Identification of Traceability Markers in Italian Unifloral Honeys of different Botanical Origin

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Abstract

In this study we carried out the analysis of three varieties of unifloral honey of acacia (Robinia pseudoacacia), chestnut (Castanea sativa) and sulla (Hedysarum coronarium), in order to assess physicochemical and biochemical parameters, nutraceutical properties and antioxidant capacities.

The three different varieties of unifloral honey showed clear differences in some parameters, such as the concentration of polyphenols, flavonoids, mineral elements, as well as different profiles of volatile molecules. These parameters allow differentiating the three varieties of unifloral honey investigated.

The different concentration of polyphenols and flavonoids, the profile of volatile compounds and the presence of different concentration of some mineral elements allow discriminating different varieties of honey, and could be used as markers of product traceability. Moreover, the high concentration of some macro elements such as potassium, remarkably high in the chestnut honey, makes it especially useful in individuals with deficiency of these mineral or practicing sports.

Keywords: Honey quality; Traceability; Flavor; Elements; Phenols

Introduction

Honey consists essentially of different sugars, predominantly fructose and glucose, as well as other substances such as organic acids, enzymes and solid particles derived from honey collection. The color of honey varies from nearly colorless to dark brown. The consistency can be fluid, viscous or partly to entirely crystallized. The flavor and aroma vary, as they are derived from the plant origin [1]. Honey is part of the traditional medicine in many cultures and, currently, is considered a functional food. A food can be regarded as ‘functional’ if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way which is relevant to either the state of well-being and health or the reduction of the risk of a pathologic process or a disease [2]. A functional food may be natural or components may be added to food to make it “functional”. Among such components we can mention ω-3 fatty acids, vitamins, probiotics, prebiotics, fibers, phytochemicals and bioactive peptides [3].

In addition to numerous properties of honey, in recent years, the interest of researchers and consumers has been focusing on product traceability. In fact, consumers claim to know the provenance of foods. Thus, food retailers are inquiring to food scientist to find parameters allowing the assessment of the provenance of foods, able to distinguish among the same products arising from different regions [4]. The nature, amount, and combination of the various components endow each honey with individual organoleptic character and nutraceutical properties. These compounds represent a fingerprint of a specific honey and therefore could be used to differentiate honeys of different botanical and also geographic origins [5]. The classical approach to the evaluation of botanical origin is based on the integration of pollen, metal content, specific volatile compounds analysis and determination of some physicochemical parameters such as color, pH, sugar contents, and diastase activity [6].

Microscopy analysis, especially the identification and quantification of pollen grains in honey sediment, is the reference method used to determine the botanical origin of sample honeys. Normally, honeys are classified as monofloral, when the pollen frequency of one plant is over 45 [7].

Mineral composition has also been employed to discriminate honeys arising from different geographical areas. Minerals seem to be good candidates for a classification system, mainly because they are stable and can be associated to the soil where melliferous flora grows. Mineral contents range from about 0.04% in pale honeys to 0.2% in some dark honeys [7].

Specific volatile compounds derived from original nectar sources are in all likelihood responsible for the specific aroma of monofloral honeys. These volatile compounds have been proved to be adequate to authenticate floral origin of honeys and some of them are real markers.

In this work the attention has been focused on the analysis of three varieties of monofloral honey of acacia, chestnut and sulla, in order to verify their quality in terms of assessment of physicochemical and biochemical parameters, nutraceutical properties and antioxidant capacity. In particular, the investigations were carried out to verify the qualitative differences between the three types of honey and to bring out the peculiarities. In particular, the analyses were carried out to
evaluate: (1) chemical and physical parameters, (2) nutritional peculiarities of each honey, (3) modification of the properties of each cultivar related the freshness and quality, (4) antioxidant activity, (5) micro and macro nutrients, (6) specific polyphenols by high-performance liquid chromatography (HPLC) reverse phase (RP) and (7) profile of volatile molecules by means of GC-MS.

**Experimental**

Solvents and chemicals were of the highest commercially available purity: Methanol, HPLC-MS grade acetonitrile (ACN) and concentrated HCl were provided by Carlo Erba (Milan, Italy). Trifluoroacetic acid (TFA), chlorogenic acid, gallic acid, 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) and Folin-Ciocalteu’s phenol reagent were from Sigma (St. Louis, MO, USA).

