Saudi Journal of Biological Sciences 26 (2019) 252–255

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Freeze-drying duration influences the amino acid and rutin content in honeybee-collected chestnut pollen

Annamaria Ranieri [a,b], Giovanni Benelli [a,*], Antonella Castagna [a], Cristina Sgherri [a], Francesca Signorini [c], Matteo Bientinesi [c], Cristiano Nicolella [c,d], Angelo Canale [a,b]

[a] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy
[b] Interdepartmental Research Center "Nutraceuticals and Food for Health", University of Pisa, Pisa, Italy
[c] Consorzio Polo Tecnologico Magona, via Magona snc, Cecina, Livorno, Italy
[d] Dipartimento di Ingegneria Civile e Industriale, Università di Pisa, Largo Lucio Lazzarino 2, Pisa, Italy

ARTICLE INFO

Article history:
Received 4 May 2017
Revised 16 August 2017
Accepted 17 August 2017
Available online 19 August 2017

Keywords:
Apis mellifera
Castanea sativa
Freeze-drying
Proline
Rutin

ABSTRACT

Honeybee-collected pollen is gaining attention as functional food for human consumption, due to antiproliferative, antiallergic, antibiotic, antidiarrheic and antioxidant activities. Among the different bioactive compounds, flavonoids from bee-collected pollen are currently recognised as powerful antioxidant and antiradical molecules. Traditional conservation methods influence pollen organoleptic properties as well as the contents of nutrients and nutraceutical compounds. Here, freeze-drying (FD) was proposed as a novel conservation method, estimating its adequacy as drying process by the evaluation of changes in free and total amino acids and proline as well as in their ratios. Honeybee-collected chestnut pollen was taken into consideration and the level of rutin, as main flavonoid, was considered as marker compound highlighting the maintenance of pollen nutraceutical properties. Results showed that FD influenced rutin level, depending on the FD duration. However, the free proline to free amino acid ratio was always below 80%, and the free amino acid to total amino acid ratio remained unaltered indicating the adequacy of the FD treatment, which did not affect the nutritional value of chestnut pollen. Overall, this study shed light on the nutraceutical profile of honeybee-collected chestnut pollen, highlighting the promising potential of FD as a novel method to treat pollen for human consumption.

1. Introduction

Beekeeping leads to the production of important foods for human consumption (Buratti et al., 2007; Benelli et al., 2014; Canale et al., 2014a,b; El-Aidy et al., 2015; Khan et al., 2018; Meo et al., 2017; Zoccali et al., 2017). In particular, honeybee-collected pollen is gaining attention as functional food, due to antiproliferative, antiallergic, antibiotic, antidiarrheic and antioxidant activities (Graikou et al., 2011; Marghitas et al., 2009; Medeiros et al., 2008). This bioactive potential mainly depends on high content of several compounds, such as essential amino acids, antioxidants, vitamins and lipids (Soares de Arruda et al., 2013; Krystyjan et al., 2015; Almeida et al., 2016; Campos et al., 2003, 2008; Soares de Arruda et al., 2013; Mărgăoan et al., 2014; Conte et al., 2017). Among the different bioactive compounds, flavonoids from bee-collected pollen are recognised as powerful antioxidant and antiradical molecules (Almaraz-Abarca et al., 2007; Leja et al., 2007; Silva et al., 2006).

Honeybee-collected pollen contains a relatively high amount of water (i.e. from 15 to 30%, w:w), which should be lowered in order to enhance its physicochemical stability and reduce the risk of microbial development. The nutritional value of fresh pollen can be altered by conditioning processes (i.e. artificial drying) carried out with poorly standardized methods (Canale et al., 2016). To avoid the risk of formation of Maillard's compounds pollen-drying process should be done at low temperatures, with short exposure times (Collin et al., 1995). Moreover, the drying process could negatively affect antioxidants, with consequent reduction of the functional value of the final product (Serra Bonvehì et al., 2001).

* Corresponding author.
E-mail address: benelli.giovanni@gmail.com (G. Benelli).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2017.08.011
1319-562X/

Therefore, novel and reliable technologies are needed to prolong the pollen shelf life, leading to high quality food with significant levels of bioactive compounds.

