.03	5.00	2.11	100	10.65
	Max	9.50	3.00	5.07	5.76	2.73	248	28.00
	Range	9.49	2.89	5.04	0.76	0.62	148	17.35
Fir	Mean	1.75	9.67	1.21	0.56	0.003	289	38.39
$N = 27$	SD	2.82	22.90	5.60	1.61	0.014	89	18.34
	Min	0.12	2.23	0.04	5.04	0.04	173	17.85
	Max	15.05	93.11	29.22	5.05	0.06	621	73.70
	Range	14.93	90.88	29.18	0.01	0.02	448	55.85

N = number of honey samples, Mean = average value, SD = standard deviation, Min = minimum value, Max = maximum value, Range = maximum value − minimum value. ANOVA in comparison of values ($p < 0.05$). The reported values are the mean of three replicates ($n = 3$)

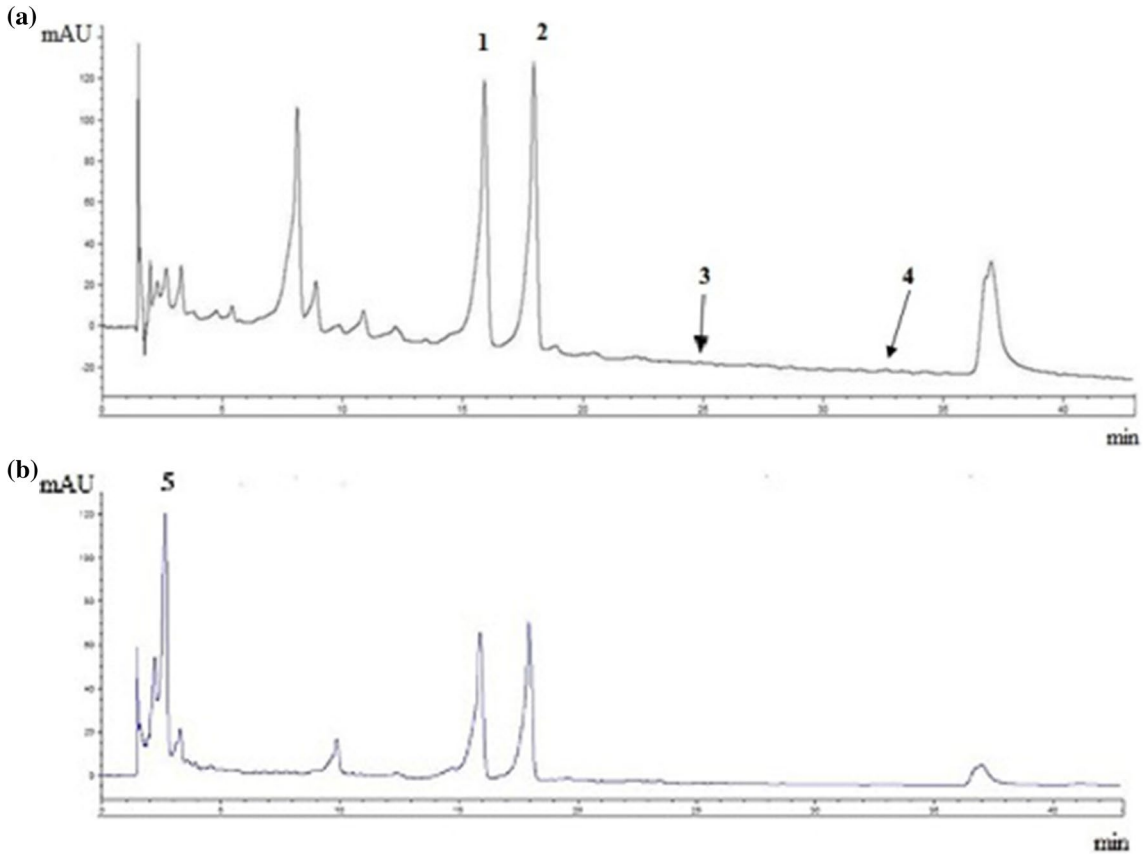


Fig. 2 **a** A typical HPLC–DAD chromatogram at λ = 254 nm of fir honey (no. 3) from Messinia. *Peak 1* myricetin, *peak 2* quercetin, *peak 3* kaempferol, *peak 4* chrysin. **b** A typical HPLC–DAD chromatogram at λ = 280 nm of fir honey (no.3) from Messinia. *Peak 5* syringic acid

