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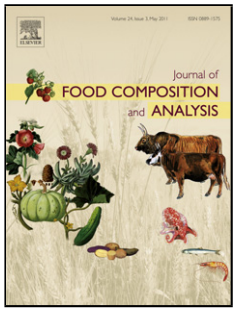

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Original Research Article

Lipid characterization of chestnut and willow honeybee-collected pollen: impact of freeze-drying and microwave-assisted drying

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Highlights

- We studied the impact of two novel conditioning methods on bee pollen lipids
- High levels of ω-6 and ω-3 fatty acids characterized chestnut and willow pollen
- Freeze-drying and microwave-assisted drying offer advantages for pollen conservation
- However, microwave-assisted treatment led to reduction of tocopherols

Abstract

Honeybee-collected pollen is gaining attention as functional food, due to its high content of bioactive compounds, such as essential amino acids, antioxidants, vitamins and lipids. Traditional conservation methods include drying in a hot air chamber or freezing. These techniques influence the pollen organoleptic properties and its content of nutraceutical compounds. We analysed the lipid component of two honeybee-collected pollens, *Castanea sativa* and *Salix alba*. Chestnut pollen was characterised by a higher level of omega-6 fatty acids, while willow pollen showed a higher concentration of omega-3 fatty acids and carotenoids. Furthermore, two novel conservation methods, freeze-drying and microwave-assisted drying, were proposed and their impact on the pollen lipid profile was assessed. Only the microwave treatment showed a damaging action on antioxidant compounds (i.e. reduction of tocopherols). Overall, this research shed light on the lipid profile of honeybee-collected pollen, highlighting the nutraceutical importance of pollen as a source of omega-6 and omega-3 polyunsaturated fatty acids.

Keywords: food analysis; *Apis mellifera; Castanea sativa*; *Salix alba,* freeze-drying; microwaves; PUFA

1. Introduction

Honeybee-collected pollen is gaining attention as functional food for human consumption, due to its high content of bioactive compounds, such as essential amino acids, antioxidants, vitamins and lipids (Quan et al., 2008; Soares de Arruda et al., 2013; Krystyjan et al., 2015; Almeida et al., 2016). Pollen is the basis of bee food. Nurse bees are major consumers of pollen, from which they produce jelly in their food glands with which they feed the larvae. Adult bees also consume some pollen (Craane, 1990; Pernal and Currie, 2000; Brodschneider and Crailsheim 2010; Di Pasquale et al., 2013). Besides the nitrogenous substances, present in the form of complex proteins or free amino acids, the bee-collected pollen is composed by water, sugars, vitamins of B group, provitamin A, folic acid, minerals and lipids (Leja et al., 2007; Campos et al., 2003, 2008; Nicolson, 2011; Soares de Arruda et al., 2013).

Currently, many countries, as Brazil, Poland, Bulgaria and Switzerland, have established guidelines about the physical, chemical and microbiological standards that the pollen for human consumption must fit (Canale et al., 2016). Besides this, each type of pollen has specific nutritional characteristics that reflect its botanical origin (Serra Bonvehì, 1988). However, while extensive research focused on the proteins and antioxidants available in honeybee-collected pollen, its lipid profile has been scarcely investigated. Frequently, analyses of the chemical composition have concentrated on pollen from plants of interest to apiculture. Relatively few plant species pollens have been studied for fatty acid composition. Most of the research work on pollen lipids has concentrated on the sterols, e.g. cholesterol, as they are also essential for honeybees (Manning, 2001). Some works studied the fatty acid (FA) content of different pollen

types and found unsaturated and saturated fatty acids (Battaglini and Bosi, 1965, 1968; Mărgăoan et al., 2014). Differences between the pollen types were concerned much more with the proportions of FA than to their diversity.

The commercial collection of pollen is carried out by beekeepers, using special traps placed at the entrance of the hives (Contessi, 2009). It is worthy to note that the nutraceutical quality of honeybee-collected pollen decline over time (Pernal and Currie, 2000). In addition, the conditioning carried out on the fresh pollen collected by bees before storage for human consumption changes its nutritional and functional value. To reduce the water content, the honeybee-collected fresh pollen is usually conditioned with little standardized methods (Serra Bonvehì et al., 1986; Canale et al., 2016).

From a technological point of view, the knowledge of the different factors contributing to the production of high quality dried pollen is scarce. Currently, the drying process is conducted at low temperatures, with short exposure times, in order to avoid the risk of formation of Maillard's compounds (Collin et al., 1995). Furthermore, a detrimental effect of the drying process on the content of antioxidants, such as polyphenols and flavonoids, has been highlighted. This leads to a lower functional value of bee-collected pollen for human consumption (Serra Bonvehì et al., 2001).

Knowledge reported above outlined that the optimization and standardization of the technologies employed to process the bee-collected fresh pollen is crucial (Canale et al., 2016). In this research, we analysed the lipid component of two honeybee-collected pollens, *Castanea sativa* and *Salix alba*, shedding light on the abundance of omega-6 and omega-3 polyunsaturated fatty acids, sterols, carotenoids and tocopherols. Furthermore, two novel conservation methods, freeze-drying and microwave-assisted

drying, were proposed and their impact on the complete lipid profile of the two pollens was assessed.

