Nutrients, phytochemicals and botanical origin of commercial bee pollen from different geographical areas

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Abbreviations

AA: amino acid; BP: bee pollen; DBP: dried bee pollen; MUFA: monounsaturated fatty acids; PA: polyamine; SFA: saturated fatty acids; TL: total lipid; UFA: unsaturated fatty acids

ABSTRACT

This work evaluated the nutritional, phytochemical composition and botanical origin of commercial bee pollen from three different countries. Fructose (17-23%) was the most abundant sugar in all samples, followed by glucose (14-16 %) and sucrose (5-6%). The protein content in Colombian (24%) and Italian (22%) pollen was higher compared to Spanish sample (14%). The total lipid contents were higher for the Spanish (6%) and Colombian pollens (6%) than the Italian sample (2.5%). Twenty-one fatty acids were identified, and the most abundant were palmitic, α-linolenic, linoleic and oleic acid. Colombian pollen was rich in n-3 fatty acids, while Italian and Spanish samples contained high amounts of n-6 fatty acids. Polyphenols and carotenoids were identified by UHPLC-DAD-Orbitrap mass spectrometry detection. Thirty-nine polyphenols were identified, and the dominant compounds were tri-caffeoyl- and caffeoyl-di-*p*-coumaroyl spermidine derivatives. Di-lauryl-zeaxanthin was the main carotenoid detected in all the samples analysed. Colombian pollen contained traces of lutein, zeaxanthin, β-carotene and phytoene, while only β-carotene was present in the Spanish and Italian samples. After saponification, the average total amount of carotenoids was 57, 25 and 221 μg g^{-1} in pollen from Spain, Italy and Colombia, respectively. The free proline to total free amino acid ratio was 53, 59 and 78 for pollen from Spain, Italy and Colombia, respectively.

Keywords

Food analysis, Food composition, Pollen, Mass spectrometry, Nutrients, Polyamines, Polyphenols, Carotenoids

1. Introduction

The chemical composition of bee pollen depends on several factors, such as plant source, geographical origin, seasonal conditions and bee activities (Nogueira et al., 2012). Furthermore, the content of nutrients can change based on the processing methods, steps and storage conditions (Bogdanov, 2004).

Pollen contains proteins, amino acids, carbohydrates, lipids, vitamins and minerals (Yang et al., 2013), polyphenols (Mihajlovic et al., 2015), phytosterols (Wang et al., 2015) and carotenoids (Bunea et al., 2014). In particular, pollen contains 23 % of protein on average, including 10 % of essential amino acids (AA) such as methionine, lysine, threonine, histidine, leucine, isoleucine, valine, phenylalanine, and tryptophan (Almeida-Muradian et al., 2005). The total lipid (TL) content of bee pollen (BP) is typically lower than 10% of the dry weight (DW) (Nicolson, 2011) and the most abundant are linoleic (18:2n-6), α-linolenic (18:3n-3) and palmitic (16:0) acids (Szczęsna, 2006). Sugar occurs in pollen loads in the amount of 40 % on average, ranging from 15 to 24% for fructose, 11-18% for glucose and 4-9% for sucrose. Other sugars such as arabinose, ribose, isomaltose and melibiose accounted for about 1% (Szczęsna et al., 2002).

The yellow-red appearance of pollen loads, which are formed by the aggregation of microscopic granules, is determined mainly by the presence of pigments, such as flavonoids and carotenoids (Stanley and Linskens, 1974).

The principal carotenoids found in bee pollen after saponification are lutein and β-cryptoxanthin, while β-carotene is detected in small or trace amounts (Mărgăoan et al., 2014). Carotenoids have important roles in human health. β-Carotene, for example, constitutes the principal source of vitamin A, and its dietary intake could lower the risk of various types of degenerative diseases (Milani et al., 2017).

69 The glycosides of quercetin, kaempferol and isorhamnetin are the predominant flavonoids
70 found in BP. The total amount ranges between 0.3–1.1% (Bonhevi et al., 2001; Han et al.,
71 2012).
72 Bee pollen also contains polyamines (PAs) and PAs linked to cinnamic acid derivatives,
73 such as *p*-coumaric acid, caffeic acid and ferulic acid. The biosynthesis of PAs occurs by the
74 addition of one or two aminopropyl groups to putrescine, to form spermidine and spermine,
75 respectively. Covalent binding of PAs to *p*-coumaric acid, caffeic acid and ferulic acid,
76 abundant in many plant families, gives rise to hydroxyl-cinnamic acid (HCA) amides (Aloisi
77 et al., 2016). These are involved in the organization of the cell wall, and most of them are
78 associated with fertility (Grienenberger et al., 2009). Moreover, the conjugated PAs are non-
79 peptide antagonists of tachykinin neurokinin1 receptors and may benefit depression and
80 anxiety (Yamamoto et al., 2002).
81 Bee pollen may exert a wide range of biological activities, including antifungal,
82 antimicrobial, antiviral, immunostimulating, local analgesic, hypolipidaemic, anti-
83 atherosclerotic, anti-inflammatory and cerebrovascular (Denisow and Denisow-Pietrzyk,
84 2016; Pascoal et al., 2014).
85 The use of the geographical indication for bee products has been proposed because of a
86 higher demand from consumers for traced and well-characterized products. Thus, the
87 evaluation of the nutritional composition and quality parameters of bee pollen from different
88 geographical areas could lead useful information to both producers and consumers. Also in
89 view of the increased number of studies that have highlighted bee pollen as a functional food
90 (Komosinska-Vassev et al., 2015).
91 Given this considerations, the main objective of this work was to determine and
92 compare the nutritional and phytochemical composition of commercial BP produced in

apiaries from Colombia, Italy and Spain. Regarding nutritional parameters, the moisture, ash, lipid, carbohydrate, amino acid and protein contents were determined.

The Proline Index, the ratio of proline/total free amino acids, has been determined as an indicator of the freshness of the pollen. For fresh products, the proline index must be less than 80 % (Serra Bonvehí and Escolá Jorda, 1997).

As phytochemicals, the profiles of the carotenoids, polyphenols and PAs were evaluated by ultra-performance liquid chromatography coupled to diode-array detector and high-resolution mass spectrometer.

Principal component analysis (PCA) was carried out to check for similarity between samples according to the nutrients, phytochemicals and geographical area of origin.

Finally, botanical origin were identified through palynological analysis.

2. Materials and methods

2.1. Chemicals

Astaxanthin, lutein, zeaxanthin, β-cryptoxanthin, violaxanthin, echinenone (internal standard), α-carotene and β-carotene were provided by Hoffmann-La Roche (Basel, Switzerland). The fatty acid methyl esters (FAME) were purchased from Supelco (Bellefonte, PA, USA). Fructose, glucose, sucrose, acetonitrile, methanol, formic acid, chloroform, ethanol, tetrahydrofuran (THF), butylated hydroxytoluene, hexane, norvaline (IS), glutamine, cysteine, asparagine and tryptophan were procured from Sigma-Aldrich (St. Louis, MO, USA). ACCQ*TAG Ultra Derivatization kit for the AA determination was purchased from Waters (Milford, USA). The kit contained AA standard (Alanine, arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine,

valine, aspartic acid and glutamic acid), derivatising reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate), eluents and a sub-2 µm column for AA analysis. Water was supplied through a Milli-Q apparatus (Millipore, Milford, MA, USA).

2.2. Samples

In this study commercial bee pollen samples from Italy (n=1), Spain (n=1) and Colombia (n=1) were evaluated for their content in nutrients, phytochemicals and botanical origin. Blended BP from southern Spain was harvested by beekeepers in the province of Córdoba (37°50′ N, 4°45′ W) between April and July 2017 and stored at −20 °C.

Italian sample resulted from the blend of pollen loads collected by the beekeeper in the municipality of Montaldeo (44°40′05 N, 8°43′52 E) in Alessandria province (Piedmont) between April and July 2017 and immediately frozen.

No information is available regarding the technological treatment carried out on Italian and Spanish pollen. The samples were vacuum-packed and available in 100 g packs.

Colombian pollen comes from the native forest of the Colombian Cundi-boyacence high pleateau zone at about 2200m above sea level (5°55′45 N, 75°59′02 W), where about 90% of the bee-pollen domestic production is concentrated. The bee pollen was gathered, sun dehydrated for 2 days and packed. The drying of the pollen by directly exposing to the sun is the most used method by farmers because Cundi-boyacence high plateau is one of the zones with highest solar radiation in Colombia.