**Honey samples**

Three different honey samples were used for this study: acacia, chestnut and sulla. The samples were collected between May and July 2014 in the South of Italy (Benevento area) and they were stored in the dark at room temperature (20°C) until analyses.

**Chemical-physical analysis**

**Moisture:** The water content was determined through the carbonization of honey. 5 g of sample were accurately weighed and placed in a crucible, whose weight had been previously recorded. Each honey sample was placed on the plate at about 200°C, checking the weight every three hours. When the weight did not vary it was considered as the final weight, to which the weight of the empty crucible was subtracted.

**pH:** 10 g of honey were weighed into a flask and 50 mL of distilled water were added [8]. The solution was thoroughly mixed until homogeneity. A pH-meter Seven Easy S20 (Mettler-Toledo, Columbus OH, USA), with an accuracy of ±0.002 pH units, was used for the measurement.

**°Brix and refractive index:** A refractometer (Optical Technology, Munchen, Germany) was used. On the plate of the instrument a smear of each honey sample was placed and the values in °Bx and the refractive index were read.

**Color measurements:** 1 g of honey was weighed and added to 10 mL of water (FD = 10) in a volumetric flask of 10 mL. The mixture was placed in a cuvette and the absorbance measured in a spectrophotometer UV/VIS (Backman Coulter).

**Determination of the content of mineral by icp-oes:** The honeys show very different colors, ranging from white or yellow to dark red or black pale. The mineral content influences the color and the taste of honey, the greater the amount of metals, the darker is the color.

About 1 g of honey sample was weighed in a crucible and placed on a carbonizing plate. Later it was put in a muffle at 550°C for six hours, until ashing. Nitric acid at 67% was added to the ashes and the sample was returned on the plate. Once dried, the plate was placed still for another six hours. The white ashes obtained were brought to volume in a 10 mL flask with water Mg acidified with nitric acid to 1%. The sample was stored at +4°C and, immediately before injecting the ICP spectrometer, it was further diluted (1: 10=0.8: 10 mL) [10]. Analyses were conducted with an ICP-OES spectrometer (mod. ICAP 7000, Thermo Scientific) equipped with auto sampler ASX-520. The calibration lines were built using a standard multi-element (Trace Cert, Fluka). The readings were taken in triplicate for each sample. The instrumental conditions were: transmitted power 1.15 kW, argon plasma flow of 12 L min⁻¹, flow auxiliary gas 0.50 L min⁻¹, 40 s integration times. The correction of the background noise on both sides was carried out.

**Gas chromatography-mass spectrometry:** The gas chromatography analysis was conducted using an Agilent 7890 GC combined with a quadrupole mass spectrometer equipped with Agilent 5975 auto sampler Gerstel MPS2. It was used a capillary column HP-INNO Wax (Agilent technologies) and helium as the carrier gas. Samples were injected at a temperature of 40°C for 1 minute. The temperature was increased in four steps: 40-60°C at 2°C min⁻¹, 60-150°C to 3°C min⁻¹, 150-200°C at 10°C min⁻¹ and 200-240°C min⁻¹, the final temperature was maintained for 7 minutes. The injector, the quadrupole, the source and the temperature of the transfer line were maintained respectively at 240°C, 150°C, 230°C and 200°C. The electrons ionized in full scan mode were recorded at 70 eV in the range of 40-300 amu. Peaks were identified using libraries NIST 98 and Wiley. The quantification was performed using the relative concentration in mg Kg⁻¹ of the internal standard, calculated as the ratio between the area of each compound and the area of the internal standard.

**Estimation of total phenolic and flavonoid contents:** 5 g of sample were added to a 50 mL of distilled water. Subsequently, each sample was agitated by vortex.

After 5 minutes, the solutions were then carefully filtered through Whatman No.42 filter paper and the extracts recovered.

The concentration of total polyphenols (TPC) in the extracts were determined by the colorimetric method Folin-Ciocalteau [11] according to Meda et al. [12]. Briefly, 0.5 mL of extract were added to 2.5 mL of Folin 0.2 N and, each sample was stored away from light for 5 minutes. After 5 minutes 2 mL of Na₂CO₃ 7.5% were added to the mix. Each sample was left for 2 hours in the dark at room temperature and the absorbance was determined by spectrophotometry at a fixed wavelength of 760 nm. Gallic acid (Sigma-Aldrich Chemie, Steinheim, Germany) (0-200 mg L⁻¹) was used as standard to produce the calibration curve. The mean of three readings was used and the total phenolic content was expressed in mg of gallic acid equivalents (GAE) 100 g⁻¹ of honey.