Recently, Canale et al. (2016) showed that microwave-assisted drying offers important advantages for the conservation of bee pollen. In this framework, here we analysed the potential of freeze-drying (FD) as a novel conservation method to reduce water content in chestnut pollen for human consumption. Different times of drying were taken into consideration. Markers of quality for honeybee pollen are needed to warrant the nutritional and biological properties required in the European market. In this context, minimum levels of rutin, a glycosylated flavonoid, free amino acids and proline have been suggested to standardise the commercial quality of honeybee-collected pollen (Serra Bonvehì et al., 2001). Accordingly, the adequacy of FD process of chestnut pollen was evaluated determining the changes in free and total amino acids and proline as well as in their ratios and the level of rutin.

2. Materials and methods

2.1. Pollen freeze-drying

Honeybee-collected chestnut pollen was collected by a beekeeper during July 2015 in chestnut orchards (44 °06′22.7″N 10 °24′02.7″E, Castelnuovo Garfagnana, Lucca, Italy), using a pollen trap (A. Metalori, Italy). Pollen samples were frozen (−20 °C) and transferred to the laboratories within 2 h for conditioning. Pollen floral origin was identified by colour and light microscope examination (Erdtman, 1969). Chestnut pollen was identified by comparison with pollen atlas databases (Erdtman, 1969; Mărghitaş et al., 2009). The presence of chestnut pollen grains in the collected pollen samples was 84.60 ± 3.85% (mean ± SD, n = 20, 5 replicates). After freeze-drying, analytical results were compared with the fresh untreated pollen sample (UP).

Pollen FD was carried out using a lyophiliser Heto PowerDry® LL1500 following the method recently described by Conte et al. (2017). During the whole FD process, the temperature inside the condensation chamber was −115 °C, with full vacuum. After FD treatment for 270, 420 or 540 min, a thermal gravimetric analysis (TGA) was carried out, to evaluate the residual water content. Then, the samples were stored in freezer at −20 °C, waiting for the analyses.

2.2. Rutin quantification by HPLC analysis

The extraction was performed as reported by Canale et al. (2016). Pollen samples (0.5 g) were sonicated with 80% methanol for 30 min, followed by 30 min stirring at 4 °C and 15 min centrifugation at 14000g, 4 °C. The pellet was extracted two additional times without sonication. A total of 15 ml of 80% methanol was used. The extracts were pooled together and filtered (0.45 μm Minisart filters, Sartorius Stedim Biotech, Goettingen, Germany).

Rutin was quantified by HPLC (Spectra System P4000 and UV 6000 LP photodiode array detector, Thermo Fisher Scientific, Waltham, MA) using a Prodigy LC-18 RP column (5 μm particle size, 250 × 4.6 mm, Phenomenex Italia, Castel Maggiore, Italy). Elution was carried out using 5% formic acid in water (solvent A) and 5% formic acid in methanol (solvent B). The flow rate was set at 1 ml min^{-1}, and gradient was as follows: 0–5 min 95% solvent A, 5–10 min 95–88% solvent A, 10–13 min 88% solvent A, 13–35 min 88–71% solvent A, 35–50 min 71–54% solvent A, 50–52 min 54–20% solvent A, 52–57 min 20% solvent A, 57–60 min 20–0% solvent A, 60–70 min 0–95% solvent A, followed by 5 min re-equilibration in the initial condition before the next injection. Rutin was quantified at 340 nm using a curve of commercial standard in the range 1–100 ppm (Sigma Aldrich Chemical Co., St. Louis, MO).

2.3. Total and free amino acid extraction

Free amino acid fraction was obtained as previously reported in Canale et al. (2016). Briefly, 0.1 g bee pollen was homogenized with 80% ethanol by mortar and pestle, followed by a sonication for 5 min. and a centrifugation at 12100 g for 15 min. The supernatants of two subsequent extractions were collected and vacuum dried. Samples, dissolved in MilliQ water, were filtered and used for the determination of free proline and free amino acids.

Total amino acid fraction was obtained incubating bee pollen (0.1 g) with 6 N HCl at 110 °C for 24 h. After cooling, samples were filtered and vacuum dried as previously described (Canale et al., 2016). After re-suspension in MilliQ water, the extracts were used for the determination of total proline and total amino acids.