139 *2.3. Botanical origin of the pollen loads*

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141 Approximately 2 g of each pollen load was grouped into subsamples, according to their
142 colour and each subsample was weighed and analysed by a Nikon Eclipse 50i optic
143 microscope at 1000 and 400× for identification and counting, respectively. The total
144 percentage of each pollen was catalogued as described by Barth (2004) into dominant pollen
145 (PD, >45% of the total), secondary pollen (PS, 16–45%), important pollen (PI, 3–15%) and
146 occasional pollen (PO, <3%). A reference collection of the Fojanini Foundation (Sondrio, IT)
147 and various pollen morphology guides were used to identify the pollen types.

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149 *2.4. Moisture and ash determination*

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151 The vacuum packs of pollen, 100g, have been opened and aliquots of 3g were weighed
152 and heated at 65 °C for 24 h in a ventilated stove. The samples were then cooled in the drier
153 to laboratory temperature, about 24 °C, and weighed. Moisture content was obtained by
154 difference. Ash determination was made through gravimetry after incineration in a muffle
155 furnace at 600°C until constant weight (AOAC, 2005).

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157 *2.5. Determination of the TL and fatty acids*

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159 The Soxhlet extraction was achieved with 5 g of BP powder using a Soxtec HT 1043
160 system (Foss, Milan, IT) containing 180 mL of a solution ethyl ether:petroleum ether (2:1,
161 v/v). The BP was extracted at 140°C for 6h, followed by a 30 min solvent rinse and solvent
162 evaporation. The weighed residue was considered as the lipid content (% DW). Subsequently,
163 the fatty acids were analysed by GC-FID. Chromatographic separations were achieved using

an Omegawax 320 capillary column (30 m × 0.32 mm i.d.; Supelco, Milan, IT), under the following conditions: initial isotherm, 140°C for 5 min; temperature gradient, 2°C min^{-1} to 210°C; final isotherm, 210°C for 20 min. The injector temperature was 250°C. The injection volume was 1 µL, with a 1/100 split ratio and the FID temperature was 250°C. The carrier and makeup gas were H_2 and N_2, respectively. Fatty acid retention times were obtained by injecting the Omegawax test mix as the standard. The fatty acid content was expressed as a percentage of the total fatty acids using the following formula:

FA % = (Peak area / Total area) x 100, where Peak area= area of the fatty acid and Total area= sum of the areas of the individual fatty acids detected in the chromatogram.

2.6. Protein determination

The nitrogen (N) content was determined by conventional acid hydrolysis and Kjeldahl digestion, using a copper catalyst in 2 g of pulverised pollen. The ammonia was distilled and collected in a solution of boric acid, which was then titrated against standard acid. Digestion and distillation were carried out using a Kjeltec 1002 apparatus (Foss, Milan, IT). Protein content was calculated as total N×5.6.

2.7. Carotenoid determination

2.7.1. Extraction

Carotenoids were extracted from 10 g of BP with 30 mL of hexane. Extraction was conducted under agitation for 2 min and diminished light. The procedure was repeated until the BP became colourless. The extracts were combined and dried. The residue was dissolved

189 in 50 mL of methanol:THF (80:20, v/v). An aliquot was then used for the determination of
190 free carotenoids by LC-DAD.
191
192 2.7.2. Saponification
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194 Hydrolysis of esterified carotenoids was carried out according to Riso and Porrini
195 (1997), with slight modifications. Briefly, 40 mL of each extract was saponified with 10 %
196 methanolic KOH (9:1, v/v), at room temperature, in the dark, under an N_2 stream for 2 h, with
197 a 5-mL aliquot removed every 20 min. For the removal of soaps and alkalis, petroleum ether
198 was added, and the solution washed with a saturated NaCl solution to achieve neutrality. The
199 organic layer containing carotenoids was dried over anhydrous sodium sulphate, evaporated
200 to dryness and the resultant samples were then kept under N_2 at -80°C until utilisation.
201
202 2.7.3. Quantitative analysis
203
204 The quantitative determination of the non-esterified carotenoids was performed
205 according to Riso and Porrini (1997), with slight modifications. The chromatographic system
206 was an Alliance 2695 (Waters, Milford, US) coupled to a DAD 2998 (Waters) and a Vydac
207 201TP54 column (C_{18}, 250 × 4.6 mm i.d., 5 μm; Esperia, CA, US). The flow-rate was 1.5 mL
208 min^{-1}, and 50 μL was injected. The column and sample were maintained at 25 and 15 °C,
209 respectively. The eluents were methanol (A) and THF (B).Chromatographic separations were
210 carried out in gradient elution mode, performed as follows: 0 % B for 3 min, 0 % B to 5 % B
211 in 0.1 min, 5% B for 5 min, 5% B to 10 % B in 0.1 min, 10% B for 5 min, 10% B to 20% B in
212 0.1 min and then 20% B for 10 min. Spectra were acquired in the range 220–700 nm and the
213 chromatograms integrated at 445 nm. Acquisition and quantification were performed using

Empower software (Waters). Calibration curves for lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene ranged from 0.1–2.0 µg/mL, and the results were expressed as micrograms per gram of DBP. The total carotenoids content was also estimated spectrophotometrically at 450nm from saponified samples and the data reported as µg zeaxanthin/g DBP (zeaxanthin $\varepsilon^{\%}$ 2540, Lombeida et al., 2016).

2.7.4. Determination of esterified carotenoids by UHPLC-DAD-Orbitrap mass spectrometry

The esterified carotenoids were analyzed by using an Acquity UHPLC (Waters), coupled to a DAD eLambda (Waters) and an Exactive Orbitrap (Thermo Scientific, San Jose, CA), equipped with a HESI-II probe for electrospray ionization (ESI). The column was a BEH Shield C_{18} (150 × 2.1 mm i.d., 1.7 µm; Waters) and the flow-rate was 0.45 mL min^{-1}. The column and sample were maintained at 45 and 15°C, respectively. Five µL was injected. The eluents were methanol (A) and THF (B). Separations were performed in gradient mode and the profile was: 0% B for 10 min, 0% B to 30% B in 10 min, and then 30% B for 10 min. Data were acquired over the wavelength range 220–700 nm and the chromatograms were plotted in the range 300–550 nm. The operative conditions were as follows: spray voltage +5 kV, sheath gas flow-rate 60 (arbitrary units), auxiliary gas flow-rate 20 (arbitrary units), capillary temperature 275°C, capillary voltage +37.5 V, tube lens +125 V, skimmer +26 V, and heather temperature 120°C. The analytes were identified in positive ESI mode by full-scan acquisition (m/z^+ 200–1000 u), using an isolation window of ±2 ppm. The automatic gain control (AGC) target, injection time, mass resolution and collision energy were 1×10^6, 100 ms, 50 K and 50 eV respectively. The MS data were processed using Xcalibur software (Thermo Scientific).

2.8. Carbohydrate determination

Approximately 20 mg of BP were dispersed in 10 mL of deionised water, and the suspension was then sonicated for 10 min, centrifuged at 1000 ×*g* for 5 min, and the supernatant recovered. The residue was extracted with 10 mL of water and treated as described above. The supernatants were combined, and then the final volume was adjusted to 50 mL with acetonitrile. The sugar content was assessed by using an Acquity UHPLC (Waters) coupled to an Exactive Orbitrap (Thermo Scientific), equipped with a HESI-II probe for ESI. The column was a BEH amide C_{18} (150 × 2.1 mm i.d., 1.7 µm; Waters) and the flow-rate was 0.2 mL min^{-1}. One µL was injected. The column and sample were kept at 25 and 20°C, respectively. The eluents were 0.02% NH_4OH in water:0.02% NH_4OH in acetonitrile (72:28, v/v). The operative conditions were as follows: spray voltage 3.0 kV, sheath gas flow-rate 40 (arbitrary units), auxiliary gas flow-rate 10 (arbitrary units), capillary temperature 300°C, capillary voltage -37.5 V, tube lens -125 V, skimmer -26 V, and heather temperature 300°C. The analytes were identified in negative ESI mode by full-scan acquisition (m/z^- 100–600 u), using an isolation window of ±2 ppm. The AGC target, injection time and mass resolution were 1×10^6, 100 ms and 50 K, respectively. The MS data were processed using Xcalibur software (Thermo Scientific). The peak identity was ascertained by evaluation of both the accurate mass and the retention time. Calibration curves for fructose, glucose and sucrose were constructed in the range 2–50 µg mL^{-1}, and the results were expressed as percentages.

