Short Communication

High hydroxycinnamic acids contents in fennel honey produced in Southern Italy

Antonio Vella\textsuperscript{a}, Gaetano Cammilleri\textsuperscript{a,b}, Andrea Pulvirenti\textsuperscript{b}, Francesco Galluzzo\textsuperscript{a}, Barbara Randisi\textsuperscript{a}, Giuseppe Giangrosso\textsuperscript{a}, Andrea Macaluso\textsuperscript{a}, Samuele Gennaro\textsuperscript{c}, Gabriele Ciaccio\textsuperscript{a}, Nicola Cicero\textsuperscript{d} and Vincenzo Ferrantelli\textsuperscript{a}

\textsuperscript{a}Istituto Zooprofilattico Sperimentale della Sicilia “A. Mirri”, Palermo, Italy; \textsuperscript{b}Dipartimento Scienze della Vita, Università degli studi di Modena e Reggio Emilia, Modena, Italy; \textsuperscript{c}Freelance prevention technician; \textsuperscript{d}Dipartimento SASTAS, Università degli studi di Messina, Polo Universitario dell’Annunziata, Messina, Italy

\textbf{Abstract}

A total of 122 honey samples (\textit{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\textsuperscript{TM} -MS/MS method. The melissopalynological examination confirmed all the samples examined as fennel (\textit{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.
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 focuses 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™–liquid chromatography Orbitrap™ high-resolution mass spectrometry method (LC–ESI-Orbitrap™ -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. 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 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 average 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; Manaa et al. 2015). Furthermore, recent studies have indicated the usefulness 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.

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

| 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     |
3. Experimental

3.1. 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 (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. (2019).

3.2. 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: $3 \times 10^6$; maximum IT: 100 ms; negative resolution: 35,000; automatic gain control (AGC) target: $1 \times 10^6$; 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).

Disclosure statement

No potential conflict of interest was reported by the author(s).
References

Bogdanov S, Jurendic T, Sieber R, Gallmann P. 2008. Honey for nutrition and health: a review. J Amer Coll Nutr. 27(6):677–689.
Campone L, Celano R, Piccinelli AL, Pagano I, Cicero N, Sanzo RD, Carabetta S, Russo M, Rastrelli L. 2019. Ultrasound assisted dispersive liquid–liquid microextraction for fast and accurate analysis of chloramphenicol in honey. Food Res Int. 115:572–579.
Cicero N, Naccari C, Cammilleri G, Giangrosso G, Cicero A, Gervasi T, Tropea A, Albergamo A, Ferrantelli V. 2017. Monitoring of neonicotinoid pesticides in beekeeping. Nat Prod Res. 31(11):1258–1262.
Cwikla C, Schmidt K, Matthias A, Bone KM, Lehmann R, Tiralongo E. 2010. Investigations into the antibacterial activities of phytotherapeutics against Helicobacter pylori and Campylobacter jejuni. Phytother Res. 24(5):649–656.
Dalili P, Santini A, Novellino E. 2018. A decade of nutraceutical patents: where are we now in 2018?. Expert Opin Ther Pat. 28(12):875–882.
Durazzo A, Lucarini M, Souto EB, Cicala C, Ciaizzo E, Izzo AA, Novellino E, Santini A. 2019. Polyphenols: a concise overview on the chemistry, occurrence, and human health. Phytotherapy Research. 33(9):2221–2243.
Giaccone V, Cammilleri G, Di Stefano V, Pitonzo R, Vella A, Pulvirenti A, Lo Dico GM, Ferrantelli V, Macaluso A. 2017. First report on the presence of Alloxan in bleached flour by LC-MS/MS method. J Cereal Sci. 77:120–125.
Giaccone V, Cammilleri G, Macaluso A, Pulvirenti A, Vella A, Ferrantelli V. 2018. A LC-HRMS After QuEChERS cleanup method for the rapid determination of dye residues in fish products. Food Anal Methods. 11(3):625–634.
Helsel DR. 2005. Nondetects and data analysis. Statistics for censored environmental data. Nondetects and Data Analysis Statistics for Censored Environmental Data. [accessed 2018 Aug 27]. https://www.cabdirect.org/cabdirect/abstract/20053102639.
Llewellyn GC, Mooney RL, Cheatle TF, Flannigan B. 1992. Mycotoxin contamination of spices — an update. Int Biodeter Biodegr. 29(2):111–121.
Lo Dico G M, Galvano F, Dugo G, D’ascenzi C, Macaluso A, Vella A, Giangrosso G, Cammilleri G, Ferrantelli V. 2018. Toxic metal levels in cocoa powder and chocolate by ICP-MS method after microwave-assisted digestion. Food Chem. 245:1163–1168.
Lo Dico G M, Ulrici A, Pulvirenti A, Cammilleri G, Macaluso A, Vella A, Giaccone V, Lo Cascio G, Graci S, Scuto M, et al. 2019. Multivariate statistical analysis of the polyphenols content for the discrimination of honey produced in Sicily (Southern Italy). J Food Compos Anal. 82:103225.
Mannaa F, Ibrahim NA, Soliman I, Abdel-Wahhab KG, Hassan N, Mohammed SG. 2015. Preventive role of chamomile flowers and fennel seeds extracts against liver injury and oxidative stress induced by an immunosuppressant drug in rats. Hepatoma Res. 1(3):125–135.
Manyi-Loh CE, Ndip RN, Clarke AM. 2011. Volatile compounds in honey: a review on their involvement in aroma, botanical origin determination and potential biomedical activities. IJMS. 12(12):9514–9532.
Maurizio A. 1962. From the raw material to the finished product: honey. Bee World. 43(3):66–81.
Mikušová P, Šrobárová A, Sulyok M, Santini A. 2013. Fusarium fungi and associated metabolites presence on grapes from Slovakia. Mycotoxin Res. 29(2):97–102.
Mohamad RH, El-Bastawesy AM, Abdel-Monem MG, Noor AM, Al-Mehdar HAR, Sharawy SM, El-Merzabani MM. 2011. Antioxidant and anticarcinogenic effects of methanolic extract and volatile oil of fennel seeds (Foeniculum vulgare). J Med Food. 14(9):986–1001.
Ohe WVD, Oddo LP, Piana ML, Morlot M, Martin P. 2004. Harmonized methods of melissopalynology. Apidologie. 35(Suppl. 1):S18–S25.