The total flavonoid content was determined using the Dowd method as adapted by Arvouet-Grand et al. [13]. Briefly, 5 mL of 2% aluminum trichloride (AlCl₃) in methanol were mixed with the same volume of the honey solution (0.01 or 0.02 mg mL⁻¹). Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 5 mL honey solution with 5 mL methanol without AlCl₃. The total flavonoid content was determined using a standard curve with quercetin (Sigma-Aldrich Chemie, Steinheim, Germany) (0-50 mg L⁻¹)
as a standard. The mean of three readings was used and expressed as mg of quercetin equivalents (QE) 100 g⁻¹ of honey. 

DPPH assay: The antioxidant activity (in vitro) of the extracts examined was determined by measuring the inhibitory activity on the production of free radicals. The colorimetric assay involves the use of free radical synthesis 2,2-diphenyl-1-picrylhydrazyl (DPPH•), generated in situ, commonly used to test the antioxidant activity [14]. 250 mg of honey sample were weighed and mixed with 5 mL of methanol. After agitation, 0.300 mL of extract was added to 2.7 mL of DPPH• (6 × 10⁻⁵ M) [15]. Percentage of inhibition of DPPH radical produced by a standard solution mixture at a concentration equivalent to the amount (mg) of honey calculated in the DPPH assay of honey samples. The mixture was shaken vigorously and incubated for 2 hours in the dark. Methanol was used as the blank. The absorbance was measured at 517 nm. The antiradical activity was expressed as percentage of inhibition (I%) of the sample (As) compared to the initial concentration of DPPH• (Ac) according to the formula: I%=(Ac × As/Ac) × 100.

Results and Discussion

Chemical-physical analysis

Table 1 reports means and standard deviations of the physicochemical parameters analyzed: moisture, pH, °Brix and ash content. The water content of honey is related to its botanical origin, atmospheric and environmental conditions during the production and storage conditions. When the water content is greater than 20%, it may cause fermentation. The optimal value of the water ranges around 17%, although there are honeys with a percentage of water even higher than 21% [16]. In this work it was found a difference between the chestnut honey and the other two types. In fact, the first has a value of moisture of 21.2 ± 1.79, while the other two types have a moisture value near to the optimal one, that is of 16.4 ± 1.81 for the honey of acacia and 17.6 ± 1.78 for the honey of sulla.

Table 1: Physicochemical characteristics of 3 honey samples. aStandard Deviation.

| Sample  | Acacia | Chestnut | Sulla | Mean Range |
|---------|--------|----------|-------|------------|
| Moisture(g 100 g⁻¹) | 16.4 ± 1.81 | 21.2 ± 1.79 | 17.6 ± 1.78 | ±21 |
| pH | 3.5 ± 0.02 | 6.7 ± 0.02 | 3.4 ± 0.02 | 3.5-4.5 |
| Brix (°Bx) | 78.2 ± 0.8 | 75.1 ± 0.7 | 78.7 ± 0.8 | 76-81 |
| Ash (g 100 g⁻¹) | 0.19 ± 0.02 | 0.27 ± 0.05 | 0.45 ± 0.05 | ±0.6 |

The color of honey is one of the factors that determine its price on the world market, and also its acceptability by consumers. During storage, the darkening may cause the darkening of honey and changes in its organoleptic properties, with consequent adverse effects on its quality that can mask its original aroma. The rate of darkening has been correlated to the composition of honey and the storage temperature. Lynn et al. [18] indicated that the main causes of darkening in honey could be: (a) Maillard reaction; (b) combination of tannates and other oxidized polyphenols with iron salts; (c) the instability of fructose (caramelization reaction). The content and the type of natural polyphenols (such as flavonoids) affect the color of the fresh honey, and their degradation reactions can cause variations in color during storage [19].

In Figure 1 are reported the spectra of the three types of honey. As it can see, chestnut honey shows absorption peaks at wavelengths higher than those present in acacia and sulla honey. This outcome is likely due to its darker color with respect to the other two types of honey.