The equations of calibration curve for fructose (Fru, 2.0-50.5 µg mL^{-1}), glucose (Glu, 2.0-49.5 µg mL^{-1}) and sucrose (Suc, 2.1-51.5 µg mL^{-1}) were as follows:

Fru: $Y = 29.3\ X + 5.2$, $R^2=0.997$, n=5

Glu: $Y = 27.8\ X – 3.2$, $R^2=0.995$, n=5

Suc: $Y = 12.8\ X + 2.4$, $R^2=0.995$, n=5

Where Y=peak area x 10^{-3}, X=µg/mL.

2.9. Polyphenol and PA determination

The BP samples were finely powdered by milling, and 200 mg extracted with methanol (20 mL) at 70°C for 2 h. The mixture was cooled, filtered and the solid residue was extracted using the same methanol volume. The resulting solutions were mixed and methanol added to adjust the volume to 50 mL. The solution was centrifuged at 1000 ×*g* for 2 min, and 5 µL injected into the UHPLC system. The analysis was performed on an Acquity UHPLC (Waters) coupled with an eLambda DAD (Waters) and a high-resolution Fourier transform Orbitrap mass spectrometer (Exactive, Thermo Scientific), equipped with a HESI-II probe for ESI and a collision cell (HCD). The operative conditions were as follows: spray voltage -3.0 kV, sheath gas flow-rate 55 (arbitrary units), auxiliary gas flow-rate 20 (arbitrary units), capillary temperature 350°C, capillary voltage -37.5 V, tube lens -125 V, skimmer -26 V, and heather temperature 130°C. The injection volume was 5 µL. A BEH Shield C_{18} column (150 × 2.1 mm, 1.7 µm; Waters) was used for the separation. The column was maintained at 50°C. The flow-rate was 0.45 mL/min, and the eluents were 0.05% formic acid in water (A) and acetonitrile (B). The UHPLC separation was achieved by the following linear elution gradient: 5–35% of B in 10 min, which was then increased from 35 to 80% B in 10 min. The acquisition was made in the full-scan mode in the range $(m/z)^-$ 80–1000 u, using an isolation window of ±2 ppm. The AGC target, injection time, mass resolution, energy and gas in the collision cell were 1×10^6, 100 ms, 50 K, 20-40-60 V and N_2 respectively. The MS data were processed using Xcalibur software (Thermo Scientific). The peak identity was ascertained by

evaluating the accurate mass, the fragments obtained in the collision cell and the on-line UV spectra (200–450 nm).

2.10. Amino acid determination

Free amino acid fraction was obtained as reported by Serra Bonvehí and Escolá Jorda, (1997) with a slight modification. Briefly, 1 g of pulverized pollen was vortexed in 20 mL of water, centrifuged at 6000 x g for 10 min at 4 °C and the supernatant transferred into a 50 mL volumetric flask. The residue was extracted with 20 ml of water, treated a described above and the final volume adjusted to 50 mL with water.

Amino acid derivation with AccQ·Tag reagents was conducted according to the manufacturer's protocol. Briefly, amino acids were derivatized by adding 80 µL of AccQ•Tag ultra borate buffer, 10 µL of IS (0.3 mg mL^{-1}) and 20 µL of derivatising reagent to 10 µL of bee pollen extract or standard solution. The mixture was then incubated for 10 min at 55 °C. The 10 min heating was an important step when converted the phenolic side chain of tyrosine to free phenol, so tyrosine could become a major mono-derivatized compound like the rest of the amino acids. Heating the samples has no other significant effect. Amino acid derivatives in room temperature were stable for as long as 1 week.

Liquid chromatographic separation was performed on an Acquity UPLC system (Waters) coupled with an eLambda DAD (Waters). The injection volume was 2 µL. The derivatives were separated on an AccQ•Tag Ultra Column (1.7 µm BEH, 100 x 2.1 mm) maintained at 50°C. The flow-rate was 0.7 ml min^{-1}. The eluents and nonlinear separation gradient used were as reported by Armenta et al. (2010). The data were acquired in the range 200-450 nm and chromatogram integrated at 260 nm.

311 The stock solution contained a 2.5 mM concentration of each amino acid with the
312 exception of cysteine with the concentration 1.25 mM. Calibration curves were in the range
313 2.2-51 μg mL^{-1}.
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315 *2.11. Statistical analysis*
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317 Statistical analysis was performed using Statistica software (Statsoft, Tulsa, OK, USA).
318 Results were expressed as mean values ± standard deviation. Differences between bee pollen
319 samples were analysed by one-way analysis of variance (ANOVA). $P< 0.05$ was considered
320 statistically significant. Principal component analysis (PCA) was performed using R statistical
321 software 3.1.2 by means the function PRCOMP.
322
323 **3. Results and discussion**
324
325 The chemical composition of the pollen samples is given in Table 1. No difference
326 among the groups was found for moisture and ash content ($p>0.05$). With regard to moisture,
327 fresh bee pollen contains about 20-30% of water, which are favorable conditions for the
328 development of bacteria, yeasts and molds. Thus, for preventing bacterial contaminations and
329 preserve the quality, pollen must be harvested daily and frozen suddenly. Freezing caused no
330 substantial changes in the chemical composition of the pollen loads. After thawing, pollen
331 may be processed by different methods such as desiccation, lyophilization, freeze-drying and
332 microwave-assisted drying. Lyophilisation and drying at 40 ºC seem to decrease some
333 nutrients like ascorbic acid (Campos et al., 2005), while microwave-assisted drying seems to
334 reduce some antioxidant compounds such as tocopherols (Conte et al., 2017). After drying,

335 the humidity level should be in the range of 4-8%. In this condition pollen retains its
336 nutritional quality and health safety.
337 In all the samples analyzed the humidity was about 15%. This was most likely due at
338 mild thermal treatment activity of the producers. Despite this relatively high water content,
339 microbiological and chemical analysis have not showed relevant concerns and violations of
340 the safety regulations. In particular, mesophilic microbe content was less than 10^5 cfu g^{-1} *S.*
341 *aureus, E. coli* and *Salmonella spp*. absents and yeasts were $2x10^3$, $5x10^3$ and $2x10^4$ cfu g^{-1} in
342 the pollen from Italy, Spain and Colombia, respectively (data not showed). Aflatoxins and
343 ochratoxin were not detected (<2 µg Kg^{-1}, data not showed).
344
345 *3.1. Nutrients*
346
347 Three main sugars were identified and quantified. Fructose was the most abundant,
348 followed by glucose and sucrose (Table 1), and the fructose/glucose ratio varied between 1.20
349 and 1.5. The sugar contents of the various pollen samples were not significantly different and
350 in agreement with literature values (Conti et al., 2016; Szczęsna et al., 2002).
351 The bee pollen protein content ranged from 12.3–21.6 g $100g^{-1}$ DBP, evaluated by
352 multiplying the N content by a factor of 5.6. These results were similar to those obtained by
353 Fuenmayor et al. (2014) and Gabriele et al. (2015). The protein contents of the Colombian
354 and Italian pollens were higher compared to Spanish pollen. The differences were likely due
355 to their botanical origins.
356 The content of TL, the relative percentage of saturated fatty acids (SFAs) and
357 unsaturated fatty acids (UFAs), n-3, n-6, and the UFA/SFA ratios of the TLs from the
358 Colombian, Italian and Spanish honeybee-collected pollen is reported in Table 1. The fatty
359 acid compositions are provided in Table 2. The TL content was highly variable and ranged

