Influence of geographic origin on the profile and level of phenolic compounds in Italian strawberry tree (*Arbutus unedo* L.) honey

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Summary
Strawberry tree honey is attracting attention for its high nutritional value and several beneficial effects of human health. These properties have been related to its high content in phenolic compounds and antioxidant activity. Strawberry tree honey is not commonly available, because it is harvested only in autumn and in very few locations, therefore its market price is higher than that of other unifloral honeys. The profile and content of 15 phenolic compounds: *cis*,*trans*-abscisic acid, rutin, luteolin, quercetin, apigenin, kaempferol, naringenin, hesperetin chrysin, gallic acid, ellagic acid, syringic acid, caffeic acid, chlorogenic acid and coumaric acid were studied in strawberry tree honey samples from two Italian regions, Sicily and Sardinia. The samples demonstrated a high content of phenolic compounds and antioxidant properties, with a particularly high levels of abscisic, chlorogenic, coumaric and syringic acids, together with luteolin, rutin and kaempferol. The geographical origin of honey significantly influenced the parameters studied, confirming that strawberry tree honey properties are attributable to both its botanical and geographical origin, which should be valorized.

Keywords
strawberry tree honey**;** antioxidant capacity; polyphenol; principal component analysis

Strawberry tree honey is a honey of a characteristic bitter taste that comes from the nectar of strawberry tree (*Arbutus unedo*), which is an evergreen shrub of the Ericaceae family. Strawberry trees are not very common, they grow in Italy, Spain, France, Albania, Greece, Croatia, Serbia, Bosnia and Herzegovina, Macedonia, Montenegro and Slovenia [1]. Since the plant blooms between September and November, it follows that the Italian production of this unifloral honey is very rare and is produced only in Sardinia in relevant quantity and quality, while only in a limited amount and if there are certain environmental conditions, in Tuscany and Sicily [2].

The medicinal and health-promoting properties of honey have been largely reported since the times of Greeks and Romans, and it is traditionally used as a natural drug in many Italian regions [2]. In fact, together with high nutritional value, it shows anti-inflammatory and antibacterial properties as to be a useful remedy for skin wounds and gastrointestinal disorders. Furthermore, it has been observed that strawberry tree honey is able to stimulate the immune system and can exert promising chemoprotective effect on cancer and metastasis prevention [2–4]. The low production and the reputation as a functional food for its therapeutic value have made strawberry tree honey one of the most expensive and researched honey on the market [5]. The biological properties of strawberry tree honey have been attributed to its high antioxidant properties that are highly correlated to its high content of phenolic compounds [2, 6].

Floral sources, geographical origins, seasonal and environmental factors have a significant impact on the antioxidant potential of honey [6]. Up till now, research on the bioactive compounds composition of strawberry tree honey has been focused on phenolics determined in a group of compounds and on its antioxidant properties [7–10]. In these studies, strawberry tree honey emerged as the most active and the richest honey in total phenols in comparison to other unifloral honeys, manuka honey included.

Regarding phenolic compounds, research was focused on homogentisic acid [1, 10, 11–14] together with unedone (2-(1,2-dihydroxypropyl)-4,8,8-trimethyl-1-oxaspiro[2.5]oct-4-en-6-one),

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(±)-2-*cis*,4-*trans*-abscisic acid (*c,t*-ABA) and (±)-2-*trans*,4-*trans*-abscisic acid (*t,t*-ABA), which had been considered as chemical markers of the botanical origin of strawberry tree honey [9, 15]. Osés et al. [5] studied strawberry tree honeys from various southern European countries, analysing arbutin, groups of polyphenols, the profiles of volatile and semivolatile compounds, Trolox-equivalent antioxidant capacity, antioxidant activities against hydroxyl and superoxide radicals and oxygen radical absorbance capacity in view of recognition of possible health claims that should be independent of geographical origin but depend on the botanical origin of honey [5].