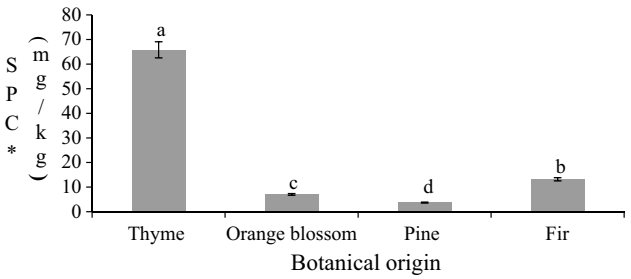


Fig. 3 Sum of polyphenol content (mg/kg) of Greek honeys according to botanical origin, in the ethyl acetate extract of honey. *Different letters* (*a*, *b*, *c*, *d*) indicate statistical significant differences ($p < 0.05$)

determined phenolic compound (mg/kg) as well as the minimum, maximum, SD, and range values are given in Table 1. Figure 2a, b show a typical HPLC–DAD chromatogram of fir honey sample (no. 3) from Messinia, pointing out the phenolic compounds quantified.

Figure 3 presents the sum (mg/kg) of the five phenolic compounds (SPC) for honeys of different botanical origin. Respective order was thyme (65.81) > fir (13.22) > orange blossom (7.04) > pine (3.73). It is clear that thyme honey is substantially richer in the selected polyphenolic compounds as compared to the rest of the honeys.

Using ANOVA, significant differences ($F(3, 169) = 7.157$, $p = 0.000 < 0.05$) were observed in the syringic acid content (mg/kg) of unifloral honey samples. Tukey's multiple comparison tests showed that thyme honey samples differ significantly in syringic acid content, among pine, orange blossom and fir honey samples that possessed lower mean values. Syringic acid values were 20.25 ± 38.26, 0.94 ± 1.54, 1.57 ± 2.93, and 1.75 ± 2.82 for thyme, pine, orange blossom, and fir honey, respectively.

Furthermore, myricetin ($F(3, 169) = 3.819$, $p = 0.011 < 0.05$) varied significantly according to botanical origin. Tukey's multiple comparison tests showed that thyme honey samples possessed the higher myricetin mean value (mg/kg), followed by fir honey samples. Pine and orange blossom honeys possessed lower mean values and did not differ significantly. Myricetin values were 19.60 ± 45.00, 1.87 ± 5.55, 0.57 ± 0.96, and 9.67 ± 22.90 for thyme, pine, orange blossom, and fir honey, respectively.

Significant differences in quercetin content ($F(3, 169) = 7.372$, $p = 0.000 < 0.05$) were observed, using ANOVA and Tukey's multiple comparison tests, between thyme honey samples and all the rest which did not differ significantly among them. Quercetin values were 11.17 ± 20.94, 0.47 ± 1.69, 1.71 ± 1.50, and 1.21 ± 5.60 for thyme, pine, orange blossom, and fir honey, respectively.

Kaempferol differed significantly among the honeys tested of botanical origins ($F(3, 169) = 9.496$, $p = 0.000 < 0.05$). The higher kaempferol values (mg/kg) were recorded for thyme honey samples, followed by those of orange blossom honey samples, while pine and fir honey samples possessed lower mean values and did not differ significantly between them. Kaempferol values were 14.44 ± 23.79, 0.32 ± 1.25, 2.82 ± 2.73, and 0.56 ± 1.61 for thyme, pine, orange blossom, and fir honey, respectively.

Finally, chrysin did not differ significantly for all the tested botanical origins ($F(3, 169) = 1.911$, $p = 0.130 > 0.05$). Chrysin values were 0.35 ± 1.00, 0.13 ± 0.33, 0.37 ± 0.92, and 0.003 ± 0.014 for thyme, pine, orange blossom, and fir honey, respectively.

As shown in previous studies [9, 11, 20], in very good agreement with present results, the polyphenol content of Greek unifloral honeys may vary according to botanical and geographical origin.

For instance, Tsiapara et al. [9] using the Folin-Ciocalteu assay, reported variations in the total phenolic content of 15 Greek unifloral honeys. The respective order regarding total phenolic content (average values, mg/kg) among the honey types investigated was thyme (300 mg/kg) > pine (292 mg/kg) > fir (114 mg/kg).