360 from 2.0–6.5%, with samples from Colombia and Spain containing significant greater TL
361 amounts relative to the Italian bee pollen. Colombian pollen was rich in n-3 fatty acids, while
362 Italian and Spanish samples contained high amounts of n-6 fatty acids.
363 The Colombian pollen has an excellent n-6/n-3 ratio (0.3), which allows an n-3 supply
364 of about 0.4 g dose^{-1} (15 g), considering that the sample contains 6% lipid. The Italian product
365 has a good n-6/n-3 ratio (1.4) but a low lipid content (2.5%). Thus, 15 g pollen provide only
366 0.09 g of n-3. On the contrary, Spanish sample has a high lipid content (5.9%) but a poor n-
367 6/n-3 ratio (2.1), and this brings to get an n-3 intake of 0.16 g dose^{-1}. Overall, Colombian
368 pollen provides an amount of n-3 fatty acids 4.4 and 2.5 times higher than that provided by
369 the Italian and Spanish pollen, respectively.
370 Considering that in Italy the n-3 target daily intake must be in the range 1.4-5.5 g
371 (LARN, 2014), pollens with high lipid content and low n-6/n-3 ratio could be an attractive
372 alternative to increase n-3 intake.
373 Regarding the SFA content and UFA/SFA ratio, no significant differences among the
374 pollen samples were observed. On the contrary, the monounsaturated fatty acid (MUFA)
375 content was higher in the Italian pollen than the Colombian and Spanish specimens. Twenty-
376 one fatty acids were identified in the pollen extracts. The most abundant were α-linolenic
377 (18:3n-3), linoleic (18:2n-6), palmitic (16:0) and oleic acid (18:1n-9). In particular,
378 Colombian pollen was high in α-linolenic (44.1% of fatty acids found), while Italian and
379 Spanish pollen were rich in linoleic acid, at 29.4 % and 37.5%, respectively (Table 2).
380 Free amino acid composition is reported in Table 3, with values expressed in mg g^{-1} of
381 DBP. The sample from Spain, Italy and Colombia contained 20, 20 and 18 amino acids,
382 respectively, and the most abundant was proline. Arginine was the second most abundant AA
383 constituting on average 15.1, 12.9 and 3.8% of the total amino acids in the Spanish, Italian
384 and Colombian pollen, respectively. Thus, proline and arginine constituted about 68, 72 and

385 82% of the total AA in the Spain, Italy and Colombia pollen (Table 3). One interesting
386 finding was that arginine was the second most predominant amino acid; other bee-collected
387 pollen originating from Poland, South Korea and China showed a high content of glutamic
388 acid, aspartic acid, leucine and lysine (Szczęsna, 2006).
389 The amount of some essential amino acids such as leucine, isoleucine, lysine, and
390 phenylalanine in Spanish and Italian pollen was higher compared to Colombian sample.
391 Tryptophan (0.11-0.15 mg g^{-1}) and cysteine (0.07-0.09 mg g^{-1}) were present in low amount in
392 pollen from Spain and Italy and were not detected in Colombian one. The Spanish pollen was
393 the richest in threonine and valine. Spanish and Italian pollen presented percentages of total
394 free amino acid significantly different ($P < 0.05$) from Colombia sample. The differences in
395 the total and individual amino acid content were probably due to the different botanical
396 composition.
397 Proline (16-20 mg g^{-1}) was the main amino acid in all the sample, reaching
398 approximately a level of the 53, 59 and 78% of the total free AA content in pollen from Spain,
399 Italy and Colombia, respectively.
400 It has been reported that proline is the main free AA in the well dried and stored pollen
401 while glutamic acid is the most abundant in freshly collected pollen. When the temperature is
402 high and the drying process period excessively long, the free AA content decreases (<2 %)
403 and consequently the ratio proline/total free AA (Proline Index) increases (>80 %).
404 Conversely, if the drying process is carried out properly, the free AA content remains high
405 (>2.5 %) and the proline index is less than 80 %.
406 A minimum quantity of 2 % of free AA content is suggested to standardize the
407 commercial honeybee-collected pollen in the European market (Serra Bonvehí and Escolá
408 Jorda, 1997), and the Proline Index can be used as an “indicator” of the pollen freshness. The
409 latter must be less than 80%.

3.2. Identification of PA-conjugates by their fragmentation patterns

Pollen extract represents a complex mixture of variable composition. Thus, an untargeted analysis was performed using an UHPLC coupled to a DAD and Orbitrap-MS. High mass resolution (50 K) and high mass accuracy (2 ppm) allow obtaining the formula of parent and product ions. Due to these features, together with the enhanced efficiency of the UHPLC technique, this system is a powerful tool for the identification of unknown analytes in the pollen extracts. Untargeted analysis, however, cannot be done based on elemental composition data alone. Additional information is required, such as the UV spectrum and fragmentation pattern with collision-induced dissociation (CID) of the parent ion. Given the relatively high proton affinities of amine-N atoms, detection in positive ion mode is initially preferred for these compounds. After this step, analyses were also conducted in the negative ion mode, for the improved elucidation of the HCAs conjugated to the PAs. Examples of the UHPLC profiles of the pollens examined in this study, over the 240–440 nm range, are shown in Figure 1. Table 4 reports the on-line UV spectra, deprotonated ion and fragments of the main flavonoids and PAs, such as spermidine and spermine conjugates to HCAs detected in the DBP. Regarding PAs linked to HCAs, the fragmentation starts with a single cleavage of the terminal amide bond (N^1) of the spermidine and spermine cores, resulting in the formation of di- or tri-substituted fragments, respectively. On the contrary, the cleavage of the amide bond at N^5 for spermidine and N^5 or N^{10} for spermine was obtained at higher collision energies. Thus, the fragmentation depends on the HCA moiety substitution position in the PA core. As an example, Figure 2A displays the fragmentation pattern of the N^1-caffeoyl-$N^{5,10}$-di-*p*-coumaroyl-spermidine (*m/z* 598.2558 u). The main product ion, *m/z* 462.2036 u, corresponds to the loss of caffeic acid and formation of the isocyanate group, respectively. The simultaneous loss of caffeic acid and *p*-coumaric acid produces the ion with *m/z* 342.1459

u, which contains two isocyanate groups. The fragments at *m/z* 135.0450and 119.0500 u have been attributed to decarboxylated caffeic acid and *p*-coumaric acid, respectively. *p*-Coumaric acid also undergoes loss of a water molecule, with the formation of the ion at *m/z* 145.0292 u. Analogously, $N^{1,10}$-di-*p*-coumaroyl-N^5-caffeoyl-spermidine (*m/z* 598.2558 u) mainly generated fragments at *m/z* 478.1980 u (Figure 2B),corresponding to the loss of *p*-coumaric acid and formation of the isocyanate group. Instead, the abundance of the ions generated at low-energy CID, by the loss of N^5-caffeic acid (*m/z* 462.2036 u) was low. Conversely, they were present at high collision energy (25 eV). Likewise, peak 2 had the formula $C_{25}H_{30}N_3O_4$, $[M-H]^-$ 436.2247u, which corresponds to $N^{1,10}$-di-*p*-courmaroyl-spermidine or $N^{1,5}$-di-*p*-coumaroyl-spermidine. After CID at different energies, the only fragments found had *m/z* 316.1666 and 119.0502 u, corresponding to the loss of *p*-coumaric acid at N^1 (-120.0575). The ions with *m/z* 290.1874, corresponding to the loss of *p*-coumaric acid at N^5, were not detected (Figure 3). Overall, PAs linked to HCA easily lose the moiety bound to the primary N and with much more difficulty, which joined to the secondary N. Thus, loss of 120.0575, 136.0524, 150.0681, 180.0787 and 166.0630 corresponded to the presence of *p*-coumaric acid, caffeic acid, ferulic acid, syringic acid and 4-methyl-gallic acid residue in position N^1 of spermine or spermidine, respectively.

3.3. Carotenoid determination

The total carotenoid content in the saponified pollen samples was determined spectrophotometrically at 450 nm, using the zeaxanthin $\varepsilon^{\%}$ and by HPLC analysis by using an authentic standard. The equations of calibration curve for lutein (Lut, 0.14-1.83 μg mL^{-1}), zeaxanthin (Zea, 0.11-1.64 μg mL^{-1}), β-cryptoxanthin (β-Cry, 0.14-1.69 μg mL^{-1}), echinenone

(Ech, 0.11-1.34 μg mL^{-1}), α-carotene (α-Car, 0.11-1.58 μg mL^{-1}) and β-carotene (β-Car, 0.16-1.87 μg mL^{-1}) were as follows:

Lut: Y = 125.7 X – 2.7, R^2=0.999, n=5

Zea: Y = 129.1 X – 1.9, R^2=0.999, n=5

β-Cry: Y = 132.2 X – 1.5, R^2=0.999, n=5

Ech: Y = 124.1 X – 2.0, R^2=0.997, n=5

α-Car: Y = 116.0 X – 2.1, R^2=0.998, n=5

β-Car: Y = 101.2 X – 1.5, R^2=0.998, n=5

Where Y=peak area x 10^{-3}, X=μg/mL

The total carotenoid content, determined by spectrophotometry, was 221.4, 56.9 and 24.7 μg g^{-1} for DBP from Colombia, Spain and Italy, respectively. According to the Student's *t*-test, there was no significant difference (p=0.591) between the methods used.