Only a limited research has been conducted on the detailed phenolics profile of this bitter honey. Jurič et al. [16], identified 52 polyphenols by ultra-high-performance liquid chromatography coupled to a linear ion trap high resolution Orbitrap mass spectrometry system without carrying out quantification, which is useful for complete characterization. Petretto et al. [17] compared strawberry tree honey to other unifloral Sardinian honeys for total phenolics content, antioxidant capacity and determined 5 phenolic acids and 9 flavonoids in them. In our previous research, we quantified the levels of 6 phenolic acids, 8 flavonoids, abscisic acid and antioxidant properties of 9 unifloral honeys including strawberry tree honey samples from Sardinia [6].

Given the scarce knowledge of individual phenolic compounds composition and their levels in strawberry tree honey, the present work aimed to study total phenolics content, antioxidant capacity polyphenols profile and polyphenols contents in strawberry tree honey samples originating from two Italian islands, namely, Sicily and Sardinia. The aims was to understand the influence of the geographic origin on the antioxidant compounds and bioactive properties. Although homogentisic acid represents the main phenolic compound in strawberry tree honey, a synergistic antioxidant effect with other phenolic compounds present in this honey could not be excluded [3]. Therefore, complete characterization of the phenolics profile of strawberry tree honey could expand the knowledge on this health-promoting food. Physico-chemical parameters were also determined to assess the overall quality of honey.

MATERIALS AND METHODS

Samples

A total of 10 commercial honey samples were collected from market in Rome, Italy, with strawberry tree as the declared origin on the label. Regarding the geographical origin, five samples were from Sicily and five from Sardinia. Year of production was 2019 for all. Samples were stored at room temperature (25 °C) in a dark place for a maximum of a month.

Chemicals and materials

Acetonitrile, methanol, hydrochloric acid, potassium persulphate, sodium carbonate and Folin–Ciocalteu reagent were all of analytical grade and obtained from Merck (Darmstadt, Germany). Ultrapure water (electrical resistivity 18.2 MΩ·cm^{-1}) was purified in a Milli-Q system (Millipore, Bedford, Massachusetts, USA). The samples were concentrated and purified on Supelclean C18 cartridges (500 mg/3 ml) obtained from Supelco (Bellafonte, Pennsylvania, USA).

Standards of 15 phenolic compounds (*cis*,*trans*-abscisic acid, rutin, luteolin, quercetin, apigenin, kaempferol, naringenin, hesperetin chrysin, gallic acid, ellagic acid, syringic acid, caffeic acid, chlorogenic acid and coumaric acid) as well as the two radicals (2,2-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH)) were all purchased from Sigma–Aldrich (St. Louis, Missouri, USA).

The extracted samples and standard solutions were filtered through a PTFE membrane filter with a pore size of 0.45 μm (Millipore).

Physico-chemical parameters

Acidity and pH

The acidity and pH of samples were determined by a pH meter HI2210-02 (Hanna Instruments, Woonsocket, Rhode Island, USA) [18].

Total acidity was determined by the volumetric method [19]. Free acidity, lactones and total acidity were calculated as follows:

$$FA = \frac{N_t(V_t - V_0)}{g} \times 1000 \quad (1)$$

where FA is free acidity (expressed in milliequivalents of NaOH per kilogram), N_t is the normality of the titrant solution (expressed as number of gram equivalents per litre), while V_t and V_0 are volumes of NaOH (in millilitres) added to bring the solution to pH 8.5 for the honey sample and for the blank, g is the mass of the test sample.

$$L = \frac{N_{ta}(10 - V_{ta})}{g} \times 1000 \quad (2)$$

where L are lactones (expressed in milliequivalents of NaOH per kilogram), N_{ta} is the normality of the titrant solution (expressed as number of

gram equivalents per litre) and V_{ta} is the volume of HCl (in millilitres) added to bring the solution to pH 8.3, g is the mass of the test sample.

$$TA = FA + L \quad (3)$$

where TA is total acidity (expressed in milliequivalents of NaOH per kilogram), calculated as the sum of FA and L.

Hydroxymethylfurfural

Hydroxymethylfurfural (HMF) in honey was determined by the high-performance liquid chromatography (HPLC) method according to Bogdanov [20]. A Shimadzu HPLC system LC-10AT (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector SPD-M20A (Shimadzu) and a C18 Kinetex analytical column (150 mm × 4.6 mm, 5 μm particle size, Phenomenex, Torrance, California, USA). Honey samples (5 g each) were diluted up to 50 ml with distilled water, filtered through a membrane filter (pore size 0.45 μm) and immediately injected to the chromatograph. The mobile phase used was 90% water and 10% acetonitrile, flow rate was 1 ml·min^{-1} and injection volume was 20 μl. The wavelength range was 220–660 nm and the chromatograms were monitored at 285 nm.