Determination of the content of mineral by icp-oes

Figure 2 shows potassium values in the three types of honey. In the chestnut honey the potassium value was much higher than in the other two types of honey (acacia: 84.4 ± 4.26; Sulla: 430.47 ± 7.75 mg kg⁻¹). The value of 2761 ± 37.75 mg kg⁻¹ of potassium found in the chestnut honey exceeds even the average value reported in literature [20]. According to Miret et al. [20], potassium is present in honey in significant amount. In fact, it is the mineral with the highest concentration present in honey, with an average value of 634 mg kg⁻¹ (78%).

The high concentration of potassium in chestnut honey is a feature that can be used to differentiate it from the other type of honey.
Additional elements detected in high concentrations, are sodium, calcium and magnesium (Table 2).

The values of Na, Mg and Ca found in this study are slightly different from those reported in the literature. In chestnut honey Miret et al. [20] report Na values ranging from 77.10 to 116.65 mg kg\(^{-1}\), for Mg values ranging from 47.40 to 110.81 mg Kg\(^{-1}\) and for Ca values ranging from 66.37 to 135.25 mg Kg\(^{-1}\). In acacia honey Marghitas et al. [21] report values of Na, Mg and Ca of respectively of 13.02 ± 5.89, 5.70 ± 1.11 and 3.05 ± 1.80. In Sulla honey Pisani et al. [22] report values of Ca of 172 ± 44 mg Kg\(^{-1}\). These discrepancies could be due to differences in the matrix, production area and environmental conditions.

Table 3 reports a series of minor constituents in the three types of honey. It is worth mentioning that low levels of iron (<2.79 mg kg\(^{-1}\)) were found in acacia and sulla honeys, while the levels of this metal in chestnut honey is higher (>4.26 mg kg\(^{-1}\)).

This is in agreement with the characteristically low levels of iron in clear honeys, differently from dark honeys that contain higher levels of such metal. The results obtained by Lynn et al. [18] confirm that the iron salts are the main compounds responsible for the obscuration of the honey.

Contaminants are reported in Table 4. The percentage of contaminants in honey must be such that it does not represent a danger to the human health. The analyses carried out in this work evaluated the quality of the honey from the point of view of toxic residues. Among these, particularly important is the concentration of lead for which, despite the lack of legislative limits in Italy, there are specific indications of the O.M.S. on the Provisional Tolerable Weekly Intake.
Intake (P.T.W.I.), corresponding to 3 mg for an adult; this value is limited to 10 times lower for children [23]. Lead is the contaminant found in greater quantities in all three types of honey, with values of 0.86 ± 0.24 mg kg$^{-1}$ for acacia honey, 0.96 ± 0.16 mg kg$^{-1}$ for chestnut honey and 0.99 ± 0.29 mg kg$^{-1}$ for sulla honey. However, these values do not represent a danger to the human health, as mentioned.

| Sample | Acacia | Chestnut | Sulla |
|--------|--------|----------|-------|
| Pb     | 0.86 ± 0.24 | 0.96 ± 0.16 | 0.99 ± 0.29 |
| Cr     | 0.24 ± 0.10 | 0.03 ± 0.04 | 0.00 ± 0.09 |
| Ni     | 0.14 ± 0.02 | 0.05 ± 0.02 | 0.06 ± 0.00 |
| As     | 0.17 ± 0.10 | 0.01 ± 0.06 | 0.00 ± 0.06 |

Table 4: Values of contaminants in honey. *Values are expressed as mean (mg kg$^{-1}$ honey). bStandard Deviation.

Gas chromatography-mass spectrometry

The evaluation of the authenticity of honey in terms of botanical origin is a major issue for consumers and for the industry. In fact, consumers require a product whose characteristics correspond to those reported in the label. Authentication of the product is essential from an economic point of view, in order to prevent unfair competition. In recent years much attention has been dedicated to the development of alternative analytical methods for evaluating the botanical origin of the honey [24]. The characterization of the volatile profile has been shown to be effective for the evaluation of the botanical origin of honey. In fact, the volatile profile represents the chemical fingerprint of honey of different botanical origin, since the nature and quantity of the volatile compounds depend on the floral source [25]. Specific volatile compounds derived from original nectar sources are in all likelihood responsible for the specific aroma of unifloral honeys. These volatile compounds have been proven to be adequate to authenticate floral origin of honeys. The Table 5 shows the amount of the volatile molecules detected in the three samples of honey.