Characterization of the free and esterified carotenoids is necessary to obtain reliable compositional data. Thus, we developed a UHPLC-DAD-ESI-HR-MS method for the identification of the carotenoid composition in pollen from three different countries. Free carotenoids were identified by comparing the retention time (RT), UV-vis spectra and accurate mass (2 ppm) with those of authentic standards. Non-esterified carotenoids were detected in small amounts (< the limit of quantification) in all pollens. Notably, the Colombian pollen contained traces of lutein, zeaxanthin, β-carotene and phytoene, while only β-carotene was present in the Spanish and Italian samples. In contrast, all samples contained esterified carotenoids. Figure 4A and 4B are the chromatograms, integrated over the wavelength range 254–600 nm, relating to non-saponified and saponified extracts from Colombian pollen. The principal peaks found in all samples had *m/z* 932.7610 and fragment

ions at *m/z* 750.5939 and 532.4072 u, corresponding to the molecular ion $[M]^{\bullet+}$, namely, the loss of one molecule of lauric acid $[M\text{-}C_{11}CO]^{\bullet+}$and two residues of lauric acid and water $[M\text{-}2C_{11}CO\text{-}2H_2O]^{\bullet+}$, respectively. Basic hydrolysis initially produced two peaks, with different intensities, at *m/z* 750.5939 u and after 2 h the dominant peak in the chromatogram had *m/z* 568.4275 u $[M]^{\bullet+}$ and a lower signal at 550.4178 u, corresponding to the loss of water $[M\text{-}H_2O]^{\bullet+}$. Based on the RT, UV-vis and MS spectrum, the peak was identified as zeaxanthin. Thus, the primary carotenoid was zeaxanthin esterified with two molecules of lauric acid (C12:0). At the end of the hydrolysis, a second peak, with the same molecular ion as zeaxanthin but a shorter RT, was found. This compound was identified as lutein. Thus, samples contained also small amounts of the di-lauryl ester of lutein. After hydrolysis, the content of carotenoids did not increase.

3.4. Multivariate statistical analysis

PCA was applied to check for similarity between samples according to their chemical composition and geographical origin. The first two principal components (PC) were able to explain up to 95% of data variability. Bee pollen samples were differentiated in PC1 (64 %) based on the content of total lipid, SFA, MUFA, PUFA, 16:0, 18:1n9, 12:0, 24:1n9, 18:3n6, 24:0 22:0, total and single sugars, while PC2 (31 %) separated the samples according to the levels of protein, ash, carotenoid, 18:2n6, 18:3n3, 20:2n6 and 20;3n6.

Figure 5 reports two-dimensional plot and show the response variability of the experimental data obtained from the pollen from Colombia, Italy and Spain. It is noted that the three samples from three different geographical regions are largely dispersed within the graph, indicating a high diversity between them. In particular, the sample from Italy has a

greater deviation compared to Spain and Colombia on the PC1 axes (the PC with the greater power of descriptiveness of the sample).

3.5. Palynological analysis

All pollen samples were found to be heterofloral, having different pollen types and percentages. In the specimen from Spain, *Cistus ladanifer* and *Echium* were the PDs (>45%), followed by *Achillea* and Compositae types T (*Taraxacum*) and S (*Carduus, Cirsium*). The POs were *Vicia, Quercus ilex, Quercus r., Rubus*, Pinaceae, *Filipendula, Trifolium incarnatum, Trifolium pratense, Trifolium repens, Prunus, Pyrus, Malus* and *Oxalis*. The three species of Trifolium have been distinguished by their size. Indeed, in decreasing order *incarnatum, pratense* and white. Furthermore, *incarnatum* has exine with a larger mesh pattern and it is more elongated than the other two. The *pratense* is also cross-linked but with less evident mesh. The white clover is the smallest and with the smoother surface.

The PDs in the Italian sample were *Rubus ulmifolius, Parthenocissus quinquefolia* and *Ampelopsis brevipedunculata*. The PSs were *Papaver, Muscari* and *Lamium*, while *Ambrosia, Fraxinus ornus, Castanea sativa* and some Gramineae, such as *Cynodon dactylon, Phleum pratense* and *Poa pratensis* were the POs.

Pollen from Colombia contained mainly *Brassica napus, Taraxacum officinale* and *Trifolium pratense*. The POs were from Rhamnaceae (*Gouania polygama*), Rosaceae with striated resin (*Prunus sp.),* Fabaceae (*Trifolium repens, Vicia* sp.) and *Euphorbia* sp.

4. Conclusion

In this study, bee pollen samples from three different geographical areas were analysed for their nutrient contents. Moreover, the botanical origin and the phytochemical profile of each pollen was evaluated. The content of carbohydrate, moisture and ash was not significantly different among the pollens, while the amount of protein was higher in the Colombian and Italian samples compared to the Spanish pollen. Colombian pollen also contained a high amount of n-3 fatty acids relative to the Italian and Spanish pollens. Notably, Colombian pollen was rich in α-linolenic, while the Italian and Spanish samples mainly contained linoleic acid. The high α-linolenic acid and TL content may be attributed to the presence of Brassicaceae, such as *Brassica napus*. Due to the high content of essential fatty acids, pollen load could be used as a dietary supplement. The main polyphenols were coumaroyl- and caffeoyl-spermidine derivatives. Proline and arginine were the main AA in all the pollen samples. Low-energy CID allowed determining which phenolic acid was bound to the N^1 of the PA. All pollen samples contained esterified carotenoids, and the main one was zeaxanthin linked to two residues of lauric acid, while only trace amounts of non-esterified carotenoids, such as lutein, zeaxanthin and β-carotene, were detected. Therefore, the nutritional composition and the high content of bioactive compounds, such as flavonoids, carotenoids and phenolic acids linked to PAs, could make bee pollen a valuable ingredient for the food and pharmaceutical industries.

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Declaration of interest

None.

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Figure legends

Figure 1. Typical UHPLC chromatograms in the range 240–450 nm of methanolic pollen extracts from Italy (A), Spain (B) and Colombia (C). See Table 4 for peak number assignment.

Figure 2. Main fragmentation patterns of the deprotonated ion with *m/z* 598.2558 u, corresponding to (**A**) N^{1}-caffeoyl-$N^{5,10}$-di-*p*-coumaroyl-spermidine and (**B**) $N^{1,10}$-di-*p*-coumaroyl-N^{5}-caffeoyl-spermidine. The product ions were obtained by high-resolution CID at 20, 40 and 60 eV.

Figure 3. Peak 2, $[M-H]^-$ 436.2247 u, identification by fragmentation pattern evaluation.

Figure 4. Typical UHPLC chromatogram in the range 300–550 nm of Colombian pollen extracts not saponified (A) and saponified by methanolic KOH (B).
Peak a: zeaxanthin-di-lauryl ester, b: β-carotene, c: phytoene, d: zeaxanthin, e: lutein.

Figure 5. Inter-sample diversity based on nutrient and phytochemical constituents. The PC1 and PC2 were responsible for 64 and 31% respectively of the total variance among the sample from Colombia, Italy and Spain.