Materials and methods

2.1. Pollen samples

Honeybee-collected chestnut pollen was harvested by a beekeeper in July 2015 in chestnut grows located in Castelnuovo Garfagnana (44°06'22.7"N 10°24'02.7"E, Lucca, Italy), using a pollen trap (A. Metalori, Italy). Honeybee-collected willow pollen was harvested by a beekeeper in April 2015 in *Salix* orchards located in Massa Macinaia (43°47'45.6"N 10°32'03.2"E, Capannori, Lucca, Italy), using the pollen trap mentioned above. Chestnut and willow pollen samples were immediately frozen and transferred to the laboratories for further conditioning. For both honeybee-collected pollens, their monofloral origin was identified by colour and light microscopy examination (400X magnification) (Erdtman, 1969). Chestnut and willow pollen types were identified by comparison available pollen atlas databases (Erdtman, 1969; Ricciardelli d'Albore, 1998; Mărghitaş et al., 2009). Post-conditioning, all analytical results were compared with the fresh untreated pollen sample (UP). Eight pollen samples were collected for the analyses, 4 samples per species (i.e. chestnut and willow) and each sample was analysed in triplicate to reduce the individual variability. Moreover, 8 samples were collected to evaluate the effect of post-harvest conditioning: (*i*) 2 untreated samples (control), (*ii*) 2 conventionally dried samples [i.e. bee-pollen dried at 32°C for 24 h in a Northwest

Technology (Italy) cool-air dryer, http://www.northwest-technology.com/], (*iii*) 2 freeze-drying samples and 2 microwave-dried samples.

2.2. Freeze-drying

Pollen freeze-drying was carried out using a lyophiliser Heto PowerDry® LL1500 with four manifold connecting to 100 mL flasks filled with 70 g of honeybee-collected fresh pollen (previously preserved in a freezer at -20°C). During the whole process, the temperature inside the condensation chamber was -115°C, with full vacuum. The exchange of heat in this type of equipment it has by convection with the temperature of the test room, which was continuously conditioned at 25°C. The maximum temperature reached by the sample during the process is 25°C. The liquid inside the condensation chamber was sampled. The samples, at the end of the treatment, were sealed and stored at a temperature of -20 ° C for subsequent analysis.

The detailed procedure for pollen freeze-drying was carried out as follows: (*i*) keep the flasks and the samples to be treated in a freezer at -20 ° C until the time of treatment; (*ii*) switch equipment until the temperature reaches -100 ° C inside the condensation chamber and the pump power for the vacuum; (*iii*) insert the sample into the sample and determine the initial weight; (*iv*) house the flasks on the structure of the freeze-dryer; (*v*) create full vacuum in the flasks; (*vi*) treat the pollen sample for 9 h of freeze-drying (the exposure period was determined by preliminary tests followed by thermogravimetric analysis, TGA) (*vii*) determine the final weight (*viii*) seal the samples and store them at -20 ° C until analysis.

After the freeze-drying treatment for 9 h, TGA was carried on, in order to evaluate the residual water content. The sample was then stored in freezer at -20°C, waiting for the analyses. TGA conducted at 120°C showed that the residual water content was 6.0% for chestnut pollen and 6.3% for willow pollen.

2.3. Microwave-assisted drying

Microwave (MW) drying was carried out following the method described by Canale et al., (2016). The tests were conducted at the absolute pressure of 50 mbar. The MW power was 150 W and the exposure time was 30 min for both pollens). At the end of the treatment, the pollen sample was immediately weighed and its temperature measured with a K-type thermocouple. The pollen was transferred into an airtight container and stored at -20°C until the analyses. TGA conducted at 120°C showed that the residual water content was 6.4% for chestnut pollen and 10.3% for willow pollen.

2.4. Fatty acids composition

Total lipids (TL) were extracted according to Rodriguez-Estrada et al. (1997), with some modification. Briefly, 15 g of pollen were inserted in a bottle with 200 mL of chloroform:methanol (1:1) solution and homogenized by Ultraturrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany). Subsequently, samples were stored at 60 °C and then 100 mL of chloroform were added. After filtration, 100 mL of K_2CO_3 1 M solution were added and the samples were stored at 4°C overnight. The apolar phase was separated and stored a -20 °C.

Total lipids extract was separated into free fatty acids (FFA), neutral (NL) and polar lipids (PL), using a solid-phase extraction procedure described by Kaluzny et al. (1985), using Supelclean LC-NH2 SPE cartridges (Supelco, Bellefonte, USA). Briefly, 10 mg of lipid sample were applied to solid-phase extraction cartridges previously conditioned with hexane. The NL fraction was eluted with 4 mL of chloroform: isopropanol (4:1) and collected. Then the cartridges were washed with 4 mL of 2% acetic acid in diethyl ether solution for the elution of FFA, which collected in a separated tube. Finally, the PL fraction was eluted with 4 mL of methanol and collected. The three fractions were dried under nitrogen flow and then dissolved in hexane for preparation of fatty acid methyl esters (FAMEs).

FAME of TL were prepared by acid trans-methylation according Christie (1993), while NL and PL fractions were methylated by a basic trans-methylation following the procedure described by Christie (1982). On the contrary, FFA were derivated by trimethylsilyldiazomethane, according to Christie (1993). Fatty acids were quantified adding nonadecanoic acid methyl ester (Sigma Chemical Co., St. Louis, MO) as the internal standard.