| Sample                      | Acacia RPA ± SD | Chestnut RPA ± SD | Sulla RPA ± SD |
|-----------------------------|-----------------|-------------------|----------------|
| Hexane                      | 0.91 ± 0.03     | 0.41 ± 0.01       | 0.19 ± 0.00    |
| dimethyl sulfide            | 0.28 ± 0.02     | 0.86 ± 0.02       | 1.49 ± 0.04    |
| Octane                      | 1.91 ± 0.05     | 4.10 ± 0.08       | 3.13 ± 0.09    |
| 2-propanone (acetone)       |                 | 1.92 ± 0.04       |                |
| ethyl acetate               | 2.86 ± 0.06     | 0.19 ± 0.01       | 1.53 ± 0.04    |
| 2-propanol 2-methyl         |                 | 2.93 ± 0.06       |                |
| butanal 2-methyl            | 0.20 ± 0.02     | 0.39 ± 0.01       | 0.34 ± 0.01    |
| butanal 3-methyl            | 0.56 ± 0.02     | 0.72 ± 0.02       | 0.57 ± 0.02    |
| ethanol                     | 1.87 ± 0.04     | 3.29 ± 0.07       | 0.65 ± 0.02    |
| furan 2-ethyl               |                 | 0.91 ± 0.02       |                |
| 2,3-butandiol               | 0.93 ± 0.03     | 2.16 ± 0.04       | 1.01 ± 0.03    |
| 2-butanol 2-methyl          | 0.13 ± 0.02     | 3.36 ± 0.07       | 0.72 ± 0.02    |
| isobutyl acetate            | 1.30 ± 0.03     | 3.06 ± 0.07       |                |
| cloroformio                 | 0.35 ± 0.02     | 0.39 ± 0.01       | 0.28 ± 0.01    |
| 3-buten-2-ol,2-methyl       | 1.02 ± 0.03     | 0.20 ± 0.01       | 0.81 ± 0.02    |
| Toluene                     | 1.31 ± 0.03     |                   |                |
| dimethyl disulfide          |                 | 0.21 ± 0.01       |                |
| hexanal                     | 2.19 ± 0.05     | 0.44 ± 0.01       | 1.78 ± 0.05    |
| isobutanol                  | 0.30 ± 0.02     | 0.79 ± 0.02       | 0.35 ± 0.01    |
| 2-pentanol-2-methyl         | 1.80 ± 0.04     | 0.50 ± 0.01       |                |
| isoamyl acetate             | 0.43 ± 0.02     |                   |                |
| 3-penten-2-one-4-methyl     | 1.04 ± 0.02     |                   |                |
| Substance                        | First Value   | Second Value | Third Value |
|---------------------------------|---------------|--------------|-------------|
| p-xylene                        | 0.12 ± 0.02   |              | 0.21 ± 0.00 |
| M-methylpyrrole                 | 0.32 ± 0.01   |              |             |
| 1-butanol                       | 0.09 ± 0.02   | 0.11 ± 0.00  | 0.20 ± 0.00 |
| 2-hexanol                       | 0.44 ± 0.01   |              |             |
| 2-heptanone                     | 0.11 ± 0.02   | 0.07 ± 0.00  | 0.27 ± 0.01 |
| heptanal                         | 1.42 ± 0.04   | 0.38 ± 0.01  | 1.26 ± 0.04 |
| 2-methyl-2-hexanol              | 0.35 ± 0.01   |              |             |
| limonene                         | 0.22 ± 0.02   | 0.10 ± 0.00  | 0.29 ± 0.01 |
| 2-butenal-2-methyl              | 1.98 ± 0.04   | 0.72 ± 0.02  |             |
| isoamyl alcohol                 | 2.90 ± 0.06   | 4.14 ± 0.08  | 1.58 ± 0.05 |
| 2-hexenal (E)                   | 0.16 ± 0.02   |              | 0.16 ± 0.00 |
| furan 2-pentyl                   | 0.09 ± 0.02   | 0.38 ± 0.01  | 0.07 ± 0.00 |
| hexanoic acid ethyl ester       | 0.22 ± 0.02   |              | 0.14 ± 0.00 |
| 3-buten-1-ol,3-methyl           | 1.59 ± 0.04   | 6.52 ± 0.13  | 2.31 ± 0.07 |
| styrene                         | 0.17 ± 0.02   | 5.08 ± 0.10  | 0.07 ± 0.00 |
| p-cymene                        | 0.33 ± 0.02   | 0.69 ± 0.02  | 0.04 ± 0.00 |
| acetoin (2-butanone-3-hydroxy)  | 0.51 ± 0.02   | 0.22 ± 0.01  |             |
| octanal                         | 2.10 ± 0.05   | 2.01 ± 0.04  | 1.98 ± 0.06 |
| 1-pentanol-2-methyl             | 0.54 ± 0.02   | 0.72 ± 0.02  | 1.51 ± 0.04 |
| 2-buten-1-ol,3-methyl           | 1.07 ± 0.03   | 5.93 ± 0.12  | 1.44 ± 0.04 |
| 6-methyl-5-hepten-2-one         | 0.48 ± 0.02   | 0.90 ± 0.02  | 0.13 ± 0.00 |
| hexanol                         | 0.74 ± 0.03   | 4.24 ± 0.09  | 10.74 ± 0.32|
| 3-hexen-1-ol (E)                | 0.26 ± 0.02   | 1.17 ± 0.02  | 0.21 ± 0.00 |
| 3-hexen-1-ol (Z)                | 2.24 ± 0.05   |              | 0.59 ± 0.02 |
| nonanal                         | 9.37 ± 0.19   | 16.20 ± 0.32 | 15.65 ± 0.47|
| 2-octenal (E)                   | 0.37 ± 0.02   | 2.43 ± 0.05  | 0.24 ± 0.01 |
| linalool oxide                  | 9.26 ± 0.19   | 3.77 ± 0.08  | 9.41 ± 0.28 |
| 1-octen-3-ol                    | 0.87 ± 0.03   | 0.30 ± 0.01  | 0.76 ± 0.02 |