673 **Table 1** Nutritional components, moisture, ash and carotenoids of pollen load samples from

674 Colombia, Italy and Spain

675

Analyte	Colombia	Italy	Spain
Moisture (%)	15.5±0.4[a]	15.1±0.5[a]	14.9±0.4[a]
Proteins (%)	21.6±0.5[a]	19.5±0.4[a]	12.3±0.2[b]
Lipids (%)	6.0±0.2[a]	2.5±0.2[b]	5.9±0.6[a]
SFA	34.9±0.7[a]	30.9±0.4[a]	33.7±0.6[a]
MUFA	8.9±1.8[a]	14.3±2.2[b]	9.6±1.1[a]
PUFA	56.2±2.7[a]	54.8±2.4[a]	56.7±2.1[a]
n-3	44.1±5.4[b]	23.1±2.3[a]	18.5±1.7[a]
n-6	11.9±2.7[a]	31.6±0.2[b]	38.2±0.2[c]
UFA/SFA	1.9±0.1[a]	2.2±0.0[a]	2.0±0.0[a]
Ash (%)	2.1±0.2[a]	1.8±0.2[a]	1.6±0.2[a]
Carbohydrate (%)	39.1±1.9[a]	44.1±1.[a]	37.7±1.4[a]
Fructose	18.7±1.2[a]	23.1±1.1[a]	17.1±1.1[a]
Glucose	14.4±0.9[a]	15.9±0.9[a]	14.1±1.3[a]
Sucrose	6.0±0.5[a]	5.1±0.3[a]	6.2±0.3[a]
Carotenoids[1] (µg Zea g^{-1} DW)	221.4±10.6[a]	24.7±1.3[b]	56.9±1.9[c]
Carotenoids[2] (µg Zea g^{-1} DW)	207.4±9.1[a]	21.3±0.9[b]	51.3±1.2[c]

676

677

[a,b,c] Different superscript letters indicate statistical differences among the bee pollen extracts ($p<0.05$). Variables were analyzed by one-way ANOVA with pollen as dependent factors. Differences were considered significant at $p \leq 0.05$; post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with $p \leq 0.05$ as the level of statistical significance. Assays were carried out in triplicate and results were expressed as mean values ± standard deviation.Carotenoids[1]: spectrophotometric analysis after saponification; Carotenoids[2]: LC-DAD analysis after saponification. DW: dry weight; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; UFA: sum of unsaturated fatty acids (MUFA+PUFA); Zea: zeaxanthin.

687 **Table 2** Percentage composition (mean±S.D.) of long-chain fatty acids (FA) of honeybee-

688 collected pollen from Colombia (PC), Italy (PI) and Spain (PS).

FA	PC	PI	PS
12:0	1.7±0.0	0.2±0.1	1.7±0.2
14:0	1.7±0.3	0.61±0.1	0.9±0.1
15:0	0.1±0.0	0.2±0.0	0.3±0.0
16:0	**25.3±1.4**	**20.6±0.0**	**26.8±1.5**
17:0	0.2±0.0	0.5±0.1	0.2±0.0
18:0	**2.4±0.2**	**2.5±0.1**	**1.6±0.1**
20:0	1.4±0.2	1.5±0.1	0.6±0.0
22:0	0.9±0.1	2.4±0.2	0.7±0.1
23:0	0.1±0.1	0.3±0.0	0.4±0.0
24:0	0.3±0.3	2.1±0.2	0.4±0.0
18:1n9	**7.0±1.9**	**12.8±1.9**	**7.4±0.4**
16:1n9	0.0±0.1	0.1±0.0	0.1±0.0
16:1n7	0.1±0.0	0.2±0.1	0.2±0.0
18:1n7	0.5±0.0	0.5±0.0	0.8±0.0
20:1n9	0.5±0.2	0.8±0.1	0.7±0.1
24:1n9	0.6±0.2	0.0±0.0	0.5±0.0
18:2n6	**10.6±2.4**	**29.4±1.5**	**37.5±4.3**
20:2n6	1.1±0.2	0.1±0.0	0.2±0.0
18:3n6	0.3±0.4	1.9±1.3	0.1±0.0
20:3n6	0.0±0.0	0.2±0.1	0.5±0.0
18:3n3	**44.1±5.4**	**23.1±2.2**	**18.4±1.7**

689 C16:0 palmitic acid, C18:0 stearic acid, C18:1n9 oleic acid, C18:2n6 linoleic acid, C18:3n3

690 α-linolenic acid

691 **Table 3** Free amino acid content (mg g^{-1} DBP) in pollen sample from Colombia, Italy and

692 Spain

693

Amino acid	Colombia	Italy	Spain
His	0.68±0.1[a]	0.49±0.10[a]	0.59±0.03[a]
Ser	0.23±0.02[a]	0.33±0.04[b]	0.47±0.01[c]
Arg	0.96±0.08[a]	3.81±0.55[b]	4.64±0.05[c]
Gly	0.08±0.01[a]	0.14±0.04[b]	0.25±0.01[c]
Asp	0.21±0.03[a]	0.28±0.07[b]	0.46±0.01[b]
Aspg	0.29±0.04[a]	0.43±0.02[b]	0.49±0.02[b]
Glu	0.13±0.02[a]	0.25±0.06[b]	0.29±0.01[b]
Glut	0.09±0.01[a]	0.14±0.02[b]	0.23±0.01[c]
Thr	0.10±0.0 [a]	0.15±0.04[b]	0.31±0.01[c]
Ala	0.43±0.02[a]	0.98±0.10[b]	1.19±.0.02[c]
Pro	19.75±0.72[a]	17.47±1.14[b]	16.17±0.06[b]
Lys	0.39±0.06[a]	0.81±0.12[b]	0.87±0.02[b]
Tyr	0.36±0.03[a]	0.76±0.29[b]	0.89±0.04[b]
Met	0.20±0.02[a]	0.21±0.03[a]	0.19±0.01[a]
Val	0.29±0.03[a]	0.30±0.08[a]	0.70±0.01[b]
Ile	0.18±0.02[a]	0.53±0.10[b]	0.64±0.04[b]
Leu	0.47±0.05[a]	1.26±0.07[b]	1.34±0.03[b]
Phe	0.44±0.04[a]	0.86±0.09[b]	0.87±0.02[b]
Trp	n.d	0.15±0.02[a]	0.11±0.01[a]
Cys	n.d	0.09±0.01[a]	0.07±0.01[a]
Total AA	25.3±1.0[a]	29.4±0.7[b]	30.8±0.2[b]
(Pro/Total AA)*100	78[a]	59[b]	53[c]

694 Ala, alanine; Arg, arginine; Asp, aspartic acid; Aspg, asparagine; Cys, cysteine, Gly, glycine;

695 Glu, glutamic acid; Glut, glutamine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine;

696 Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp,

697 tryptophan; Tyr, tyrosine; Val, valine.

698 n.d: not detected (< LOD).

699 [a,b,c] Different superscript letters indicate statistical differences among the bee pollen extracts

700 ($p<0.05$). Variables were analyzed by one-way ANOVA with pollen as dependent factors.

701 Differences were considered significant at $p \leq 0.05$; post-hoc analysis of differences between

treatments was assessed by the Least Significant Difference (LSD) test with $p \leq 0.05$ as the level of statistical significance.

704 **Table 4** Polyamines linked to hydroxyl-cinnamic acids and flavonoids in pollen sample from