2.4. Free and total proline determination

Proline was detected in the free and total amino acid fractions essentially following Magné and Lahrer (1992). This procedure, which allows the elimination of carbohydrate interference, makes use of 1% ninhydrin in 60% acetic acid. The chromophore, developed after incubation of samples at 100 °C for 1 h, was extracted by addition of toluene, and absorbance was recorded at 520 nm. A calibration curve in the range 3–30 μg proline was applied.

2.5. Free and total amino acid determination

Free and total amino acids were measured as previously reported in Canale et al. (2016). A 4% ninhydrin solution in ethylene glycol was used. After incubation at 100 °C for 20 min, 50% isopropanol was added as a diluents and absorbance was read at 570 nm. A calibration curve in the range 3–30 μg leucine was applied. Since for this reaction, the relative color yield for proline was reported as zero, to calculate free and total amino acids in the samples it was necessary to sum the amounts derived from the detection at 570 nm with those of proline detected at 520 nm.

2.6. Data analysis

Data on rutin and amino acid content were subjected to one-way analysis of variance (ANOVA) followed by Tukey's HSD test at a 95% confidence level to evaluate the effect of the different FD treatments. We used NCSS 2000 statistical software (NCSS Statistical Software, Kaysville, UT, USA). All analyses were replicated on three independent samples.

3. Results and discussion

The TGA conducted at 120 °C on chestnut pollen freeze-dried for 270, 420 or 540 min showed that the lyophilisation progressively reduced the water content in the treated samples, leading to a residual water content values ranging from 7 to 13%, as reported in Fig. 1.

The rutin concentration was significantly affected by FD treatment ($F_{3,12} = 25.99$, $P < 0.001$), which induced a decrease in rutin level depending on the FD duration and the consequent residual water content. The longest treatment (540 min) allowed the maintenance of the initial rutin concentration, while the intermediate (420 min) and, mainly, the shortest (270 min) treatments led to pollen showing severely reduced rutin levels (−26.5 and −72.5%, respectively) (Fig. 2).

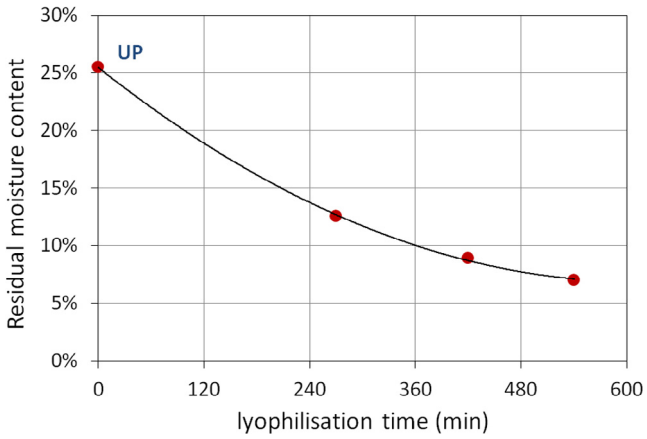

Fig. 1. Decrease of residual water content in honeybee-collected chestnut pollen after freeze-drying treatments with different duration, UP = untreated pollen.

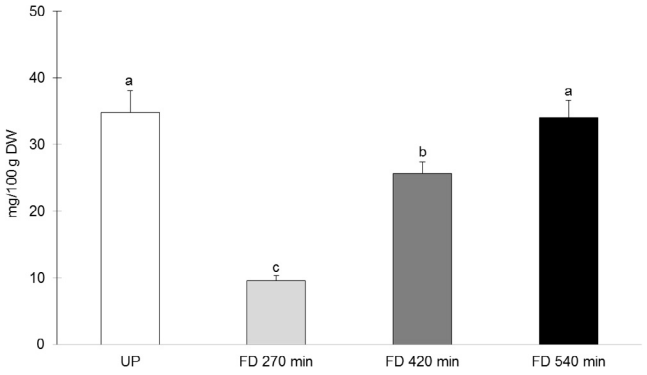

Fig. 2. Rutin concentration of honeybee-collected chestnut pollen before (UP = untreated pollen) and after the freeze-drying treatment, carried out for 270, 420 or 540 min. Data are means of three replicates ± SE. Above each column, different letters indicate significant differences among treatments (ANOVA followed by Tukey's HSD test, $P \leq 0.05$).