Spilioti et al. [11] analyzing 11 honey samples of different botanical origin (pine, fir, thyme, forest, commercial fir, citrus) by using high performance liquid chromatography indicated that protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid and *p*-coumaric acid were the major phenolic acids among honeys analysed. Conifer tree honey (from pine and fir) contained significantly higher amounts of protocatechuic and caffeic acid (mean values of 6.64 and approximately 0.40 mg/kg, respectively) than thyme and citrus honey (mean values of 0.44 for protocatechuic and approximately 0.12 mg/kg for caffeic acid, respectively), while *p*-hydroxybenzoic acid was the dominant phenolic acid in thyme honeys (mean value of 1.25 mg/kg).

Furthermore, Karabagias et al. [20] using high performance liquid chromatography reported significant variations in the phenolic content (syringic acid, quercetin, kaempferol, myricetin, chrysin) of 35 *Thymus capitatus* L. honeys according to geographical origin. Those reported values (mean ± SD) for syringic acid (45.25 ± 47.96), myricetin (40.45 ± 61.82), quercetin (20.96 ± 19.59), kaempferol (31.25 ± 26.89) and chrysin (0.87 ± 1.41) are in general agreement with the results of the present study.

In a study dealing with the antioxidant activity of 7 honey samples of different floral and geographical origin (3 commercial honeys, Langenese brand, German (*acacia* 1, polyfloral forest and meadow), 3 artisanally produced from beekeepers in Serbia (Urtica dioica, Tilia platyphyllos and acacia 2) and 1 pine (*Pinus* spp., Thassos island, Greece), using the Folin-Ciocalteu assay, Gorjanović et al. [19] reported, in very good agreement with the results of the present study, significant variations in the total phenolic content for honeys of different botanical origin. Respective average ± SD values (μg/mL) for total phenolic content ranged between 94.00 ± 3.10 for acacia honey (no. 2) to 620.70 ± 0.8 for polyfloral forest honey. The trend clearly shows the impact of botanical origin on honey's phenolic profile.

Sergiel et al. [25] used high performance liquid chromatography in combination with electro-spray ionization tandem mass spectrometry (HPLC–ESI–MS) detection to investigate the phenolic profile of 12 Polish honey samples corresponding to six different botanical origins (acacia, buckwheat heather, linden, multiflower, and rape). These authors reported significant variations in the content (ng/g) of phenolic acids (caffeic, chlorogenic, *p*-coumaric, ferulic, homogentisic, *p*-hydroxybenzoic, and vanillic acid), and flavonoids (apigenin, genistein, hesperetin, kaempferol, luteolin, rhamnetin, rutin, tricetin and quercetin) for different floral types of honey.

More specifically, the reported average value for quercetin content (converted to mg/kg) regarding 2 buckwheat and 1 heather honey sample (3.37, 3.95, 3.83 mg/kg respectively) was higher than our results for pine, orange blossom and fir honeys (0.47, 1.21 and 1.71 mg/kg, respectively), but significantly lower than present results dealing with thyme honeys (11.17 mg/kg) (Table 1). Furthermore, the reported quercetin content for the 2 acacia, 2 linden and 2 multifloral honey samples, was lower than the results of the present study for thyme, pine, orange blossom and fir honey.

Regarding kaempferol content, the reported average values (mg/kg) for 1 linden (3.96 mg/kg) and 1 multifloral (4.37 mg/kg) honey sample were higher than our results for pine (0.32 mg/kg), fir (0.56 mg/kg) and orange blossom honey samples (2.82 mg/kg), but much lower than our results for thyme honeys (14.44 mg/kg); in the case of 1 buckwheat honey, the reported average content of quercetin was in very good agreement with our results for orange blossom honey samples (1.92 vs 2.82 mg/kg, respectively).

It is worth mentioning that in the rest 9 honey samples (2 acacia, 1 buckwheat, 1 linden, 1 multifloral, 2 heather,

and 2 rape) investigated by Sergiel et al. [25], kaempferol content was lower (<0.05 mg/kg) compared to results of the present study (Table 1), whereas data for myricetin, chrysin and syringic acid were not reported.