Colour intensity

To determine the colour intensity, honey samples (5 g each) were diluted with warm distilled water (45 °C) to a volume of 10 ml, sonicated for 5 min and through a membrane filter (pore size 0.45 μm). Then, absorbance was measured at 450 nm and 720 nm using a spectrophotometer UV-1800 (Shimadzu). The net absorbance was defined as the difference between spectrophotometric absorbance at 450 nm and 720 nm [21].

Antioxidant capacity and total phenolics content

The determination of antioxidant capacity (*AC*) and total phenolics content (*TPC*) was carried out by spectrophotometric assays as described by Meda et al. [22]. Honey sample (5 g) was diluted with 15 ml ultrapure water in a 50 ml volumetric flask and filled with ultrapure water after homogenization in an ultrasonic bath for 15 min. The solution was then filtered through a polytetrafluoroethylene (PTFE) membrane filter (pore size 0.45 μm) and analysed for determination of *TPC* and *AC*. *TPC* was determined by a modified Folin–Ciocalteu method [23]. Briefly, 0.3 ml of the sample extracts and 6 ml deionized water were mixed with 0.5 ml of Folin–Ciocalteu reagent and the solution was left 6 min at room temperature (25 °C). After 30 min, 3 ml of 20% sodium carbonate was added and absorbance was determined at 765 nm. The results were expressed as milligrams of gallic acid equivalents (GAE) per kilogram of honey.

To quantify *AC* of the extracts of honey samples, the radical scavenging activity (*RSA*) of two different radical compounds, namely, DPPH and ABTS, was evaluated. A mixture of a methanolic solution of DPPH (71 mmol·l^{-1}, 3 ml) with 1.0 ml of honey extract was left for 60 min in the dark. The reduction of the DPPH radical absorbance at 515 nm was measured [24]. The radical ABTS was obtained by reacting 1 ml ABTS with 1 ml of potassium persulphate (0.005 mol·l^{-1}) in the dark for 20 h. The absorbance of the mixture of 0.1 ml of the honey extract with 1 ml of the diluted ABTS radical solution was measured at 734 nm after 6 min [25].

RSA was calculated for both the radicals as a percentage of radical discoloration using the equation:

$$RSA = \frac{(AB - AA)}{AB} \times 100 \quad (4)$$

where *AB* is the absorbance of the DPPH/ABTS solution and *AA* is the absorbance of the honey sample solution. Scavenging activities of the honey extracts were expressed as percentage of inhibition.

Individual phenolic compounds

The chromatographic determination of the phenolic compounds in the honey samples was carried out following Preti and Tarola [6], after the extraction performed according to Michalkiewicz et al. [26]. Briefly, honey samples diluted with acidified water (5 g to 5 ml) were passed through a C18 solid phase extraction (SPE) cartridge (500 mg/6 ml) previously conditioned with 3 ml of acetonitrile and 9 ml of ultrapure water, and then washed with 6 ml of acidified water. The cartridge was eluted with acetonitrile (2 ml) and then concentrated to a final volume of 1 ml by nitrogen flow.

HPLC analyses were performed on a LC-10AT system with a 20 μl sample loop, a photodiode array detector SPD-M20A (Shimadzu) and a C18 Kinetex (150 mm × 4.6 mm, 5 μm particle size) analytical column (Phenomenex). The linear gradient was from 3 % B to 45 % B in 55 min and then to 100 % B in 60 min. With 2% acetic acid in water (solvent A) and acetonitrile (solvent B), at a flow rate of 0.7 ml·min^{-1}. The photodiode array detector spectra were set between 200 nm and 600 nm and the monitoring was carried out at 280 nm, 320 nm and 350 nm.