FA composition of TL, NL and PL was determined by GC using a GC2010 Shimadzu gas chromatograph (Shimadzu, Columbia, MD, USA) equipped with a flame-ionization detector and a high polar fused-silica capillary column (Chrompack CP141 Sil88 Varian, Middelburg, the Netherlands; 100 m, 0.25mm i.d.; film thickness 0.20 µm). Hydrogen was used as the carrier gas at a flow of 1 mL/min. Split/splitless injector was used with a split ratio of 1:80. An aliquot of the sample was injected under the GC conditions, previuosly described by Conte et al., (2016). Individual FA methyl esters were identified by comparison to a standard mixture of 52 Component FAME Mix (Nu-

Chek Prep Inc., Elysian, MN) and to 77 individual pure FAME standards (Larodan Fine Chemicals, Malmo, Sweden). The identification of isomers of 18:1 was based on commercial standard mixtures (Supelco, Bellefonte PA) and published isomeric profiles (Kramer et al., 2008). FFA methyl ester were analysed under the same conditions used for the analysis of total FAME, except for the split time as the split valve was closed during the first two minutes of the GC run.

2.5. Sterols, tocopherols and carotenoids composition

Sterols, tocopherols and carotenoids profile were determined on unsaponified fraction of total lipids. Firstly, 300 mg of total lipids were dissolved in hexane/isopropanol (4:1 v/v) and directly cold saponified according to Hulshof et al., (2006) with some modifications. Briefly, 4.5 mL of KOH ethanolic solution (4.8% w/v) were added to dried total lipids and stored at RT overnight. Subsequently, 4.5 mL of dH_2O were added to the mix and then, the unsaponified fraction was extracted with 2 hexane washes (2x9 mL). The apolar phase was collected in a new tube, dried and resuspended in methanol. Before saponification, 150 μL of dihydrocholesterol in chloroform (2 mg mL^{-1}; Steraloids, Newport, RI, USA) were added to total lipids as internal standard for determination of sterols content.

Sterols were determined after silylation of purified samples and residual unsaponifiable matter. Silylation solution was composed by a pyridine solution of hexamethyldisilazane and trimethylchlorosilane (Serra et al., 2014). Trimethysilyl derivatives were identified and quantified by using a gas chromatography equipped with flame ionisation detector and an apolar 30 m×0.25 mm i.d. capillary column by

injection of 3 µL of sample. The injection and the detector were set at 325°C; the initial oven temperature was 250°C, then, with a rate of 1°C/min, the temperature was increased at 270°C; a final temperature of 350°C was reached by a rate of 10°C/min and this temperature was held for 5 min; the carrier gas flux was 1 mL/min and the run was carried out in constant pressure mode (Boselli et al., 2001). Sterols content were expressed as g/100 g of total sterols. Sterols were identified using a previous pollen sterol profile (Villette et al., 2015).

The content of carotenoids and tocopherols was determined using Prostar HPLC equipment (Varian) with a C18 reverse phase column (ChromSep HPLC column SS, 250mm× 4.6 mm, with ChromSep guard column Omnispher 5 C18). The mobile phases were (A) methanol/acetonitrile/water (10:70:20 v/v/v) and (B) methanol/ethyl acetate (70:30 v/v). The flow was 90:10 A:B at 1 mL min^{-1} for 15 min, followed by 50:50 A:B at 1 mL min^{-1} for 5 min and finally 0:100 A:B at 1.5 mL min^{-1} for 10 min. Carotenoids were detected at 450 nm, while tocopherols were detected using fluorescence at 298 nm excitation and 330 nm emission. For this analysis, the HPLC system was equipped with a UV-DAD and a fluorimeter, assembled in series to revealed carotenoids and tocopherols in the same run. Both carotenoids (neoxantin, anteraxantin, lutein, zeaxantin, α-carotene and β-carotene) and tocopherols (α-tocopherol) (Sigma-Aldrich, St. Louis, MO, USA) were quantified by an external calibration curve of respective components at concentrations ranging from 0.045 µg mL^{-1} to 7 µg mL^{-1}. All components were expressed as µg g^{-1} of honeybee pollen.

The peak of each carotenoid was confirmed by comparing the retention time and UV spectrum of each marker constituent, by the Aurora package, present in the Prostar software (Varian, Lexington, Massachusetts, USA).

2.5. Data analysis

In order to evaluate the effect of botanical origin of pollen and post-harvest treatment, data were analysed using the following linear model:

$y_i = \mu + BO_i + \varepsilon_i$ [1]

Where: yi = values of individual variables (fatty acids, tocopherols, carotenoids, sterols, fat, protein and carbohydrates content); BO_i = fixed effect of the i_{th} botanical origin of pollen (i.e. chestnut or willow); ε_i = random residual.

$y_i = \mu + T_i + \varepsilon_i$ [2]

Where: yi = values of individual variables (fatty acids, tocopherols, carotenoids, sterols, fat, protein and carbohydrates content); T_i = fixed effect of the i_{th} drying treatment of pollen (i.e. untreated pollen, cool air-dried pollen, freeze-dried pollen, microwave-dried pollen); ε_i = random residual.