Finally, in a similar study carried out on Spanish honey samples from different harvesting periods Escriche et al. [26] reported, in very good agreement with the results of the present study, variations in the content of myricetin, quercetin, kaempferol and chrysin depending on the floral type of honey. Myricetin was not detected in citrus and rosemary honeys, whereas it was determined in polyfloral and honeydew honeys [average values (mg/kg) 13 and 26, respectively]. Quercetin recorded higher average values in honeydew honeys (9 mg/kg) followed by rosemary (5 mg/kg), polyfloral (4 mg/kg), and citrus (3 mg/kg) honeys. Respective average values (mg/kg) for kaempferol followed the order: rosemary honeys (27) > polyfloral honeys (12) > citrus honeys (7) > honeydew honeys (4). Chrysin recorded higher average values in rosemary honeys (23), followed by honeydew (19), polyfloral (15), and citrus honeys (9), respectively. These reported values (mg/kg) for myricetin are in general agreement with the results of the present study dealing with thyme and fir honeys, whereas quercetin content was lower than present results regarding thyme honey, and higher than our reported results for pine, orange blossom, and fir honey, respectively.

Results of the present study dealing with the kaempferol content (mg/kg) of thyme and orange blossom honeys are in general agreement with the results reported for polyfloral and honeydew honeys by Escriche et al. [26], while chrysin content reported in the present study is significantly lower in all botanical origins as compared to Escriche et al. [26]. It should be mentioned that these authors did not detect syringic acid, while other phenolic acids such as caffeic and *p*-coumaric were identified in honey samples tested.

It should also be noted that factors such as altitude, temperature, rainfall, sunlight, harvesting period, geographical origin or even different contribution of nectar/pollen percentage (genotype) owed to different flowers present in the greater area may affect the phenolic profile of honey, i.e. Karabagias et al. [20] reported significant differences of polyphenol content of thyme honeys collected from different geographical regions in Greece. The systematic study of these factors in combination with chemometric techniques may open a new field in future research regarding honey's phytochemicals.

Colour intensity of Greek unifloral honeys

Significant variations ($F(3, 169) = 48.750, p = 0000 < 0.05$) in colour intensity of Greek honeys (thyme, pine, orange blossom, fir) were observed for samples of different botanical origin (Fig. 4), using ANOVA and Tukey's multiple comparison tests. More specifically, mean value ± SD of colour intensity ranged from 164 ± 49 (orange blossom honey) to 405 ± 135 mAU (pine honey) (Table 1).

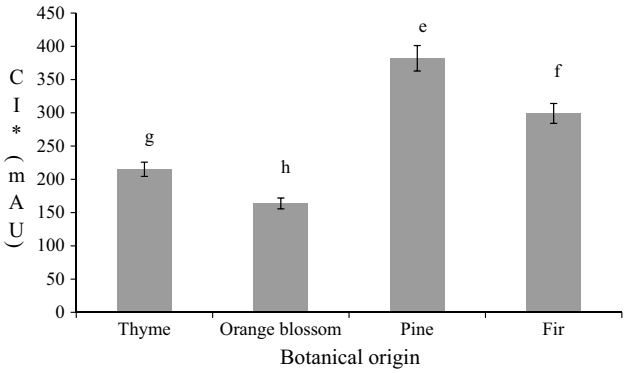


Fig. 4 Colour intensity of Greek unifloral honeys of different botanical origin. *Different letters* (*e*, *f*, *g*, *h*) indicate statistical significant differences at the $p < 0.05$ confidence level. *CI = Colour intensity (mAU) of aqueous honey solution

Colour intensity (mAU) followed the order: pine (405 ± 135) > fir (289 ± 89) > thyme (209 ± 71 > orange blossom (164 ± 49) (Fig. 4). This is the first ABS_{450} report for Greek unifloral honeys. That is, honeydew honeys such as pine and fir are usually darker than blossom honeys such as thyme and orange blossom honey. Castro et al. [27] stated that colour assessment using visual comparisons, spectrophotometric measurements and *CIE L*a*b** parameters can be a useful complementary tool for distinguishing the botanical origin of honey. Additionally, González-Miret et al. [28] classified honey samples into two groups based on their lightness value: light honeys (citrus, rosemary, lavender, eucalyptus and thyme) with $L^* > 50$ and dark honeys (honeydew, heather, chestnut and avocado) with $L^* < 50$.

