High hydroxycinnamic acids contents in fennel honey produced in Southern Italy

Antonio Vella[1], Cammilleri Gaetano*[1,2], Pulvirenti Andrea[2], Galluzzo Francesco Giuseppe[1], Randisi Barbara[1], Giangrosso Giuseppe[1], Macaluso Andrea [1], Gennaro Samuele[3], Ciaccio Gabriele[1], Cicero Nicola[4], Ferrantelli Vincenzo[1]

1 Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", via Gino Marinuzzi 3, 90142 Palermo, Italy.

2 Dipartimento Scienze della Vita, Università degli studi di Modena e Reggio Emilia, Via Università 4, 41121 Modena, Italy

3. Freelance prevention technician

4 Dipartimento SASTAS, Università degli studi di Messina, Polo Universitario dell'Annunziata, 98168 Messina, Italy.

*corresponding author: gaetano.cammilleri86@gmail.com Tel: +390916565258

Abstract

A total of 122 honey samples (*Apis mellifera* ssp. Ligustica) collected from Southern Italy were examined for floral identification by melissopalynological examination and for polyphenols detection by an LC–ESI-Orbitrap^{TM} -MS/MS method. The melissopalynological examination confirmed all the samples examined as fennel (*Foeniculum vulgare*) unifloral variety. The analytical method carried out for polyphenols detection showed satisfactory linearity and recovery values, achieved during the validation of the method. Very high amounts of flavonols (kaempferol and quercetin) and hydroxycinnamic acids (caffeic acid, chlorogenic acid and ferulic acid), were found in all the samples examined. Among the hydroxycinnamic acids group, caffeic acid showed the highest mean contents (865.90±67.07 µg/kg). The results of this work confirmed the high presence of phenolic acids with strong free radical–scavenging activity in fennel products such as honey, suggesting their use to reduce oxidative stress.

Keywords: *honey; polyphenols; fennel; high resolution mass spectrometry*

1. Introduction

Honey is a natural mixture that contains nutrients and bioactive compounds (Cicero et al. 2017; Campone et al. 2019). The sugar spectrum of ripe honey is the end product of a whole series of enzymatic processes, to which various plant and animal enzymes (such as carbohydrases) contribute (Maurizio 1962). Some of nutrients present in honey, such as polyphenols, are considered the major responsible for a wide range of biochemical activities. (Bogdanov et al. 2008; Tenore et al. 2012; Santini & Novellino 2017; Santini et al. 2018; Daliu et al. 2018; Durazzo et al. 2019). Several flavonoids present in honey, such as apigenin, myricetin, naringenin, quercetin, hesperetin and pinocembrin, due to their antioxidant activity, have neuroprotective, anti-inflammatory and anticarcinogenic properties. The characterisation

and quantification of polyphenolic compounds and other components in honey, that might be responsible for their nutraceutical properties, are essential to improve our knowledge about the honey potential effects for the human health (Lo Dico et al. 2019). Honey composition is closely related to its botanical origin and to the environmental conditions of the area (Manyi-Loh et al. 2011). At present, the literature is mainly focused on the nutraceutical properties of the principal unifloral production, such as Fabaceae, Rutaceae and Rosaceae; very few studies are focues on the chemical characterisation of Apiaceae unifloral varieties (Ramos et al. 1999; Ramos et al. 2002). The Apiaceae family is very rich in plant species; at this family belong herbs, rich in aromatic substances, with umbel and fruit inflorescences composed of two achenes welded along a central axis. The banner function is carried out by the entire inflorescence, the small flowers are nectariferous and visited by diptera, lepidoptera, beetles and different species of hymenoptera, including honey bees. The floral diversity of Sicilian territory (Southern Italy) is well suited to the production of various types of unifloral honey. However, Sicilian honeys are not guaranteed by EU quality labels (PDOs and PGIs), probably due to the lack of data regarding their physical and chemical properties that do not allow to have comprehensive comparison. In this work we examined samples of unifloral honey produced in western Sicily (Southern Italy) for the detection of polyphenols content by an automated TurboFlow^{TM} –liquid chromatography Orbitrap TM high-resolution mass spectrometry method (LC–ESI-Orbitrap^{TM} -MS/MS) in order to have detailed information on the nutraceutical properties of this product.

2. Results and Discussion

All the honey samples examined correspond to fennel (*Foeniculum vulgare*) unifloral variety as confirmed by the melissopalynological examination, showing a relative frequency of *Foeniculum vulgare* pollen over 45%. The melissopalynological examination also verified the presence of Brassicaceae pollens such as *Diplotaxis* erucoides etc. The validation of the

method produced satisfactory linearity and recovery values, confirming the reliability of the method proposed. A total of 12 phenolic compounds were detected in all the honey samples examined (Table 1). The highest contents found corresponded to the flavonols and hydroxycinnamic acids groups, accounting for 88% of the total polyphenols contents. In particular, kaempferol and quercetin showed the highest mean contents, with maximum values of 5486.23 µg/kg and 3620.8 µg/kg, respectively. Of the three compounds examined in the hydroxycinnamic acids group (caffeic acid, chlorogenic acid and ferulic acid), caffeic acid presented the highest avereage content in all the samples analysed (865.90 ± 67.07 µg/kg), followed by chlorogenic acid (210.90 ± 15.51 µg/kg). Among the hydroxybenzoic acids, only vanillic and syringic acid were found, showing maximum values of 483.10 µg/kg and 521.65 µg/kg, respectively. The results of this work showed that Mediterranean insular climates can facilitate the growth of Apiaceae family plants. In Sicily and Sardinia is occasionally produced honey in which pollen analysis shows a strong prevalence of species belonging to this family. In this case, it is often difficult to trace the original species, due to the difficulty in identifying the pollen grains (the Apiaceae are a very homogeneous group from the palynological point of view, with small differences between the species) and for the lack of precise information from part of the producers. Spanish studies have found *Foeniculum vulgare*-type pollen in all the samples of multifloral honeys from different apiaries (Ramos et al. 1999; Ramos et al. 2002; Ramos & Ferreras 2006).