Due to rutin sensitivity to long storage periods and to excessive heating during drying process, this flavonoid is considered a marker of quality for honeybee pollen (Canale et al., 2016). Serra Bonvehì et al. (2001) suggest that a minimum quantity of 20 mg rutin /100 g is needed to meet the nutraceutical and biological properties and to standardize the commercial quality of pollen in the European market. The untreated *C. sativa* pollen contained a good amount of rutin (about 32 mg/100 g). Despite a significant reduction, samples freeze-dried for 420 min retained appreciable rutin levels, higher than the reported quality limit. Conversely, a shorter FD (270 min) did not allow the maintenance of the required quality standard. Differences among the treatments could depend on the different residual water content, which negatively affects rutin content. Canale et al. (2016) recently dehydrated *C. sativa* pollen by microwave-assisted drying. Despite the pollen residual moisture (4–15%) was like that obtained in the present experiment, rutin did not showed significant variations, irrespective of microwave power and treatment time (Canale et al., 2016). Factors other than residual moisture are probably involved in accounting for this different response. An opposite effect of different post-harvest conditioning technologies was also observed on pollen lipids. Conte et al. (2017) indeed observed no damaging action due to FD on bee-collected chestnut and willow pollen, while microwave-based treatments led to a reduction of pollen tocopherols.

Maillard's reactions, which lead to a decrease in organoleptic value of pollen, can be avoided if a correct drying is performed. The use of FD allowed maintaining in all samples a minimum quantity of 2% of free amino acid content (Table 1) as required for the standardization of the commercial pollen in the European market (Serra Bonvehì et al., 2001). Even if the percentage of free amino acid to total amino acid ratio did not change with FD, the amounts of free amino acids almost doubled at the intermediate (420 min) and longest (540 min) treatment (Table 1) indicating the higher presence of highly assimilable proteins since, in the form of free amino acids, they are ready to be used directly by the body. Moreover, the free proline to free amino acid ratio was always below 80%, indicating that this treatment did not affect the nutritional value of the bee pollen (Serra Bonvehì and Lòpez Alegret, 1986) as previously observed when a microwave-assisted drying was applied (Canale et al., 2016). In contrast with the microwave-assisted drying treatment, with FD free proline increased and total proline was subjected to a decrease in comparison with the control levels only in the sample with the highest moisture content (270 min). The maintenance of the free amino acids to total amino acids ratio is indicative of the adequacy of FD as drying process which preserved proteins from proteolytic events (Table 1). Total amino acids were quantified at 30 g/100 g DW (data not shown), a value three times higher the minimum estimated for proteins of dried pollen by Campos et al. (2008) and indicative of the high nutritional properties of chestnut pollen.

4. Conclusions

Overall, bee-collected pollen is gaining attention as functional food for human consumption. Among the different bioactive compounds, flavonoids of bee-collected pollen are recognised as powerful antioxidant and antiradical molecules. This research shed light on FD as an alternative drying method to treat honeybee-collected chestnut pollen for human consumption. Honeybee-collected chestnut pollen was taken into consideration and the level of rutin, as main flavonoid, was considered in the maintenance of its nutraceutical properties. We observed that the FD treatments for 420 and 540 min maintained rutin and amino acid parameters in the range required for the standardization of the commercial pollen in the European market. The fact that pollen can contribute significantly to human protein needs, together with the health benefits due to the presence of flavonoids, with special reference to rutin, explains the more and more attention due to pollen as a possible superfood.

Compliance with ethical standards

All national guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Acknowledgments

Two anonymous reviewers kindly improved our work with constructive comments. We would like to thank the Apicoltura Biologica Metalori Aldo (Lucca), which kindly provided chestnut pollen analysed in this study.

Conflict of interest

The Authors declare no conflict of interest.

Table 1
Impact of freeze-drying on honeybee-collected chestnut pollen: free proline, total proline, free amino acids and total amino acids content after freeze-drying treatment lasting 270, 420 or 540 min.