Statistical analysis

All the analyses were determined in triplicate and results were expressed as mean values ± standard deviations. Analysis of variance (ANOVA) was used to evaluate significant differences in phenolic compounds profile and bioactive properties among honeys. Tukey's test at $p \leq 0.05$ was performed to discriminate the geographical origin of honey. A principal component analysis (PCA) on the data matrix of the phenolic compounds was conducted using V-PARVUS [27] to highlight the grouping of the samples in relation to their similarities and differences. Pearson's correlation coefficients between parameters were also calculated.

RESULTS AND DISCUSSION

Physico-chemical parameters

For an overall quality control of the samples. pH, free acidity, lactones and total acidity, HMF and colour were investigated and the results are shown in Tab. 1. Average pH values were pH 4.24 for Sardinian and pH 4.26 for Sicilian honeys, in accordance with those previously reported in Italian [18] and Portuguese [14] honeys. The results of free acidity, total acidity and lactones were within the limits set by EU Directive 2014/63 [28], confirming the absence of fermentative processes and good preservation of the samples. Sicilian honeys contained higher quantities of glucono-δ-lactone that hydrolyses to gluconic acid, which is the major contributor to honey acidity [18]. On the contrary, HMF content was higher in Sardinian samples but no sample exceeded the European Union legal limit of 40 mg·kg^{-1}. HMF is formed by degradation of fructose caused by poor storage and overheating [29], so it can be explained by poor processing practices.

Colour intensity of honey has been evaluated both as a quality parameter, since its presence is linked to the formation of Maillard reaction products during storage, but also because it has been associated to honey's total phenolics content and antioxidant capacity, with darker coloured honeys often having stronger antioxidant properties [30]. Strawberry tree honey has a typical dark colour that reached a medium colour intensity of 376.57 mAU in Sicilian honeys.

Total phenolics and antioxidant capacity assays

The results of *TPC* and *AC*, measured as the radical scavenging power of two radicals ABTS and DPPH, are displayed in Fig. 1 and Fig. 2. The differences between the values for Sicilian and Sardinian honey samples were all statistically significant ($p < 0.05$). Previous research found strawberry tree honey as the richest in *TPC* and having the highest *AC* among honeys from various floral sources [8, 17, 21]. Values reported were in the range of 389–972 mg·kg^{-1} (expressed as GAE). Our results were in the range of those reported in the literature, with Sardinian honeys that showed higher values than the Sicilian ones with 1035.3 mg·kg^{-1} vs 956.4 mg·kg^{-1} (expressed as GAE). Becerril-Sánchez et al. [3] observed that *TPC* and total flavonoids content were different in honeys of the same floral origin but from different geographical locations, because their presence depended on their pollen pattern related to climate and altitude [2].

The content of phenolic and flavonoid compounds enabled to differentiate Chilean honeys according to their geographical origin [31]. Also, unifloral honeys from various regions of China showed significant differences in *TPC* in relation to their provenance [32].

The antioxidant activity of honey is generally associated with its phenolics content. In this study,

Tab. 1. Physico-chemical properties of Sicilian and Sardinian strawberry tree honeys.

Parameters	Sicily ($n = 5$)	Sardinia ($n = 5$)
Free acidity [meq·kg^{-1}]	29.56 ± 3.04 ^{a}	24.34 ± 2.81 ^{b}
Lactone [meq·kg^{-1}]	10.16 ± 1.09 ^{a}	7.60 ± 0.92 ^{b}
Total acidity [meq·kg^{-1}]	39.72 ± 3.11 ^{a}	30.81 ± 3.63 ^{b}
pH	4.26 ± 0.10 ^{a}	4.24 ± 0.10 ^{a}
HMF (mg·kg^{-1})	11.10 ± 1.05 ^{a}	19.63 ± 4.65 ^{b}
Colour intensity [mAU]	376.57 ± 14.52 ^{a}	328.43 ± 19.16 ^{b}

Values represent mean ± standard deviation of three replicates per honey sample. Within each row, means followed by different superscript letters are significantly different at $p \leq 0.05$ (ANOVA, Tukey's test).
HMF – hydroxymethylfurfural; nd – not detected.