| 2,3,5-trimethylfuran            | 0.21 ± 0.01   |              |             |
| acetic acid                     | 3.98 ± 0.08   | 1.47 ± 0.03  | 3.02 ± 0.09 |
| furfural                        | 2.13 ± 0.05   | 0.46 ± 0.01  | 2.96 ± 0.09 |
| trans linalool oxide            | 2.77 ± 0.06   | 1.86 ± 0.04  | 3.98 ± 0.12 |
| 1-hexanol,2-ethyl               | 2.85 ± 0.06   | 0.62 ± 0.01  | 1.75 ± 0.05 |
| decanal                         | 1.91 ± 0.05   | 3.77 ± 0.08  | 2.54 ± 0.07 |
| 2-acetyl furan                  | 0.66 ± 0.02   |              | 0.55 ± 0.01 |
| benzaldehyde                    | 4.52 ± 0.10   | 10.67 ± 0.21 | 3.17 ± 0.09 |
| Compound                        | Value 1 (± Standard Deviation) | Value 2 (± Standard Deviation) |
|--------------------------------|--------------------------------|--------------------------------|
| 2-nonenal                      | 0.65 ± 0.02                    | 0.55 ± 0.01                    |
| lilac aldehyde (isomer I)      | 0.64 ± 0.02                    | 0.41 ± 0.01                    |
| linalool                       | 2.34 ± 0.05                    | 1.48 ± 0.03                    | 1.40 ± 0.04 |
| 1-octanol                      | 1.19 ± 0.03                    | 3.50 ± 0.07                    | 1.27 ± 0.04 |
| lilac aldehyde (isomer II)     | 0.57 ± 0.02                    | 0.43 ± 0.01                    |
| 2,3-butandiol                  | 1.35 ± 0.04                    | 0.25 ± 0.01                    |
| lilac aldehyde (isomer III)    | 0.82 ± 0.03                    | 0.20 ± 0.01                    | 0.44 ± 0.01 |
| Alfa isosopherone              | 0.64 ± 0.02                    | 0.49 ± 0.01                    | 0.34 ± 0.01 |
| (4-terpineol)                  |                                | 0.78 ± 0.02                    |
| hotrienol                      | 4.51 ± 0.10                    | 0.46 ± 0.01                    | 17.11 ± 0.52 |
| benzaldehyde 2-methyl          |                                | 0.81 ± 0.02                    |
| p-menth-1-en-9-al              | 0.66 ± 0.02                    | 1.05 ± 0.03                    |
| methyl benzoate                | 0.63 ± 0.02                    | 0.82 ± 0.02                    | 0.75 ± 0.02 |
| ethyl decanoate                | 0.42 ± 0.02                    | 0.27 ± 0.01                    |
| benzene acetaldehyde           | 3.07 ± 0.07                    | 2.07 ± 0.04                    | 2.28 ± 0.07 |
| acetophenone                   |                                | 10.25 ± 0.20                   |
| 1-nonanol                      |                                | 6.42 ± 0.13                    |
| benzaldehyde,3-methyl          | 1.11 ± 0.03                    | 1.63 ± 0.05                    |
| 2(3H)-furanone,5-ethenylidihydro-5-methyl | 1.57 ± 0.04 | 0.10 ± 0.00 |
| butanoic acid 2-methyl         | 1.00 ± 0.03                    | 0.41 ± 0.01                    |
| 4-hexen-1-ol 5-methyl-2-(1-methylethenyl) | 0.26 ± 0.02 | 1.08 ± 0.02 |
| benzaldehyde 2-hydroxy         |                                | 0.18 ± 0.00                    |
| 4-oxo isophorone               | 0.70 ± 0.02                    | 0.35 ± 0.01                    |
| alfa terpineol                 | 0.50 ± 0.02                    | 0.37 ± 0.01                    | 0.35 ± 0.01 |
| borneol                        | 3.41 ± 0.07                    | 4.38 ± 0.09                    | 0.06 ± 0.00 |
| dodecanal                      | 0.92 ± 0.03                    | 0.24 ± 0.01                    | 1.72 ± 0.05 |
| 2-furan-methanol,5-methyl      | 0.20 ± 0.02                    | 0.27 ± 0.01                    | 0.42 ± 0.01 |
| geranial                       | 0.38 ± 0.02                    | 0.39 ± 0.01                    |
| epoxy linalol (2H-pyrans-3-ol,6-ethenyl tetrahydro-2,6,8-trimethyl) | 3.65 ± 0.08 | 0.41 ± 0.01 | 0.18 ± 0.00 |
| 2,5-hexanediol 2,5-dimethyl    | 0.31 ± 0.02                    | 1.63 ± 0.03                    | 0.08 ± 0.00 |
| 1-(2-butoxyethoxy)ethanol      | 0.43 ± 0.02                    | 0.44 ± 0.01                    |
| butanoic acid 3-methyl         | 0.41 ± 0.02                    |                                |
| octadecanal                    | 3.33 ± 0.07                    | 0.32 ± 0.01                    | 0.78 ± 0.02 |
| beta damascenone               | 0.13 ± 0.02                    | 0.48 ± 0.01                    | 0.09 ± 0.00 |
| furan 3-phenyl                 |                                |                                | 0.09 ± 0.00 |