3. Results and Discussion

3.1. Characterization of lipid fraction

Pollen lipids are an key source of energy for honeybees (Szczesna, 2002). The single components of lipids play a relevant role in the development, nutrition and reproduction of *A. mellifera*. Notably, lipids composition may include some important molecules for human nutrition, considering that the essential fatty acids and anti-oxidant substances are contained in the lipid fraction.

To the best of our knowledge, little research focused on the lipid composition of the bee-collected pollen (Manning, 2001; Margaoan et at 2014; Arien et al., 2015). In our experiments, a preliminary TLC characterization was carried out in order to achieve some basic information about the nature of lipid classes contained in the lipid fraction of honeybee samples. **Figure 1** shows the lipid profile of chestnut and willow pollen. In both type of pollen, triglycerides were the most abundant components of lipids, though we revealed the presence of diglycerides and FFA. These results showed that an intensive lipolitic process probably characterizes pollen. Analysing **Figure 1** by an image-processing program (ImageJ, a free on-line available software, https://imagej.nih.gov/ij/), we revealed that pollen was characterized by a high content of phospholipids, tocopherols and phytosterols. This lipid profile is typical of honeybee food, because thanks to this composition pollen may be attractive and stimulate the phagostimulatory activity (Manning, 2001). The attractive properties were related principally to FFA fractions as proposed by Lepage & Boch (1968), so it justified their relevant presence in the samples.

3.2. Effect of botanical origin on lipid fraction

The honeybee pollen fatty acid content and the pattern of TL, PL and NL fractions are presented in **Table 1**. The sums (SFA, MUFA and PUFA) and ratio of FA from polar, neutral and total lipids are presented in **Table 2**. The botanical origin did not affect the total FA concentration in TL and NL, while PL fraction of chestnut pollen was higher than that of willow. The FA profile of the two types of honeybee pollen

reflected the FA composition of the native pollen from *Salix* spp. (Rosenkvist & Laakso, 1991) and *Castanea* spp. (Borges et al., 2007).

The chromatographic analysis revealed 25 FA in both types of pollen. This number is higher than that reported in previously works (Saa-Otero et al., 2000), giving a more detailed profile of pollen FA. To the best of our knowledge, the FA profile in the single lipid fractions has not been previously characterized. The total FA composition was similar to data reported by Manning (2001) for honeybee pollen, except for stearic acid in willow pollen. According to Manning (2001), S. alba pollen shows a percentage of C18:0 higher than 9%. In our study, willow pollen had a level of C18:0 lower than 9%. This discrepancy is probably due to the different number of fatty acids reported in the present study if compared to previous research. To the best of our knowledge, this is the first study where a 100 m high polar column was adopted to analyse fatty acid composition of honeybee-collected pollen. Adopting this analytic approach more fatty acids were revealed and separated in the lipid fraction, including some fatty acid that co-elute when shorter column are adopted. Therefore, the concentration of stearic acid was probably more accurate.

The major FA in total lipids for both species were linoleic acid (18:2c9c12, 29% of total FA), α-linolenic acid (C18:3c9c12c15, 28.5% of total FA), palmitic acid (C16:0, 20% of total FA) and oleic acid (C18:1c9, 9%). C16:0 and C18:1c9 were not affected by the botanical origin (**Table 1**). On the contrary, linoleic acid was higher in chestnut pollen, while α-linolenic acid was higher in willow pollen. These results are in agreement with other works, which revealed high concentration of linoleic, α-linolenic and palmitic acid (Serra Bonvehi, 1986; Serra Bonvehi & Escolà Jordà, 1997).

The high level of α-linolenic acid, especially in willow pollen, is very important because increase the functional role of pollen as anti-atherogenic food (Denisow et al., 2016). This effect of bee pollen is related to the omega-3 FA, especially α-ALA, which act as a precursor for prostaglandin-3, the main inhibitor of platelet aggregation. Their role has been demonstrated in humans after pollen consumption (Yakusheva , 2010). A useful parameter to estimate the healthy properties of food is the n6/n3 ratio. Western diets typically have an n6/n3 ratio of 20–30:1, whereas the ideal ratio is thought to be 4:1 or less (Simopoulos, 1999). Pollen samples considered here showed very low values of the n6/n3 ratio (0.5-1), comparable with those reported for fish. This result was mainly due to high concentration of α-linolenic acid. This omega-3 fatty acid has a recognized role in the regulation of the blood cholesterol level, provided that the daily intake is not lower than 2 g/d (EFSA, 2011). The amount of α-linolenic acid provided by chestnut and willow pollen was 334 and 430 mg per 100 g of fresh pollen, respectively. These levels are higher than the minimum threshold necessary to obtain the claim “Source of fatty acids omega-3” (300 mg per 100 g of fresh matter), as reported in European regulation (EC 1924/06). Unfortunately, the claim is not completely achieved because the energy level (285 kcal per 100 g of fresh matter) does not allow obtaining the same amount of omega-3 fatty acids also in 100 kcal (EC 1924/06). Although this consideration, pollen may be considered an important source of omega-3 fatty acids for human diet.

Among the other fatty acids, C6:0, C12:0, C14:0, C18:0, C18:1c11 and C24:1c15, C22:5ω3 and C22:6ω3 were contained at higher concentration in willow pollen, whereas C17:0, C22:0 and C24:0 were significantly higher in chestnut samples. Notably, several fatty acids detected in honeybee samples, such as capric (C10:0), lauric

(C12:0), myristic (C14:0), linoleic acid and α-linolenic acid, showed antimicrobial activity against some important honeybee pathogens (Feldlaufer et al., 1993).