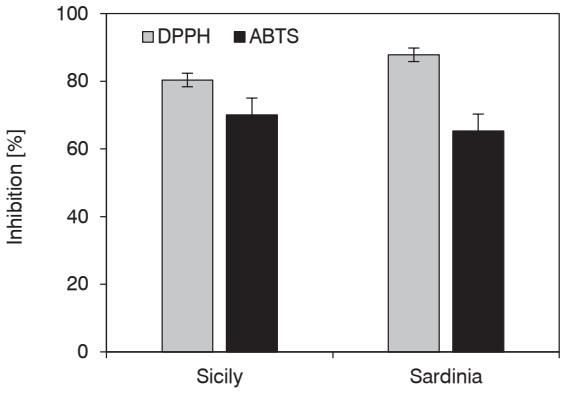

Fig. 1. Antioxidant capacity of strawberry tree honeys.

Differences between Sicilian and Sardinian data were all statistically different ($p < 0.05$, ANOVA, Tukey's test).
DPPH – antioxidant capacity determined by DPPH method, ABTS – antioxidant capacity determined by ABTS method.

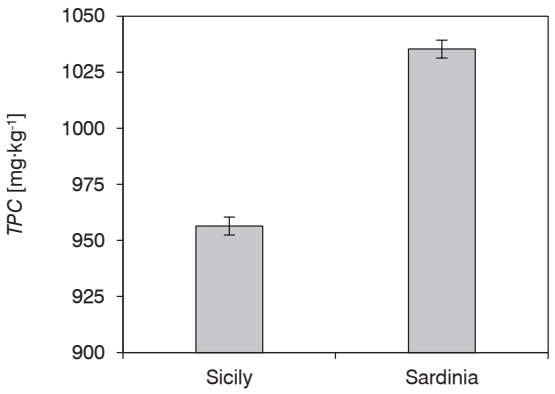

Fig. 2. Total phenolics content of strawberry tree honeys.

Differences between Sicilian and Sardinian data were all statistically different ($p < 0.05$, ANOVA, Tukey's test).
TPC – total phenolics content (expressed as milligrams of gallic acid equivalent per kilogram of honey).

the antioxidant values provided by the DPPH assay were higher in Sardinian honeys and correlated with the values of *TPC* ($r = 0.85$), while those from the ABTS assay were superior in Sicilian honeys and did not show significant correlation with *TPC*. Since values obtained by DPPH and ABTS assays showed a strong positive correlation ($r = 0.98$), these data could tentatively be explained by the different *RSA* exerted by different phenolics on the two radicals used in the two assays. Probably, different levels of certain phenolic compounds were present in honeys with different geographical origin that scavenge in different ways the two radicals. One of these compounds could be homogentisic acid, which represented approximately 50–60 % of total phenolics and whose concentration was reported to be affected by the geographical origin of strawberry tree honey [13].

Previously reported findings on the correlation between colour, *TPC* and *AC* [5, 33] were not confirmed in this study. The honey colour is closely related also to other parameters besides phenolics, such as minerals content, pollen floral origin, as well as Maillard and caramelization reactions that take place during processing and storage [6]. Therefore, the present results may suggest that, in strawberry tree honey, these factors contribute prevalently to its dark colour. These considerations were previously presented by PETRETTO et al. [17] at comparing Sardinian monofloral honeys based on total phenolics. No linear correlation was then found, as strawberry tree honey was the richest in phenolics but not the darkest among the varieties studied [17].

Individual polyphenols

The 15 individual polyphenols determined by HPLC with diode-array detection were all present in the strawberry tree honey samples studied, except for quercetin, which was never detected, and naringenin, which was determined in only two samples, one from Sicily and one from Sardinia. Results are presented in Tab. 2 as mean values ± standard deviations. Regarding the six phenolic acids, chlorogenic acid was the most abundant, followed by syringic, coumaric, gallic, ellagic, and caffeic. Chlorogenic, coumaric and gallic acids were quantified at significantly higher contents in Sicilian honeys than in the Sardinian ones. Abscisic acid reached a level of 8.5 $mg \cdot kg^{-1}$ in Sicilian honeys, much higher than that observed in Sardinian samples, but with values inferior to those reported previously [9]. Abscisic acid is a plant hormone involved in protection mechanisms at environmental stress and was studied previously as a strawberry tree honey chemical marker [13].