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hexanoic acid 0.71 ± 0.02 0.71 ± 0.02
Geranyl acetone 0.34 ± 0.02 0.39 ± 0.01
3-methyl cinnoline 0.54 ± 0.01
phenol 2-methoxy 0.24 ± 0.01
butanoic acid butyl ester 1.51 ± 0.04 0.37 ± 0.01
benzyl alcohol 0.24 ± 0.02 6.85 ± 0.14 0.82 ± 0.02
phenylethyl alcohol 1.02 ± 0.03 4.72 ± 0.09 1.43 ± 0.04
quinoline 0.08 ± 0.00
1,5-octadiene-3,7-diol-3,7-dimethyl 0.23 ± 0.01 0.88 ± 0.02
hexanoic acid 2-ethyl 0.86 ± 0.03 0.61 ± 0.02
2,4-diaminophenol 0.42 ± 0.02 1.61 ± 0.05
phenol 0.31 ± 0.02 0.39 ± 0.01 0.32 ± 0.01
2(3H) furanone dihydro-5-pentyl 0.28 ± 0.02 0.35 ± 0.01 0.41 ± 0.01
2-propenal 3-phenyl 1.73 ± 0.04 1.68 ± 0.03 0.14 ± 0.00
octanoic acid 0.30 ± 0.02 0.63 ± 0.02
2-pentadecanone,6,10,14-trimethyl 0.75 ± 0.03 0.36 ± 0.01
sesquiterpene (delta selinene) 0.20 ± 0.02 0.64 ± 0.02
2-propen-1-ol 3-phenyl 1.05 ± 0.02
phenol 2,6 dimethyl 0.20 ± 0.01
2-methoxyl-4-vinyl phenol 0.50 ± 0.02 0.16 ± 0.00
ethanone,1-(2-amino phenyl) 0.36 ± 0.02 0.12 ± 0.00 0.24 ± 0.01
sesquiterpene (alpha cadinol) 0.26 ± 0.02 0.20 ± 0.01 0.43 ± 0.01
dihydro methyl jasmonate 0.58 ± 0.02 0.17 ± 0.00
2-ethyl hexyl salicylate 0.61 ± 0.02 0.46 ± 0.01