Fatty acid composition of NL was similar to that observed for total lipids. The major FA in NL were C18:3c9c12c15 (26% of total FA), C18:2c9c12 (20% of total FA), 18:1c9 (17% of total FA) and C16:0 (17% of total FA) irrespective of the kind of pollen considered (**Table 1**). Chestnut pollen was characterized by a higher percentage of C16:0iso, C16:1t4, arachidonic acid (C20:4ω6), C22:0 and C22:6ω3. Willow pollen contained higher percentage of C12:0, C14:0, C18:0 and C18:1c11. Polar lipids were characterized by a smaller number of fatty acids than NL (12 instead of 26 fatty acids). The main FA in PL were C18:2c9c12, that was higher in chestnut pollen (45.18 vs 28.38 % of total FA), and C18:3c9c12c15, that was higher in willow pollen (42.24 vs 22.33 % of total FA). Other FA representative of the chestnut pollen were C14:0, C17:0, C18:0, C20:0, C22:2 and C24:0. Willow pollen is characterized by a higher percentage of C18:1c11 (**Table 1**).

The total SFA in pollen is higher in willow in both TL and in NL fraction, while no difference in PL were revealed. The total MUFA were unaffected by the botanical origin, averaging 12%, 18% and 2% of total FA in the TL, NL and PL respectively. Similarly to MUFA, also total PUFA content was not affected by botanical origin. PL accounted for 68% of total FA, a higher percentage than NL (55%). In the all fractions, the percentage of PUFA n-3 is higher in willow pollen, while PUFA n-6 was more representative in chestnut pollen.

The FA pattern of NL and PL fractions was characterized by a high proportion of PUFA. MUFA accumulated preferentially in NL, while their percentage in PL fraction was very low. Therefore, a selective deposition in lipid fractions was

highlighted. The FA composition of membrane phospholipids plays a key role in the modulation of membrane fluidity and, in turn, membrane function and cellular metabolism. In the pollen, the small content of MUFA balances the high level of PUFA in PL fraction. This suggests the presence of a homeoviscous adaptation mechanism, in which the degree of unsaturation of FA in membrane PL is maintained constant (Macdonald & Cossins, 1985). Further research is ongoing to evaluate the optimal operating conditions for both drying treatments. Moreover, further biological analysis will be carried out on the treated samples after 3 and 6 months, with in order to evaluate the effect of the new treatment on the pollen shelf life.

The effect of botanical origin on tocopherols and carotenoids is reported in **Table 2**. Carotenoids content ranged between 41 and 199 µg/g of pollen, with an average level of 120 µg/g of pollen. Honeybee pollen of willow showed a significantly higher content than chestnut one (approximately five fold). Chestnut pollen showed a carotenoid level comparable with that of tomato and watermelon, while willow pollen showed a level similar to pumpkin and sweet potatoes, the food sources richest in carotenoids (Pacheco et al., 2014). 15 g of pollen, approximately a teaspoon, give more than 100% of daily requirements of carotenoids. The single carotenoids revealed in both type of pollen were neaxatin, anteraxantin, lutein, zeaxantin, α- and β-carotene. All carotenoids contents were significantly higher in willow pollen. Carotenoids such as β-carotene, lutein, zeaxanthin, and antheraxanthin occur in varying proportions in several pollen species (Shult et al., 2009). Post-conditioning treatment did not affect carotenoid levels.

Tocopherols revealed in pollen were δ-tocopherol, γ-tocopherol and α-tocopherol (**Table 2**), but the level was not affected by botanical origin. Sterol

composition in the *Castanea sativa* and *Salix alba* pollens are summarized in **Table 3**. The results showed a different profile between the two types of pollen. The sterols more representative in chestnut were β-sitosterol (52%) and campesterol (22%), 24-methylencolestanol (11%) and 24-methylcolesterol (7%). On the contrary, willow pollen showed a higher level of β-sitosterol (28%), 25-dehydrositostanol (16%), isofucosterol (16%), 24-methylcolesterol (13%) and 24-methylcolestanol (13%). Cholesterol amount was revealed in both type of pollen with a percentage ranging from 0.5 to 2 %. These levels agree with data reported in pollen of different species (0-5 %) as reported by Villette et al., (2015). 24-methylenecholesterol, cholesterol, stigmasterol and 24-methylencolestanol showed similar percentage between chestnut and willow pollen. Particularly, 24-methylencholesterol is very important, since it is common in pollen and is a major sterol source for honeybees. Nearly all insects need to obtain sterol from their diet because of their inability to synthesize them directly. Sterol is the precursor for important hormones such as moulting hormone, which regulates growth because it is required at the time of each moult (Huang, 2010). Lastly, quantitative variations in the relative quantities of sterols in both collected honeybee pollens (**Figure 3**) have been detected, in agreement with Standifer (1968).