705 Colombia, Italy and Spain

706

Peak	RT	UV (nm)	$[M-H]^-$	Fragment	Compound
1	10.8	265, 350	771.1998	609.1463, 314.0434	I-(Glc)-Glc-Ara
2	11.93	296, 306	436.2247	316.1666	S-$N^{1,10}$-di-*p*C
3	12.03	265, 348	609.147	446.0854, 285.0405	L-Glc-Glc
4	12.22	258, 354	625.1417	300.0275	Q-Glc-Glc
5	12.27	258, 354	639.1572	314.0434	I-Glc-Glc
6	13.5	256, 356	595.1314	300.0277, 463.0890	Q-Glc-Ara
7	13.91	265, 346	609.1469	284.0325	K-Glc-Glc
8	14.46	254, 356	609.1468	314.0433, 284.0327	I-Glc-Ara
9	14.94	255, 354	463.0891	300.0277, 271.0614	Q-Glc-Glc
10a	15.35	272, 315	477.1044	314.0434, 299.0197	I-Glc
10b	15.49	265, 346	593.1519	284.0328	K-Glc-Rha
11	15.55	253, 353	623.1623	314.0434	I-Glc-Rha
12	15.68	253, 353	609.1471	315.0514	I-Glc-Ara
13a	16.75	309	685.3249	519.2610, 399.2037, 165.0555	SP-N^{14}-*p*C-N^{x}-*p*C-N^{1}-4mGA
13b	16.87	265, 346	447.0938	284.0328, 255.0301	K-Glc
14a	16.89	315	715.3357	549.2717, 399.2038, 165.0555	SP-N^{14}-FA-N^{x}-*p*C-N^{1}-4mGA
14b	17.1	253, 353	477.1045	314.0435	I-Glc
15	18.12	245, 377	317.0305	287.0991, 151.0035	M
16	18.94	250, 354	301.9355	151.0035	Q
17	20.34	290	403.1034	271.0612, 151.0035	P-Ara
18	20.62	245, 320	630.2459	494.1932, 468.2139, 358.1409, 372.1617, 304.1668, 135.0451	S-$N^{1,5,10}$-tri-CA
19	22.63	243, 309	614.251	478.1981, 452.2193, 358.1409, 161.0243, 135.0450	S-$N^{1,10}$-di-CA-N^{5}-*p*C
20	23.04	251, 346	285.0409	151.0035, 133.0293, 255.0299	K
21	23.51	290	271.0612	151.0035	P
22a	23.77	243, 320	879.3458	713.2806, 743.2929	SP-$N^{5,10,14}$-tri-CA-N^{1}-4mGA
22b	23.81	243, 320	849.3351	713.2830, 577.2304	SP-$N^{1,5,10,14}$-tetra-CA
23	24.42	296, 310	598.2563	462.2036, 342.1461, 135.0450, 145,0293, 478.1983	S-$N^{5,10}$-di-*p*C-N^{1}-CA
24	24.71	246, 310	598.2563	478.1983, 358.1409, 161.0242	S-$N^{1,10}$-di-*p*C-N^{5}-CA
25	25.26	294, 310	582.2615	462.2036, 342.1459, 145.0293, 119.0501	S-$N^{1,5,10}$-tri-*p*C
26	26.57	294, 310	582.2615	462.2036, 342.1459, 145.0293, 119.0501	S-$N^{1,5,10}$-tri-*p*C
27a	26.69		612.2829	492.2140, 462.2037, 372.1564, 342.1459	S-$N^{1,10}$-di-*p*C-N^{5}-FA
27b	26.74	293, 308	642.2829	492.2139, 522.2242, 372,1563, 466.2344	S-$N^{1,10}$-di-FA-N^{5}-*p*C

27c	26.81	317,290	672.2932	522.2245, 372.1563, 175.0390, 149.0605, 135.0449	S-$N^{1,5,10}$-tri-FA
28	27.49	290, 310	785.3562	665.2983, 545.2405, 145.0293	SP-$N^{1,5,10,14}$-tetra-*p*C
29	27.91	274, 310	785.3562	665.2983, 545.2405, 145.0293	SP-$N^{1,5,10,14}$-tetra-*p*C
30	28.17	290, 310	785.3562	665.2983, 545.2405, 145.0293	SP-$N^{1,5,10,14}$-tetra-*p*C
31	28.44	296, 308	785.3562	665.2983, 545.2405, 145.0293	SP-$N^{1,5,10,14}$-tetra-*p*C
32	29.21	268, 334	537.0814	151.0035, 385.0719, 443.0410	Amentoflavone
33	29.88	267, 333	537.0814	311.2234, 223.1704, 375.0511, 353.1915	bi-Apigenin

Ara: arabinose; CA: caffeic acid; FA: ferulic acid; Glc: glucose; I: isorhamnetin; K: kaempferol; L: luteolin; M: myricetin; pC: *p*-coumaric acid; P: pinobanksin; Q: quercetin; Rha: rhamnose; RT: retention time (min); S: spermidine; SP: spermine; x: unknown position; 4mGA: 4-methyl-gallic acid.

712 **Table 4** Free amino acid content (mg g^{-1} DP) in pollen sample from Colombia, Italy and

713 Spain

714

Amino acid	Colombia	Italy	Spain
His	0.68±0.1[a]	0.49±0.10[a]	0.59±0.03[a]
Ser	0.23±0.02[a]	0.33±0.04[b]	0.47±0.01[c]
Arg	0.96±0.08[a]	3.81±0.55[b]	4.64±0.05[c]
Gly	0.08±0.01[a]	0.14±0.04[b]	0.25±0.01[c]
Asp	0.21±0.03[a]	0.28±0.07[b]	0.46±0.01[b]
Aspg	0.29±0.04[a]	0.43±0.02[b]	0.49±0.02[b]
Glu	0.13±0.02[a]	0.25±0.06[b]	0.29±0.01[b]
Glut	0.09±0.01[a]	0.14±0.02[b]	0.23±0.01[c]
Thr	0.10±0.0 [a]	0.15±0.04[b]	0.31±0.01[c]
Ala	0.43±0.02[a]	0.98±0.10[b]	1.19±.0.02[c]
Pro	19.75±0.72[a]	17.47±1.14[b]	16.17±0.06[b]
Lys	0.39±0.06[a]	0.81±0.12[b]	0.87±0.02[b]
Tyr	0.36±0.03[a]	0.76±0.29[b]	0.89±0.04[b]
Met	0.20±0.02[a]	0.21±0.03[a]	0.19±0.01[a]
Val	0.29±0.03[a]	0.30±0.08[a]	0.70±0.01[b]
Ile	0.18±0.02[a]	0.53±0.10[b]	0.64±0.04[b]
Leu	0.47±0.05[a]	1.26±0.07[b]	1.34±0.03[b]
Phe	0.44±0.04[a]	0.86±0.09[b]	0.87±0.02[b]
Trp	n.d	0.15±0.02[a]	0.11±0.01[a]
Cys	n.d	0.09±0.01[a]	0.07±0.01[a]
Total AA	25.3±1.0[a]	29.4±0.7[b]	30.8±0.2[b]
Proline Index	78[a]	59[b]	53[c]

715 Ala, alanine; Arg, arginine; Asp, aspartic acid; Aspg, asparagine; Cys, cysteine, Gly, glycine;

716 Glu, glutamic acid; Glut, glutamine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine;

717 Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp,

718 tryptophan; Tyr, tyrosine; Val, valine.

719 n.d: not detected (< LOD). Proline Index= (Proline/Total AA) x 100.

720 [a,b,c] Different superscript letters indicate statistical differences among the bee pollen extracts

721 ($p<0.05$). Variables were analyzed by one-way ANOVA with pollen as dependent factors.