| Table 5: Volatile compounds of three types of honey, a Values expressed as relative peak area %, b Relative peak area, c Standard Deviation. |

About 300 aroma compounds have been separated by gas chromatography, but only 100 were well identified in chestnut, acacia and sulla honey. Specifically chestnut honey is characterized by high amounts of acetophenone. Indeed Guyot et al. [26] proposed the acetophenone as a compound ‘guide’ typical of this honey. This suggests that the chestnut honey differs from the other two types for which no specific markers of the honey botanical origin were found.

**Estimation of total phenolic and flavonoid contents**

The concentration of total polyphenols is shown in Figure 3. The polyphenol test employs the Folin-Ciocalteu, a mixture of phosphotungstic acid and phosphomolybdic acid which is reduced by the oxidation of phenols. The reagent is presented as a yellow solution after the reaction in the presence of phenolic compounds is reduced to a mixture of the blue oxides of tungsten and molybdenum.

The analysis provides a figure which corresponds to the total content of polyphenols in relation to this colorimetric change, which is measured by reading absorbance at a wavelength of 760 nm. According to literature [12], the total phenolic content in honey (mg GAE 100 g⁻¹ of honey) is comprised between 32.59 and 114.75 mg with an average of 74.38 ± 20.54 mg, using the standard curve of gallic acid (R²=0.9990). The results obtained in this work are in agreement with those reported in literature.

In fact, the concentration of the polyphenols in honey is reported to be of 134.2 ± 0.95 mg GAE 100 g⁻¹. The honey of acacia and chestnut showed values of 118.6 ± 0.87 and 95.6 ± 0.80 respectively. Interestingly, the sulla honey shows a value of polyphenols higher than the average value reported in literature.

Flavonoids in the diet can be classified as flavonols, flavanones, flavones, anthocyanidins and isoflavones. They feature a wide range of biological effects, such as antibacterial, anti-inflammatory, anti-allergic and anti-thrombotic effects. The flavonoids act as antioxidants in a variety of ways, including the direct capture of reactive oxygen species, inhibition of enzymes responsible for the production of superoxide
anions, chelation of transition metals involved in the processes of the formation of radicals and prevention of peroxidation process by reducing alkoxy radicals and peroxidic [12].

The method of aluminum chloride [27], allows the determination of the content of total flavonoids, separating their contribution from that of the polyphenols. The reagent AlCl₃ is presented as a colorless mixture that following the reaction with the flavonoids is reduced to a mixture of straw yellow color. According to Meda et al. [12], the total content of flavonoids of honey samples (mg QE 100 g⁻¹) varies between 0.17 and 8.35 mg with mean value of 2.57 ± 9.2 mg. Figure 4 reports the content of these bioactive molecules in chestnut, Sulla and acacia honey. Chestnut honey shows a content of flavonoids twice as much as the flavonoids contained in acacia and sulla honeys.

DPPH Assay

In Figure 5 the results of the antioxidant activities of the three samples are reported. The antiradical activity test, which involves the reducing activity of antioxidant molecules against the DPPH •, characterized by a dark purple color. In the presence of an antioxidant agent DPPH • loses its color, becoming a good indicator of the antioxidant activity contained in a sample. The results of this work differ slightly from those reported in the literature [28]. The major antioxidant activity is been obtained for the chestnut honey, with a percentage of inhibition equal to 31.1%.

Conclusion

In conclusion, the honey, especially that one that does not undergo the process of thermization, thus preserves its peculiarities, cannot be considered only a sweetener, but a functional food that is beneficial to the human health. In fact, the high content of bioactive molecules, the antioxidant activity and the concentration of some macro elements make this food absolutely superior compared to other sweeteners commonly used.

In this study we carried out analyses that allow the differentiation of different types of honey.

The sulla honey, for example, shows higher concentrations of polyphenols, while chestnut honey has a higher content of potassium and exhibits some volatile molecules not present in the other honeys analyzed.

The different types of honey can be discriminated by means of the profile of volatile compounds and the different concentration of some mineral elements, so some of these parameters can be used as markers of product traceability.

Moreover, the high concentration of some macro elements such as potassium, especially in the chestnut honey, makes it especially useful in individuals with deficiency of these mineral or practicing sports.

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