3.2 Effect of post-harvest method on lipid fraction composition

Here we focused on the evaluation of post-harvest treatment effects on lipid composition of pollen. Post-harvest pollen conditioning did not affect lipid composition, in comparison with control pollen samples. Tocopherol composition is the only lipid component, which showed a significantly decrease after all post conditioning treatment

(**Figure 2**). Particularly, α-tocopherol, δ-tocopherol and γ-tocopherol showed a reduction of 60%, 75% and 90% respectively with freeze-drying treatment. On the contrary, microwave-assisted treatment caused the complete oxidation of all tocopherols. This result highlighted the role played by tocopherols as antioxidant component, especially under hard stress like post conditioning treatment. It is interesting to note that microwave decrease almost completely the tocopherol content, while the effect of freeze-drying on the oxidative stress was milder (**Figure 2**). To the best of our knowledge, this is the first report about the impact of freeze-drying and microwave-assisted drying on pollen lipid composition.

4. Conclusions

Overall, this research shed light on the lipid profile of honeybee-collected pollen, highlighting that the chestnut pollen was characterised by a higher level of omega-6 fatty acids, while the willow pollen showed a higher concentration of omega-3 fatty acids and carotenoids. Notably, only the microwave treatment showed a damaging action on antioxidant compounds (i.e. reduction in the content of tocopherols), while no negative effects of freeze-drying were reported both for chestnut and willow pollen. These evidences allowed us to candidate the employ of freeze-drying as a novel and effective method for the treatment of commercial honeybee-collected pollen.

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Conflicts of interest

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Figure 1. Thin Layer Chromatography of the lipid profile characterising chestnut and willow pollen.

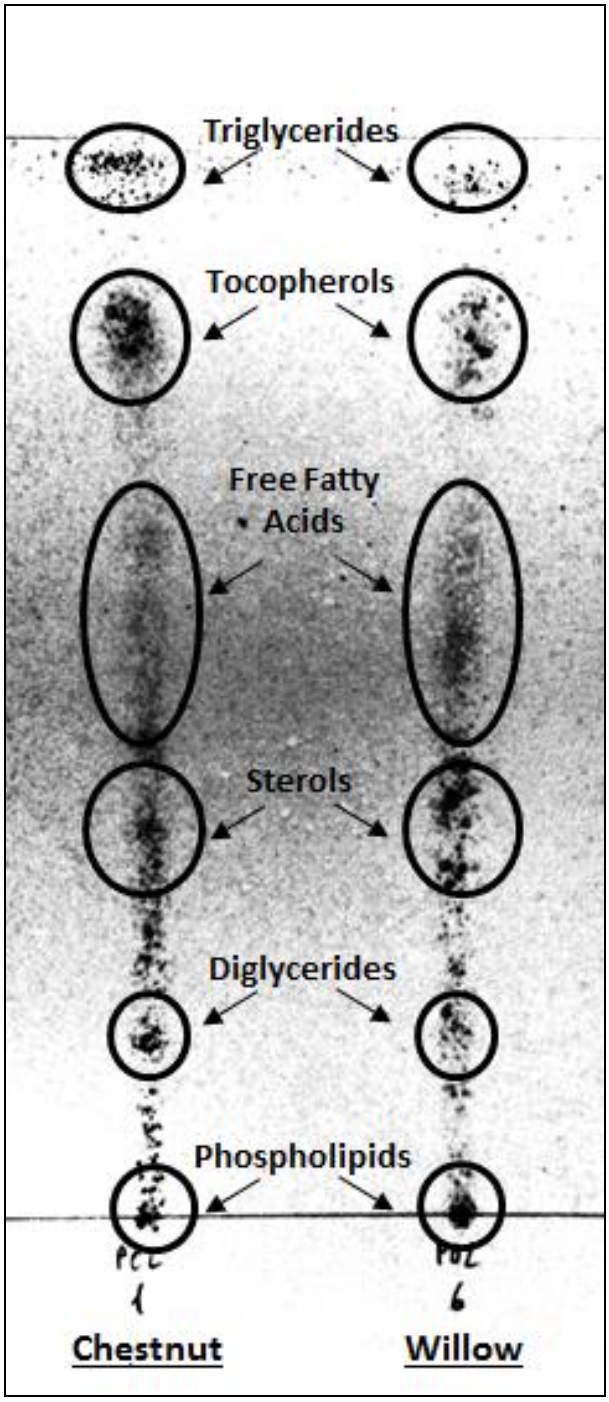

Figure 2. Effect of post-conditioning treatment on tocopherols level of pollen. Untreated pollen (black), Dried pollen (dark grey), Freeze-drying pollen (light grey) and microwave-dried pollen (white). Above each column, different letters indicate significant differences (general linear model, $P<0.05$). Eight samples (two per treatment) were considered for the analysis and each sample was analysed in triplicate.