Among the 7 flavonoids studied, luteolin presented the highest content followed by rutin and kaempferol. Significantly higher levels were determined in Sardinian honeys only for luteolin and chrysin, with the presence and quantity of chrysin in honey depending on the degree of contamination of honey with propolis [34].

The total content of the phenolics determined was superior in Sicilian honey samples, and the values were not consistent with the results of *TPC* assays, where Sicilian honeys had lower values than Sardinian honeys, with no significant correlation. These data, together with the difference in

the results of radical scavenging assays, can lead to the hypothesis that some phenolic compounds remained unexplored in this study, with high content in strawberry tree honey and different *RSA* that contributed to these differences. For example, abscisic acid showed a positive correlation with results of ABTS assay ($r = 0.79$) but negative with results of DPPH assay ($r = –0.81$).

PETRETTO et al. [17] determined luteolin, apigenin and rutin on similar levels, while reporting a much lower content of syringic acid and did not detect chlorogenic acid. These differences can be attributable to the strong contamination of pollen and nectar from other floral sources. Compared to the phenolics profile of other unifloral honeys, strawberry tree honey has a higher content of rutin, luteolin and abscisic acid, which clearly differentiated this unifloral honey from the others in our previous work [5].

Principal component analysis

PCA was applied to evaluate the effect of the geographical origin on the profile and content of the 15 phenolic compounds studied. In Fig. 3, the scores and loading resulting from PCA are displayed. Principal component 1 (PC1) explained up to 39.5 % of the total variance and PC2 explained 17.5 %. Thus, the two-dimensional graph presented was able to explain 57.0 % of the variability in the experimental data. The variables that contributed most to PC1 were abscisic acid, chrysin, luteolin, coumaric acid, chlorogenic acid and gallic acid. PC2 was associated with ellagic and caffeic acids, hesperetin and kaempferol. Evaluation of Sicilian and Sardinian strawberry tree honeys resulted in a clear separation along PC1, with Sicilian honeys characterized by the high presence of abscisic, gallic and coumaric acids, luteolin and rutin, while Sardinian honeys had high contents of syringic acid, chlorogenic acid and chrysin.

Polyphenols mostly originate from pollen and nectar collected by bees and are greatly dependent on the floral diversity and maturity, and polyphenols profiles are linked to the floral and geographical origin of honey [29]. In strawberry tree honey, pollen and nectar from other botanical species, such as *Eucalyptus* and *Echium*, is often present [3]. Furthermore, the composition of plants pollen and nectar is geographically dependent and the pollen of the same botanical origin but from different locations may have different chemical composition [35]. The bioactive compounds composition of honey may also be influenced by many other factors such as the health of the bee colonies, the beekeeping procedures as well as the storage time and conditions [36]. The

Tab. 2. Polyphenols content in strawberry tree honey from Sicily and Sardinia regions.

Phenolic compound [mg·kg^{-1}]	Sicily ($n = 5$)	Sardinia ($n = 5$)
Gallic acid	7.27 ± 0.50 ^{a}	5.59 ± 0.46 ^{b}
Caffeic acid	1.15 ± 0.42 ^{a}	1.13 ± 0.25 ^{a}
Chlorogenic acid	10.10 ± 1.07 ^{a}	13.38 ± 1.96 ^{b}
Syringic acid	9.22 ± 0.96 ^{a}	9.43 ± 1.56 ^{a}
Coumaric acid	8.02 ± 0.46 ^{a}	6.17 ± 0.49 ^{b}
Ellagic acid	6.96 ± 1.14 ^{a}	6.46 ± 0.89 ^{a}
Rutin	3.02 ± 0.45 ^{a}	2.69 ± 0.35 ^{a}
Abscisic acid	8.50 ± 0.59 ^{a}	6.61 ± 0.77 ^{b}
Quercetin	nd	nd
Luteolin	6.61 ± 0.76 ^{a}	5.32 ± 0.46 ^{b}
Naringenin	0.49 ± 1.10 ^{a}	0.19 ± 0.33 ^{a}
Hesperetin	0.80 ± 0.12 ^{a}	0.78 ± 0.15 ^{a}
Kaempferol	2.32 ± 0.94 ^{a}	1.58 ± 0.54 ^{a}
Apigenin	0.93 ± 0.19 ^{a}	0.74 ± 0.07 ^{a}
Chrysin	0.43 ± 0.13 ^{a}	0.66 ± 0.09 ^{b}
Total	65.98 ± 1.65 ^{a}	60.74 ± 1.82 ^{b}

Values represent mean ± standard deviation of three replicates per honey sample. Within each row, means followed by different superscript letters are significantly different at $p \leq 0.05$ (ANOVA, Tukey's test).
nd – not detected.