Beretta et al. [17] reported significant variations in the colour intensity for 11 commercial honey samples from different regions in Italy belonging to 11 different botanical origins (strawberry tree, buckwheat, chestnut, sulla, clover, dandelion, chicory, acacia, mountain multifloral, honeydew) as well as in 3 tropical honey samples from Burkina Faso (Africa). Respective average values (mAU) ranged from 25 for the pale-white honey (acacia honey) to 3413 for the dark-brown honey (strawberry tree honey). This finding is in excellent agreement with the results of the present study, since light blossom honeys (i.e. thyme and orange blossom) had significantly lower average values than dark-coloured honeys (i.e. pine and fir) (Table 1). Furthermore, the obtained results for thyme honeys (average value of 209 mAU) match better with the results of sylia (average value of 222 mAU) and dandelion (average value of 225 mAU) honeys, as reported by Beretta et al. [17]; the respective average values of multifloral and honeydew

samples (415 and 466 mAU) match better with our results dealing with pine honey samples (405 mAU) (Table 1).

Additionally, Bertoncelj et al. [5] reported significant variations in colour intensity of 70 Slovenian honey samples belonging to 7 different botanical origins (acacia, lime, chestnut, fir, spruce, multifloral, forest). Colour intensity (average values) varied from 70 mAU (in acacia honey) to 495 mAU (in chestnut honey). These results are in excellent agreement with the results of the present study, since light-coloured honeys (i.e. acacia and lime) had lower colour intensity values than dark-coloured honeys (i.e. chestnut, spruce, fir, forest and multifloral). The reported values (average, mAU) for fir (405), spruce (417), and forest honeys (467) match better with the present results for pine honeys (405), while the reported values for lime honeys (123) are in good agreement with present results dealing with orange blossom honeys (164) (Table 1).

The same significant variations in colour intensity [net absorbance ($Abs_{560} - Abs_{720}$)] were reported by Vela et al. [29] in a study involving 36 Spanish honeys of different floral origins (nectar and honeydew). Respective average values (mAU) were lower in nectar honeys (270) and higher in honeydew honeys (680), in excellent agreement with the results of the present study.

Finally, the reported ABS_{450} values for 7 commercial Indian, 9 Malaysian, and 4 Algerian honey samples, of different botanical origin, ranged from 524 to 1678, 170 to 741, and 724 to 1188 mAU, respectively [30–32]. The average values of colour intensity reported by Khalil et al. [31] were: 170 mAU for 1 Borneo tropical honey, 298 and 301 mAU for 2 Tualang honeys (nos. 2 and 3 respectively), and 283 mAU for 1 multifloral honey (referred as "Honey B"). These reported results are in good agreement with present results regarding orange blossom honeys (164 mAU) and fir honeys (289 mAU), respectively. Likewise, Indian and Algerian honeys showed higher colour intensity values compared to results of the present work [30, 32]. All the above research studies were carried out in different parts of the world and point out the impact of botanical origin on the colour intensity of honey.

Radical scavenging activity (% RSA) of Greek unifloral honeys

Radical scavenging activity varied significantly for honeys of different botanical origin, based on ANOVA ($F(3, 169) = 33.128$, $p = 0.000 < 0.05$). Tukey's multiple comparisons in the data set showed that the 4 botanical origins (thyme, pine, orange blossom, fir) differed significantly in the mean value of % RSA (Fig. 5). % RSA values followed the order: pine (50.38 ± 19.17) > fir (38.39 ± 18.34) > thyme (25.84 ± 11.24) > orange blossom (17.24 ± 5.50) (Table 1).

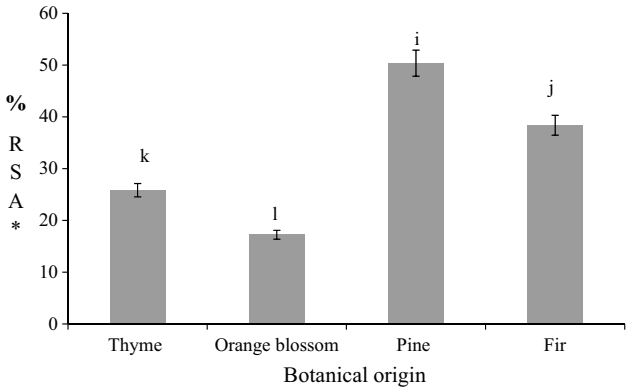


Fig. 5 % Radical scavenging activity (% RSA) of Greek unifloral honeys of different botanical origin. *Different letters* (*i*, *j*, *k*, *l*) indicate statistically significant differences at the $p < 0.05$ confidence level. *% RSA = % Radical scavenging activity of aqueous honey solution