In 2011, Parvanov et al. (2011), detected for the first time the main quality parameters of Bulgarian fennel (*Foeniculum vulgare* Mill.) bee honey; however, no information were reported about the polyphenols contents. The results of this work confirmed the high hydroxycinnamic acids contents present in fennel, according to what was found by Singh et al. (2004) revealing phenolic acids in fennel extract, all with antitumor activity. Fennel seed extract can significantly decrease serum aminotransferase (ALT), aspartate aminotransferase

(AST) and alkaline phosphatase (ALP), suggesting their hepatoprotective activity. The mechanisms by which fennel seed extracts offered their protective effects against hepatotoxicity are based on their antioxidant abilities, which may be responsible for protecting the hepatic cells against the oxidative stress, possibly by increasing the endogenous defensive capacity of the liver to combat oxidative stress (Mohamad et al. 2011; Mannaa et al. 2015). Furthermore, recent studies have indicated the usefullness of fennel extracts as antibacterial for such microorganisms as *Candida albicans*, (Parvanov et al. 2011) *Helicobacter pylori* and *Campylobacter jejuni* (Cwikla et al. 2010). However, the possible risk associated with the presence of toxic substances present in fennel such as mycotoxins should not be underestimated (Llewellyn et al. 1992; Santini et al. 2009; Mikušová et al. 2013). In conclusion, this work reported for the first time the phenolic composition of fennel honey produced in Mediterranean countries. Our results underlined that fennel honey have high nutraceutical properties that could provoke a great interest among consumers in the future.

3. Experimental

Sampling plan

A total of 122 honey samples were collected from March to December 2018 in common honeybees (*Apis mellifera* ssp. Ligustica) apiarists of Castro land, Corleone (western Sicily 37°45'51.8"N 13°17'47.8"E). The honey samples were examined for floral origin by melissopalynological analysis, according to the protocol of Ohe et al. (2004) by consulting the atlas of Ricciardelli d'Albore (Ricciardelli D'Albore 1998). The samples were stored at controlled temperature and humidity conditions (about 25 °C and 60% humidity) and protected from light and heat for the determination of polyphenols contents according to the protocol of Lo Dico et al. (Lo Dico et al. 2019).

Sample preparation and LC–ESI-MS/MS analysis

Fifty mL of 30 mM ammonium acetate (pH 5)/ methanol mixture (50:50 v/v) were added to 5 g of honey samples weighted on analytic balance. The mixture was shaken for two minutes by vortex and filtered by a PTFA syringe filter Ø 0.20 µm (Millipore, Bedford, MA, USA) to remove every impurities that could compromise the chromatographic analysis. The samples were transferred into an amber vial for LC analysis.

The samples were extracted and purified by a Cyclone P column (50 mm ×0.5 m, 60 µm particle size, 60 Å pore size, Thermo Fisher Scientific, Waltham, MA, USA); a Hypersil Gold column (2.1 mm × 100 mm, 1.7 µm particle size) was employed for the chromatographic separation. The polyphenols contents were assessed by a Q-Exactive Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an HESI (Heated Electro Spray Ionization) configured at positive and negative polarity modes. The chromatographic and Mass spectrometer conditions were according to Lo Dico et al. (Lo Dico et al. 2019). Briefly, the chromatographic conditions were as follow: column temperature, 30°C; sample temperature, 6°C; flow rate, 0.2 mL min $^{-1}$. The autosampler sample holder temperature was maintained at 7 °C. The mobile phase consisted of eluent A: 30 mM ammonium acetate (pH 5), eluent B: methanol, eluent C: water (0.5% formic acid), and eluent D: acetonitrile/acetone/2-propanol (4:3:3). The sample injection volume was 5 µL for a total run time of 18 min. The scan mode was full scan, with scan range (m/z) 100–700; microscans, 1 m/z; positive resolution: 70,000; FT automatic gain control (AGC) target: 3×10^6; maximum IT: 100 ms; negative resolution: 35,000; automatic gain control (AGC) target: 1 × 10 6 ; maximum IT: 100 ms.

The method was validated according to EN ISO/IEC 17025:2005 parameters (Giaccone et al. 2017; Giaccone et al. 2018; Lo Dico et al. 2018). All the results under the LOQ of the method were considered for the statistical analysis as half of the LOQ values, according to Helsel (2005).

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Compound	**Mean±SD (µg/Kg)**
Apigenin	386.20 ± 53.01
Kaempferol	3474.15 ± 521.00
Quercetin	1115.23 ± 233.90
Myricetin	45.56 ± 7.28
Hesperidin	10.42 ± 1.93
Naringenin	81.42 ± 9.70
Pinocembrin	351.60 ± 133.2
Caffeic acid	865.90 ± 67.07
Chlorogenic acid	210.90 ± 15.51
Ferulic acid	97.06 ± 6.70
Syringic acid	304.00±32.2
Vanillic acid	295.5 ± 43.02

Table 1: Average values of the Phenolic compounds found in the samples examined (N = 122).