Parameter	UP	FD 270 min	FD 420 min	FD 540 min
Free Pro (mg/g DW)	16.31 ± 1.35 c	22.44 ± 0.82 b	27.55 ± 0.62 a	26.16 ± 0.40 a
Total Pro (mg/g DW)	44.58 ± 1.83 b	36.74 ± 2.91 c	47.42 ± 1.70 ab	51.00 ± 1.94 a
Free Pro/Total Pro (%)	36.58 b	61.08 a	58.09 a	51.29 a
Free AA (mg/g DW)	24.67 ± 0.92 c	34.48 ± 0.71 b	43.22 ± 0.68 a	41.50 ± 0.97 a
Free AA/Total AA (%)	12.90 a	13.48 a	13.63 a	13.87 a
Free Pro/Free AA (%)	66.11 a	65.10 a	63.74 a	63.04 a

UP = untreated pollen.
FD = freeze-drying.
Pro = proline.
AA = amino acids.
Values are means ± standard errors.
Within a row, different letters indicate significant differences among treatments (ANOVA, Tukey's HSD test, $P \leq 0.05$).

Funding

This work was supported by a University of Pisa Grant (Progetti di Ricerca di Ateneo, PRA_2017_13) and Regione Toscana PROAPI (PRAF 2015). G. Benelli is sponsored by Regione Toscana PROAPI (PRAF 2015) and University of Pisa, Department of Agriculture, Food and Environment (Grant ID: COFIN2015_22). Founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

References

Almaraz-Abarca, N., Graça-Campos, M., Ávila-Reyes, J.A., Naranjo-Jiménez, N., Herrera-Corral, J., González-Valdez, L.S., 2007. Antioxidant activity of polyphenolic extract of monofloral honeybee-collected pollen from mesquite (*Prosopis juliflora*, Leguminosae). J. Food Compos. Anal. 20, 119–124.

Almeida, J.F., de Florio Almeida, J., Soares dos Reis, A., Serafini Heldt, L.F., Pereira, D., Bianchin, M., de Moura, C., Plata-Oviedo, M.V., Haminiuk, C.W.I., Ribeiro, I.S., da Luz, C.F.P., Carpes, S.T., 2016. Lyophilized bee pollen extract: A natural antioxidant source to prevent lipid oxidation in refrigerated sausages. *LWT* –. Food Sci. Technol. http://dx.doi.org/10.1016/j.lwt.2016.06.017.

Benelli, G., Benvenuti, S., Desneux, N., Canale, A., 2014. *Cephalaria transsylvanica*-based flower strips as potential food source for bees during dry periods in European Mediterranean basin countries. PloS One 9, e93153.

Buratti, S., Benedetti, S., Cosio, M.S., 2007. Evaluation of the antioxidant power of honey, propolis and royal jelly by amperometric flow injection analysis. Talanta 71 (3), 1387–1392.

Campos, M.G., Webby, R.F., Markham, K.R., Mitchell, K.A., da Cunha, A.P., 2003. Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. J. Agric. Food Chem. 51, 742–745.

Campos, M.G.R., Bogdanov, S., de Almeida-Muradian, L.G., Szczesna, T., Mancebo, Y., Frigerio, C., Ferreira, F., 2008. Pollen composition and standardization of analytical methods. J. Apic. Res. 47 (2). 156-136.

Canale, A., Canovai, R., Cosci, F., Giannotti, P., Benelli, G., 2014a. Survey of Italian honeys for the presence of foreign matter using the filth test. Food Additiv. Contamin. A 31, 905–909.

Canale, A., Cosci, F., Canovai, R., Giannotti, P., Benelli, G., 2014b. Foreign matter contaminating ethanolic extract of propolis: a filth-test survey comparing products from small beekeeping farms and industrial producers. Food Additiv. Contamin. A 31, 2022–2025.

Canale, A., Benelli, G., Castagna, A., Sgherri, C., Poli, P., Serra, A., Mele, M., Ranieri, A., Signorini, F., Bientinesi, M., Nicolella, C., 2016. Microwave-assisted drying for the conservation of honeybee pollen. Materials 9 (5), 363. http://dx.doi.org/10.3390/ma9050363.

Collin, S., Vanhavre, T., Odart, E., Bouseta, A., 1995. Heat treatment of pollens: impact on their volatile flavor constituents. J. Agric. Food Chem. 43, 444–448.

Conte, G., Benelli, G., Serra, A., Signorini, F., Bientinesi, M., Nicolella, C., Mele, M., Canale, A., 2017. Lipid characterization of chestnut and willow honeybee-collected pollen: impact of freeze-drying and microwave-assisted drying. J. Food Compos. Anal. 55, 12–19.