Parvanov P, Dinkov D, Tananaki C, Mihaylova G. 2011. Sensorial characteristics and composition of Bulgarian’s fennel (Foeniculum vulgare Mill.), bee honey: I. Quality parameters. In: [place unknown].

Ramos I-S, Ferreras C. 2006. Pollen and sensorial characterization of different honeys from El Hierro (Canary Islands). Grana. 45(2):146–159.

Ramos I-S, Pérez BM, Ferreras CG. 1999. Pollen characterization of multifloral honeys from La Palma (Canary Islands). Grana. 38(6):356–363.

Ramos I-S, Pérez BM, Ferreras CG. 2002. Pollen spectra of different unifloral honeys from La Palma (Canary Islands, Spain). Grana. 41(1):48–57.

Ricciardelli D’Albore. 1998. Mediterranean Melissopalynology. http://www.izsum.it/Melissopalynology/.

Santini A, Cammarata SM, Capone G, Ianaro A, Tenore GC, Pani L, Novellino E. 2018. Nutraceuticals: opening the debate for a regulatory framework. Br J Clin Pharmacol. 84(4):659–672.

Santini A, Ferracane R, Meca G, Ritieni A. 2009. Overview of analytical methods for beauvericin and fusaproliferin in food matrices. Anal Bioanal Chem. 395(5):1253–1260.

Santini A, Novellino E. 2017. To nutraceuticals and back: rethinking a concept. Foods. 6(9):74.

Singh UP, Singh DP, Maurya S, Maheshwari R, Singh M, Dubey RS, Singh RB. 2004. Investigation on the phenolics of some spices having pharmacotherapeutic properties. J Herbal Pharmacother. 4(4):27–42.

Tenore GC, Ritieni A, Campiglia P, Novellino E. 2012. Nutraceutical potential of monofloral honeys produced by the Sicilian black honeybees (Apis mellifera ssp. sicula). Food Chem Toxicol. 50(6):1955–1961.