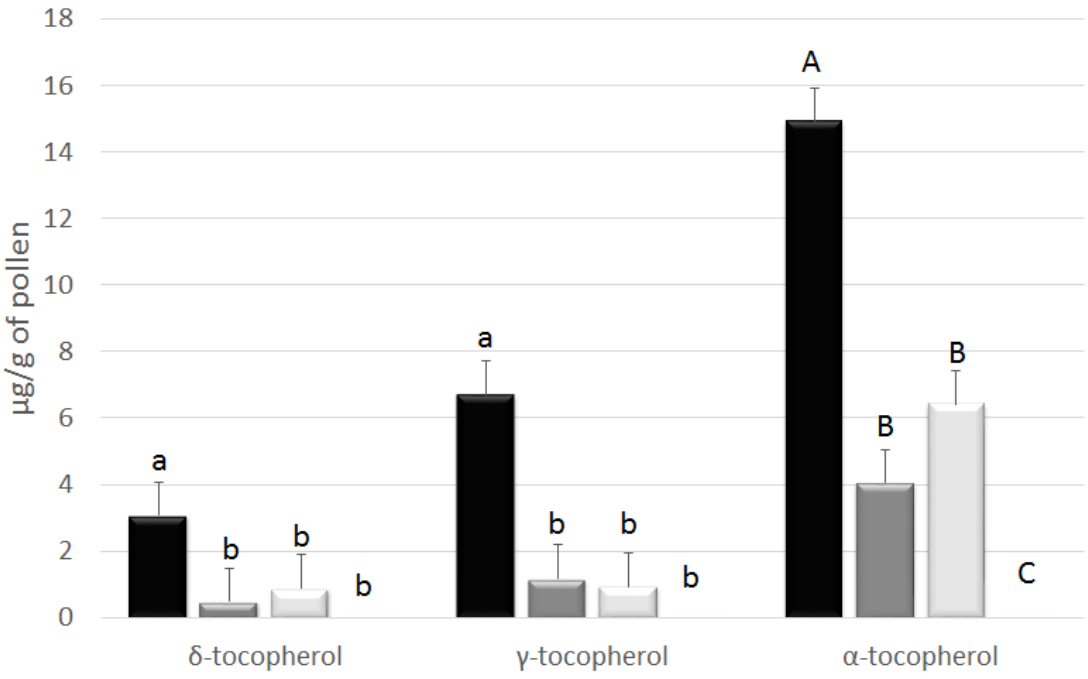

Figure 3. Sterol profile of chestnut (black line) and willow (grey line) pollen.

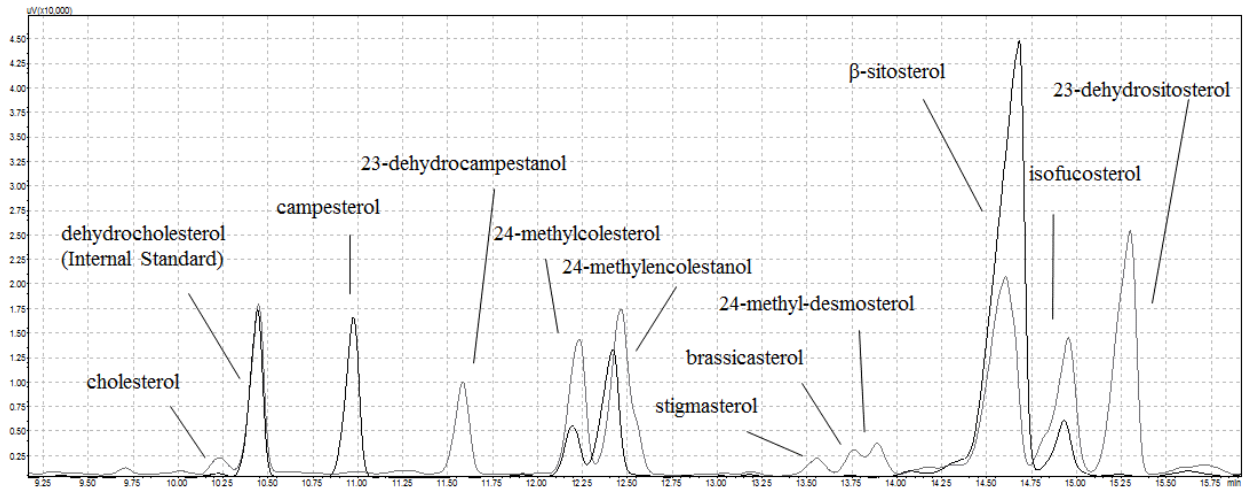

Table 1. Effect of the botanical origin on fatty acid concentration (Total = g/100 g fresh pollen) and composition (single FA = g/100 g total fatty acid) of total, neutral and polar lipids in honeybee-collected pollen.

Fatty	Total lipids				Neutral lipids				Polar lipids			
acids	Chestnut[1]	Willow[1]	SE[2]		Chestnut[1]	Willow[1]	SE[2]		Chestnut[1]	Willow[1]	SE[2]	
Total	1.38	1.30	0.48	ns	1.00	1.07	0.33	ns	0.38	0.23	0.04	*
C6:0	0.08	0.26	0.03	*	0.03	0.15	0.04	ns	-	-	-	-
C10:0	0.08	0.15	0.02	ns	0.09	0.12	0.04	ns	-	-	-	-
C12:0	0.22	1.20	0.15	*	0.36	0.97	0.23	*	-	-	-	-
C14:0	0.70	3.08	0.95	*	0.88	2.73	1.08	*	0.24	0.12	0.02	*
C16:0iso	0.17	0.14	0.03	ns	0.68	0.35	0.07	*	-	-	-	-
C16:0	19.39	20.30	0.86	ns	15.34	18.16	0.25	*	23.98	25.17	0.35	ns
C16:1 t4	0.09	0.04	0.01	ns	0.40	0.16	0.05	*	-	-	-	-
C16:1 c9	0.10	0.07	0.01	ns	0.12	0.11	0.01	ns	-	-	-	-
C17:0	0.29	0.20	0.02	*	0.11	0.11	0.01	ns	0.24	0.12	0.01	**
C18:0	1.82	5.50	0.52	*	1.29	4.15	0.35	*	1.80	0.68	0.16	*
C18:1 c9	8.53	9.85	1.03	ns	17.95	15.17	1.84	ns	1.43	1.09	0.33	ns
C18:1 c11	0.60	1.45	0.20	*	0.37	0.68	0.04	*	0.29	0.43	0.01	**
C18:2 c9c12	37.92	19.61	3.73	*	29.87	19.75	2.71	*	45.18	28.38	2.27	*
C20:0	0.99	1.49	0.16	ns	1.21	1.09	0.18	ns	0.24	0.03	0.03	*
C18:3 c9c12c15	24.26	33.06	1.56	*	22.98	28.28	1.81	ns	22.33	42.24	2.62	*
C18:4 n3	0.24	0.51	0.16	ns	0.97	3.07	0.45	*	-	-	-	-
C20:6 n6	0.05	0.08	0.01	ns	0.25	0.26	0.03	ns	-	-	-	-
C22:0	1.66	0.97	0.13	*	0.60	0.38	0.12	*	-	-	-	-