El-Aidy, W.K., Ebeid, A.A., Sallam, A.E.R.M., Muhammad, I.E., Abbas, A.T., Kamal, M. A., Sohrab, S.S., 2015. Evaluation of propolis, honey, and royal jelly in amelioration of peripheral blood leukocytes and lung inflammation in mouse conalbumin-induced asthma model. Saudi J. Biol. Sci. 22, 780–788.

Erdtman, G., 1969. Handbook of palynology – An introduction to the study of pollen grains and spores. Munksgaard, Copenhagen, pp. 1–486.

Graikou, K., Kapeta, S., Aligiannis, N., Sotiroudis, G., Chondrogianni, N., Gonos, E., Chinou, I., 2011. Chemical analysis of Greek pollen – antioxidant, antimicrobial and proteasome activation. Chem. Cent. J. 5, 3.

Khan, S.U., Anjum, S.I., Rahman, K., Ansari, M.J., Khan, W.U., Kamal, S., et al., 2018. Honey: Single food stuff comprises many drugs. Saudi J. Biol. Sci. 25, 320–325.

Krystyjan, M., Gumul, D., Ziobro, R., Korus, A., 2015. The fortification of biscuits with bee pollen and its effect on physicochemical and antioxidant properties in biscuits. *LWT* –. Food Sci. Technol. 63, 640–646.

Leja, M., Mareczek, A., Wyźgolik, G., Klepacz-Baniak, J., Czekońska, K., 2007. Antioxidative properties of bee pollen in selected plant species. Food Chem. 100, 237–240.

Magnè, C., Lahrer, F., 1992. High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. Anal. Biochem. 200 (1), 115–118.

Mărgăoan, R., Mărghitas, L.A., Dezmirean, D.S., Dulf, F.V., Bunea, A., Socaci, S.A., et al., 2014. Predominant and secondary pollen botanical origins influence the carotenoid and fatty acid profile in fresh honeybee-collected pollen. J. Agric. Food Chem. 62, 6306–6316.

Marghitas, L.A., Stanciu, O.G., Dezmirean, D.S., Bobis, O., Popescu, O., Bogdanov, S., Campos, M.G., 2009. In vitro antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania. Food Chem. 115, 878–883.

Medeiros, K.C.P., Figueiredo, C.A.V., Figueredo, T.B., Freire, K.R.L., Santos, F.A.R., Alcantara-Neves, N.M., Silva, T.M.S., Piuvezam, M.R., 2008. Anti-allergic effect of bee pollen phenolic extract and myricetin in ovalbumin-sensitized mice. J. Ethnopharmacol. 119, 41–46.

Meo, S.A., Al-Asiri, S.A., Mahesar, A.L., Ansari, M.J., 2017. Role of honey in modern medicine. Saudi J. Biol. Sci. 24, 975–978.

Serra Bonvehì, J., Lòpez Alegret, P., 1986. Microbiological studies on honeybee-collected pollen produced in Spain: total bacteria, coliforms, *Escherichia coli, Staphylococcus, Streptococcus* "D" of Lancefield, sulfite reducing *Clostridia, Bacillus*, yeasts, moulds, and the detection of aflatoxins by thinlayer chromatography (TLC). Ann. Falsific. Exp. Chim. Toxicol. 849, 259–266.

Serra Bonvehì, J., Soliva Torrento, M., Centelles Lorente, E., 2001. Evaluation of polyphenolic and flavonoid compounds in honeybee-collected pollen produced in Spain. J. Agric. Food Chem. 49 (4), 1848–1853.

Silva, T.M.S., Camara, C.A., Silva Lins, A.C., Maria Barbosa-Filho, J., Silva, E.M.S., Freitas, B.M., Santos, F.A.R., 2006. Chemical composition and free radical scavenging activity of pollen loads from stingless bee *Melipona subnitida* Ducke. J. Food Compos. Anal. 19, 507–511.

Soares de Arruda, V.A., Santos Pereira, A.A., Silva de Freitas, A., Barth, O.M., Bicudo, de Almeida-Muradian, L., 2013. Dried bee pollen: B complex vitamins, physicochemical and botanical composition. J. Food Compos. Anal. 29, 100–105.

Zoccali, P., Malacrinò, A., Campolo, O., Laudani, F., Algeri, G.M., Giunti, G., Palmeri, V., 2017. A novel GIS-based approach to assess beekeeping suitability of Mediterranean lands. Saudi J. Biol. Sci. 24, 1045–1050.