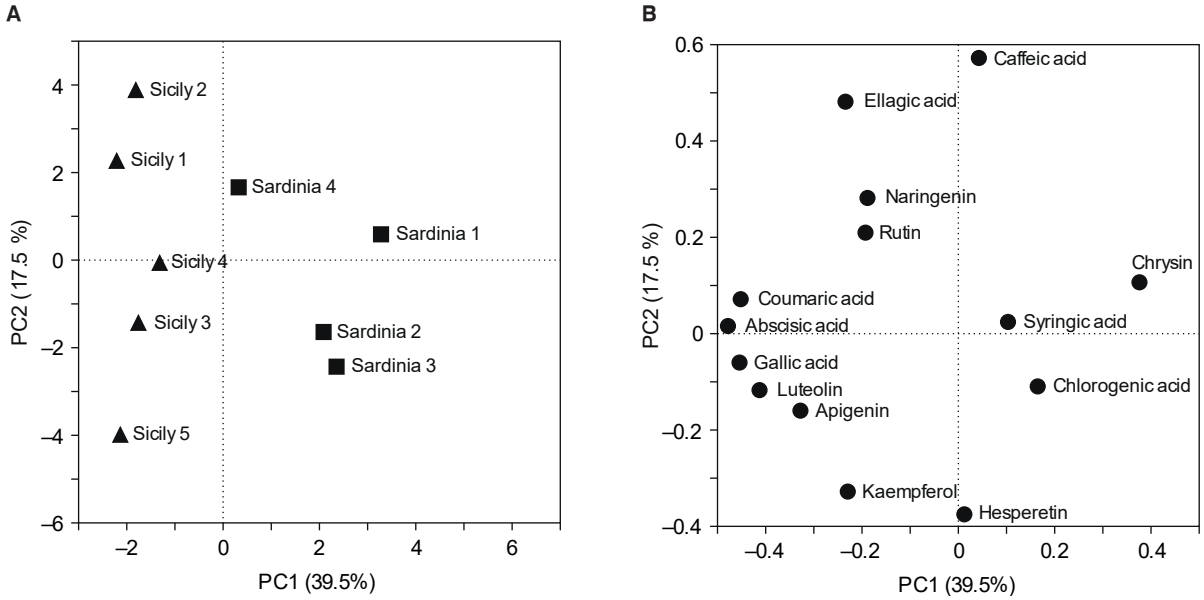

Fig. 3. Principal component analysis of the results of the analysis of phenolic compounds in honey samples. A – scores, B – loadings.

profile of phenolic compounds was significantly different for honeydew and thyme honeys from different geographic locations [37]. In *Mimosa scabrella* Bentham honeydew, significant differences in the content of phenolic acids and flavonoids from various geographical regions were observed [38].

CONCLUSIONS

The present work resulted in determination of 15 phenolic compounds in strawberry tree honeys from Sicily and Sardinia, confirming the high antioxidant capacity and the high *TPC* of strawberry tree honey, together with the high level of abscisic acid. Among the phenolic acids studied, strawberry tree honey showed high contents of chlorogenic, coumaric and syringic acids. Among flavonoids, high contents of luteolin, rutin and kaempferol were determined. The geographical origin of honey significantly influenced the parameters studied. Results showed how the content and type of phenolic compounds in honey and its antioxidant effectiveness strictly reflected the nectar and pollen chemical composition, which are highly variable among plant species and strictly dependent on the foraging geographical environment of bees. This aspect could be better explored in future studies, involving more samples and a melissopalynological characterization. Considering the growing interest for strawberry tree honey for its nutritional value, its rarity and high market value, it could be of economic interest to valorize the identity and peculiar characteristics of the local production linked to a certain territory through the recognition of geographical indication.

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