C20:4 n6	0.09	0.12	0.04	ns	3.08	1.44	0.30	*	-	-	-	-
C23:0	0.26	0.12	0.06	ns	0.33	0.18	0.04	ns	-	-	-	-
C22:2	0.06	0.09	0.01	ns	0.29	0.13	0.07	ns	1.65	0.48	0.14	*
C24:0	1.32	0.75	0.11	*	0.34	0.41	0.03	ns	1.36	0.13	0.29	*
C24:1 c15	0.13	0.31	0.02	*	0.33	0.19	0.04	ns	-	-	-	-
C22:5 n3	0.16	0.22	0.03	*	0.29	0.43	0.02	*	-	-	-	-
C22:6 n3	0.08	0.20	0.04	*	0.23	0.17	0.02	ns	-	-	-	-

[1] 4 samples per species were considered in the analysis and each sample was analysed in triplicate

[2] Standard Error

ns: not significant

*: P-value < 0.05

Table 2. Effect of the botanical origin on the sums of fatty acids (g/100 g total fatty acid) of total, polar and neutral lipids in honeybee-collected pollen.

	Chestnut[1]	Willow[1]	SE[2]	
Total lipids				
SFA	27.15	33.16	1.37	*
MUFA	9.80	12.52	1.39	ns
PUFA	63.05	54.33	2.67	ns
PUFA n6	38.25	20.05	3.71	*
PUFA n3	24.76	34.01	1.64	*
n6/n3	1.67	0.59	0.26	*
Neutral lipids				
SFA	21.75	30.47	2.58	*
MUFA	20.32	16.59	1.91	ns
PUFA	57.93	52.94	2.82	ns
PUFA n6	33.20	21.57	2.81	*
PUFA n3	24.44	31.24	1.42	*
Polar lipids				
SFA	29.70	29.57	2.12	ns
MUFA	2.21	2.62	0.27	ns
PUFA	68.10	67.81	7.81	ns
PUFA n6	45.18	28.38	2.27	*
PUFA n3	22.33	42.24	2.62	*

[1] 4 samples per species were considered in the analysis and each sample was analysed in triplicate

[2] Standard Error

ns: not significant

*: P-value < 0.05

SFA: Satured Fatty Acids; MUFA: Monounsatured Fatty Acids

PUFA: Polyunsatured Fatty Acids

Table 3. Effect of the botanical origin on the carotenoids (µg/g of fresh pollen), tocopherols (µg/g of fresh pollen) and sterols (g/100g of sterols) in honeybee-collected pollen.

	Chestnut[1]	Willow[1]	SE[2]	
Carotenoids	41.77	198.60	41.47	*
Neoxantin	0.17	1.47	0.32	*
Anteraxantin	26.88	105.53	20.50	*
Lutein	6.40	54.23	17.12	*
Zeaxantin	3.01	10.74	1.65	*
α-carotene	3.771	17.650	3.46	*
β-carotene	1.54	8.98	1.74	*
Tocopherols	8.77	10.52	1.24	ns
δ-tocopherol	1.60	0.61	0.53	ns
γ-tocopherol	2.44	1.96	0.77	ns
α-tocopherol	4.73	7.95	1.10	ns
Sterols				
Cholesterol	0.64	1.19	0.19	ns
campesterol	21.66	2.24	3.42	***
23-dehydrocampestanol	0.12	5.16	0.32	***
24-methylcolesterol	7.46	12.67	1.91	ns
Stigmasterol	0.25	0.24	0.08	ns
Brassicasterol	0.17	1.97	0.05	***

24-methylencolestanol	10.57	12.56	1.24	ns
24-methyl-desmosterol	0.30	2.42	0.74	**
β-sitosterol	51.51	27.68	5.16	*
25-dehydrositostanol	0.45	16.01	1.58	***
isofucosterol	6.66	15.83	1.23	**
23-dehydrositosterol	0.20	2.02	0.11	***

[1] 4 samples per species were considered in the analysis and each sample was analysed in triplicate

[2] Standard Error

n = number of samples

ns: not significant

*: P-value < 0.05

**: 0.01< P-value < 0.05

***: P-value < 0.001