This is the first systematic [DPPH·] free radical scavenging activity report for 170 Greek unifloral honeys using an aqueous honey solution. It was found that all the aqueous honey solutions were able to scavenge the [DPPH·] free radical. This finding is in excellent agreement with the results of Vela et al. [29] who reported higher radical scavenging activity (% RSA) for the darkest coloured honeys (honeydew honeys) (66.80 ± 18.10) compared to light-coloured honeys (nectar honeys) (28.70 ± 16.60). In our case light-coloured honeys are thyme and orange blossom, whereas honeydew honeys are pine and fir.

In a similar study, dealing with 35 Lithuanian honey samples of different botanical origin (multifloral, willow, spring rape, pine extract, linden), Baltrušaitytė et al. [33] reported significant variations in the % RSA for honey samples of different botanical origin. Respective values ranged from 31.10 ± 4.50 for linden honey to 93.00 ± 1.00 for pine extract honey. Pine extracts had higher radical scavenging activity than the present pine honey samples (Table 1).

In a study dealing with 9 Malaysian honey samples, Khalil et al. [31] reported significant variations in % radical scavenging activity for honey samples of different floral origin. Respective values ranged from 26.79 ± 1.90 for Borneo tropical honey to 81.64 ± 1.59 for Tualang honey (no. 2). The reported values for Borneo tropical honey are in very good agreement with the results of the present work regarding thyme honeys.

Furthermore, the EC_{50} values (mean ± SD, mg of water-soluble antioxidants present in/0.1 mL of honey solution added to the [DPPH·] free radical solution) were obtained from graphs (i.e. Figure 1). Respective values were: 8.56 ± 3.57, 28.54 ± 7.86, 32.28 ± 13.51, 40.88 ± 12.31 for pine, fir, thyme, and orange blossom honeys. It should be noted that the lower the EC_{50} value, the higher the antioxidant activity.

Beretta et al. [17], using a similar aqueous honey solution (1 g/10 mL vs 1.2 g/10 mL in the present study) and reaching a final [DPPH·] concentration in the reaction medium equal to 83.3 μmol/L, reported EC_{50} values for chestnut and honeydew honey to be 7.93 ± 0.04 and 8.48 ± 0.24, respectively. These values are in very good agreement with those obtained for pine honeys in the present study.

Additionally, in a study carried out on 20 Portuguese honey samples produced from nectar of *Lavandula*, *Echium* and *Erica* plants, Estevinho et al. [34] using a methanolic honey extract (50 mg/mL) and reaching a final [DPPH·] concentration in the reaction medium equal to 54 μmol/L reported different EC_{50} values (mg/mL) for samples of different floral origin (i.e. 68.17 for *Lavandula* honeys and 27.24 for *Erica* honeys). The reported values for *Erica* honeys (dark honeys) are in good agreement with our results regarding fir honeys (28.54 ± 7.86). Even though floral origin of above honeys is different, they both belong to dark-coloured honeys show similarities regarding EC_{50} values.

More recently, Sant'Ana et al. [23] evaluated the antioxidant activity of 21 honey samples from 3 different regions in Brazil using the DPPH assay. These authors using an aqueous honey solution (0.025 g/mL) and reaching a final [DPPH·] concentration equal to 87 μmol/L reported significant variations in the EC_{50} values (mg/mL). Respective values ranged from 10.19 ± 1.65 for Eucalyptus honey to 67.69 ± 1.24 for morráo candeia honey. The same applies here with regard to dark-coloured honeys stated above.

Taking into consideration, the EC_{50} values for commercial antioxidants (i.e. BHA = 3.6 mg/mL and a-tocopherol = 8.6 mg/mL, respectively) [34] present results mark a satisfactory antioxidant activity for Greek unifloral honeys and highlight their prospective health benefits as part of a regular diet.

Conclusion

The present study demonstrates remarkable variations in antioxidant properties of Greek unifloral honeys of different botanical origin. These variations should be considered when using honey as a source of natural dietary antioxidants, especially in a simple aqueous medium. Because of the health benefits of natural dietary antioxidants, floral origin should comprise an important factor in evaluating the potential of honey as an antioxidant agent. The in vitro antioxidant activity of a simple simulant (aqueous honey solution), the pigment and polyphenol content, highlight the prospective health benefits of Greek unifloral honey and support previous work in the literature by Gheldof et al. [2], who reported that the water-soluble fraction of honey contained most of the antioxidant components (protein, gluconic acid, ascorbic acid, and the combined activities of the enzymes glucose oxidase, catalase and peroxidase), compared to non-polar fraction (i.e. phenolic). Along the same line of reasoning, Bogdanov et al. [35] reported numerous beneficial nutritional and health effects of honey through daily consumption (antimicrobial, antioxidant, antiviral, antiparasitic, antiinflammatory, antimutagenic, anticancer and immunosuppressive activities or even enhanced gastroenterological and cardiovascular health), depending on its botanical origin.

Based on the present data, we propose the daily consumption of 30 g of honey, especially dark-coloured honey (i.e. pine or fir) as a part of a well-balanced diet.

To our knowledge, this is the first study in Greece carried out on such a large number of honey samples, providing analytical data on antioxidant properties in a simple/and or reproducible way for unifloral honeys; this constituting the novelty of the present work.

Finally, this approach is the first step (physico-chemical analysis) that may provide a trace for further analytical investigations on the profile of selected phytochemicals and/or the water-soluble antioxidants of Greek unifloral honey so that more rigorous in vivo studies can be carried out.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

References

1. Erdman Jr JW, Balentine D, Arab L, et al. (2007) Flavonoids and heart health. In: Proceedings of the ILSI North America Flavonoids Workshop, May 31–June 1 2005, Washington, DC. J Nutr 137(3):718–737
2. Gheldof N, Wang XH, Engeseth NJ (2002) Identification and quantification of antioxidant components of honeys from various floral sources. J Agric Food Chem 50:5870–5877
3. Wang XH, Gheldof N, Engeseth NJ (2004) Effect of processing and storage on antioxidant capacity of honey. J Food Sci 69:96–101
4. Turkmen N, Sari F, Poyrazoglu ES, Velioglu YS (2006) Effects of prolonged heating on antioxidant activity and colour of honey. Food Chem 95:653–657
5. Bertoncelj J, Dobersek U, Jamnik M, Golob T (2007) Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. Food Chem 105:822–828
6. Schramm DD, Karim M, Schrader HR, Holt RR, Cardetti M, Keen C (2003) Honey with high levels of antioxidants can provide protection to healthy human subjects. J Agric Food Chem 51(6):1732–1735

7. Al-Waili NS (2003) Identification of nitric oxide metabolites in various honeys: effects of intravenous honey on plasma and urinary nitric oxide metabolites concentrations. J Med Food 6(4):359–364
8. Al-Mamary M, Al-Meeri A, Al-Habori M (2002) Antioxidant activities and total phenolics of different types of honey. Nutr Res 22(9):1041–1047
9. Tsiapara AV, Jaakkola M, Chinou I, Graikou K, Tolonen T, Virtanen V, Moutsatsou P (2009) Bioactivity of Greek honey extracts on breast cancer (MCF-7), prostate cancer (PC-3) and endometrial cancer (Ishikawa) cells: profile analysis of extracts. Food Chem 116:702–708
10. Kassim M, Achoui M, Mohd-Rais M, Ali-Mohd M, Kamaruddin MY (2010) Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. Nutr Res 30:650–659
11. Spilioti E, Jaakkola M, Tolonen T, Lipponen M, Virtanen V, Chinou I, Kassi E, Karabournioti S, Moutsatsou P (2014) Phenolic acid composition, antiatherogenic and anticancer potential of honeys derived from various regions in Greece. PLoS One 9(4):e94860
12. Anklam E (1998) A review of the analytical methods to determine the geographical and botanical origin of honey. Food Chem 63:549–562
13. Latorre MJ, Peña R, García S, Herrero C (2000) Authentication of Galician (N.W. Spain) honeys by multivariate techniques based on metal content data. Analyst 125:307–312
14. Popek S (2000) A procedure to identify a honey type. Food Chem 79:401–406
15. Alissandrakis E, Tarantilis PA, Pappas C, Harizanis PC, Polissiou M (2009) Ultrasound-assisted extraction gas chromatography-mass spectrometry analysis of volatile compounds in unifloral thyme honey from Greece. Eur Food Res Technol 229:365–373
16. Antony SM, Han IY, Rieck JR, Dawson PL (2000) Antioxidative effect of Maillard reaction products formed from honey at different reaction times. J Agric Food Chem 48:3985–3989
17. Beretta G, Granata P, Ferrero M, Orioli M, Facino RM (2005) Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. Anal Chim Acta 533:185–191
18. Blasa M, Candiracci M, Accorsi A, Piacentini MP, Albertini MC, Piatt E (2006) Raw millefiori honey is packed full of antioxidants. Food Chem 97:217–222
19. Gorjanović SZ, Alvarez-Suarez JMA, Novaković MM, Pastor FT, Pezo L, Battino M, Sužnjević DZ (2013) Comparative analysis of antioxidant activity of honey of different floral sources using recently developed polarographic and various spectrophotometric assay. J Food Comp Anal 30:13–18
20. Karabagias IK, Vavoura MV, Badeka A, Kontakos S, Kontominas MG (2014) Differentiation of Greek thyme honeys according to geographical origin based on the combination of phenolic compounds and conventional quality parameters using chemometrics. Food Anal Method 7:2113–2121
21. Council Directive 2001/110/EC relating to honey. Official Journal of the European Communities, L 10, 47–52
22. IHC Working Group" Authenticity of Bee Products". Summary Report of the annual meeting, 1 October 2013 (during the 43th Apimondia Conference in Kiev, Ukraine)
23. Sant'Ana LDO, Sousa JPLM, Salgueiro FB, Lorenzon MCA, Castro RN (2012) Characterization of monofloral honeys with multivariate analysis of their chemical profile and antioxidant activity. J Food Sci 71(1):135–140
24. SPPS, v.22.0 (2013) IBM
25. Sergiel I, Pohl P, Biesaga M (2014) Characterisation of honeys according to their content of phenolic compounds using high performance liquid chromatography/tandem mass spectrometry. Food Chem 145:404–408
26. Escriche I, Kadar M, Juan-Borrás M, Domenech E (2014) Suitability of antioxidant capacity, flavonoids and phenolic acids for floral authentication of honey. Impact of industrial thermal treatment. Food Chem 142:135–143
27. Castro RM, Escamilla MJ, Reig FB (1992) Evaluation of the color of some unifloral honey types as a characterization parameter. J AOAC Int 75:537–542
28. González-Miret ML, Terrab A, Hernanz D, Fernández-Recamales MA, Heredia FJ (2005) Multivariate correlation between color and mineral composition of honeys and by their botanical origin. J Agric Food Chem 53:2574–2580
29. Vela L, de Lorenzo C, Pérez RA (2007) Antioxidant capacity of Spanish honeys and its correlation with polyphenol content and other physicochemical properties. J Sci Food Agric 87:1069–1075
30. Saxena S, Gautam S, Sharma A (2010) Physical, biochemical and antioxidant properties of some Indian honeys. Food Chem 118:391–397
31. Khalil MI, Mahaneem M, Jamalullail SMS, Alam N, Sulaiman SA (2011) Evaluation of radical scavenging activity and colour intensity of nine Malaysian honeys of different origin. J Apiprod ApiMed Sci 3(1):4–11
32. Khalil MdI, Moniruzzaman M, Boukraâ L, Benhanifia MdM, Islam A, Islam MdN, Sulaiman SA, Hua Gan S (2012) Physicochemical and antioxidant properties of Algerian honey. Molecules 17:11199–11215
33. Baltrušaitytė V, Venskutonis PR, Čeksterytė V (2007) Radical scavenging activity of different floral origin honey and beebread phenolic extracts. Food Chem 101:502–514
34. Estevinho L, Pereira AP, Moreira L, Dias LG, Pereira E (2008) Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. Food Chem Toxicol 46:3774–3779
35. Bogdanov S, Jurendic T, Sieber R, Gallmann P (2008) Honey for nutrition and health: a review. J Am Coll Nutr 27:677–689