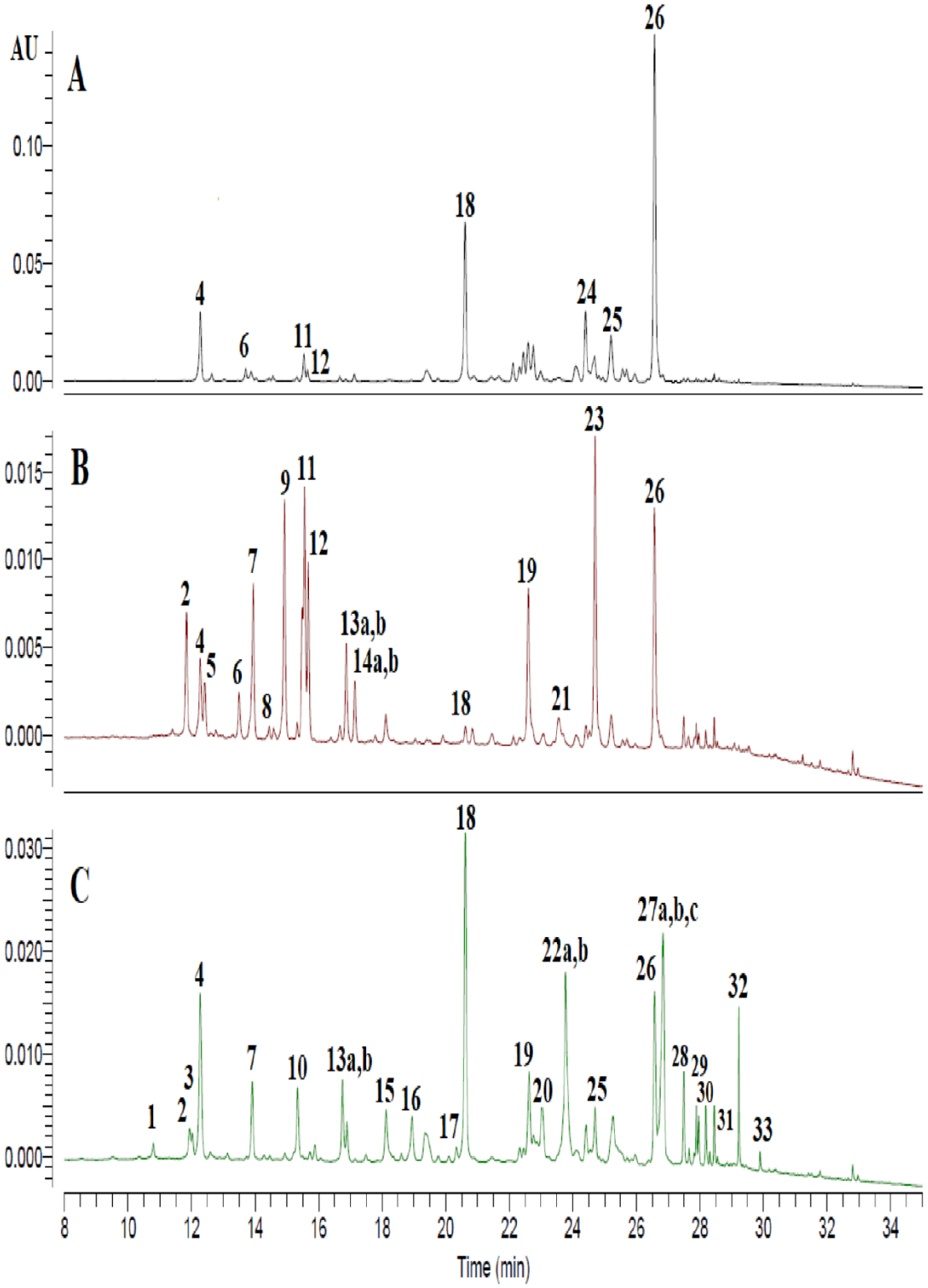
722 Differences were considered significant at $p \leq 0.05$; post-hoc analysis of differences between

723 treatments was assessed by the Least Significant Difference (LSD) test with $p \leq 0.05$ as the

724 level of statistical significance.

725

Figure 1

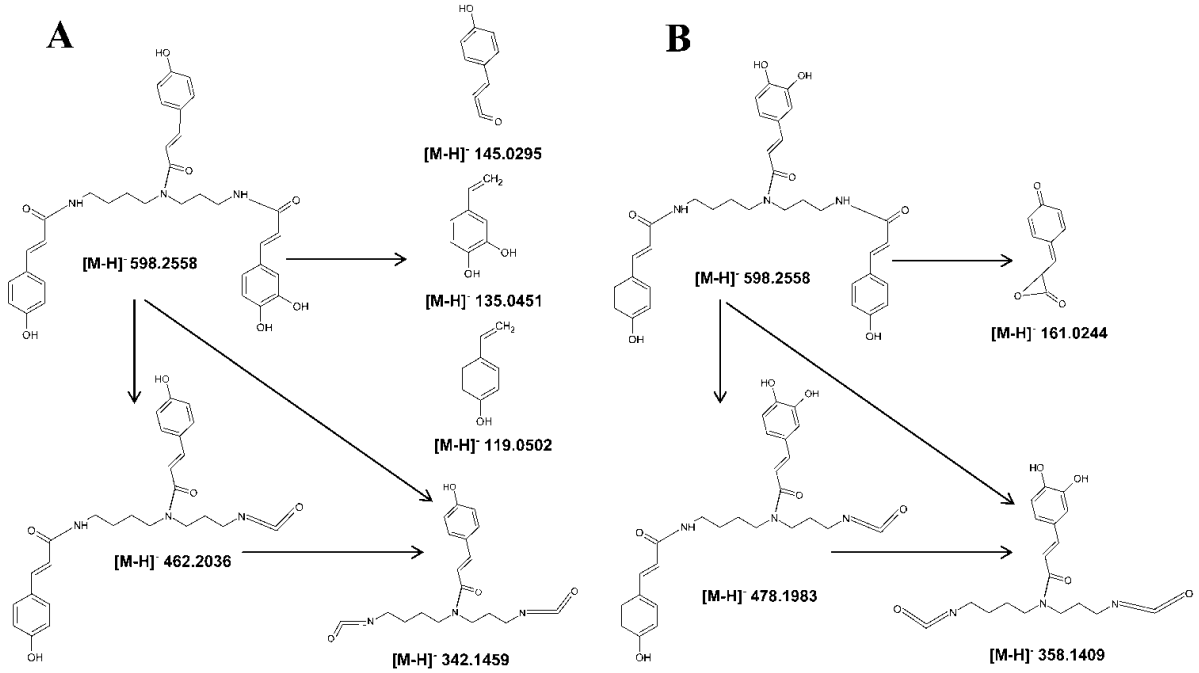


726

727 **Figure 2**

728

729



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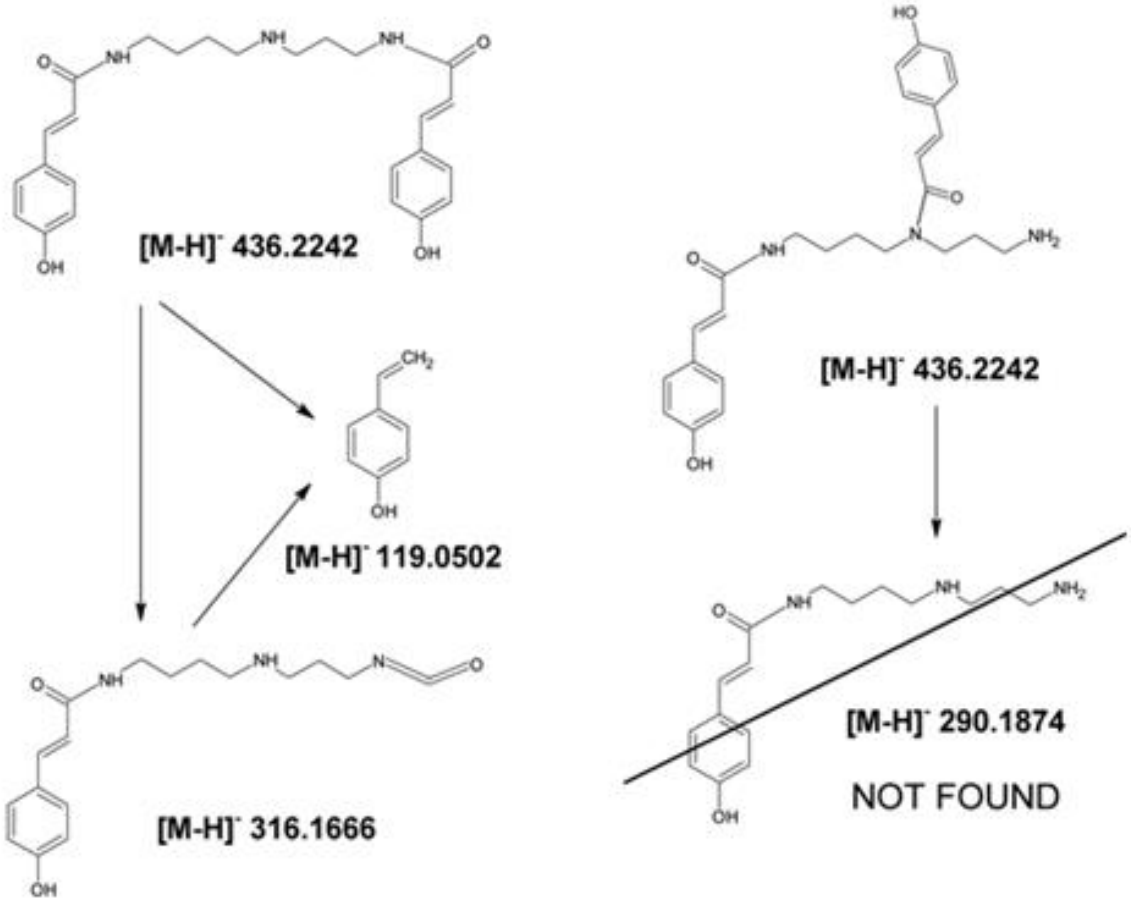
731

Figure 3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[M-H]⁻ 119.0502
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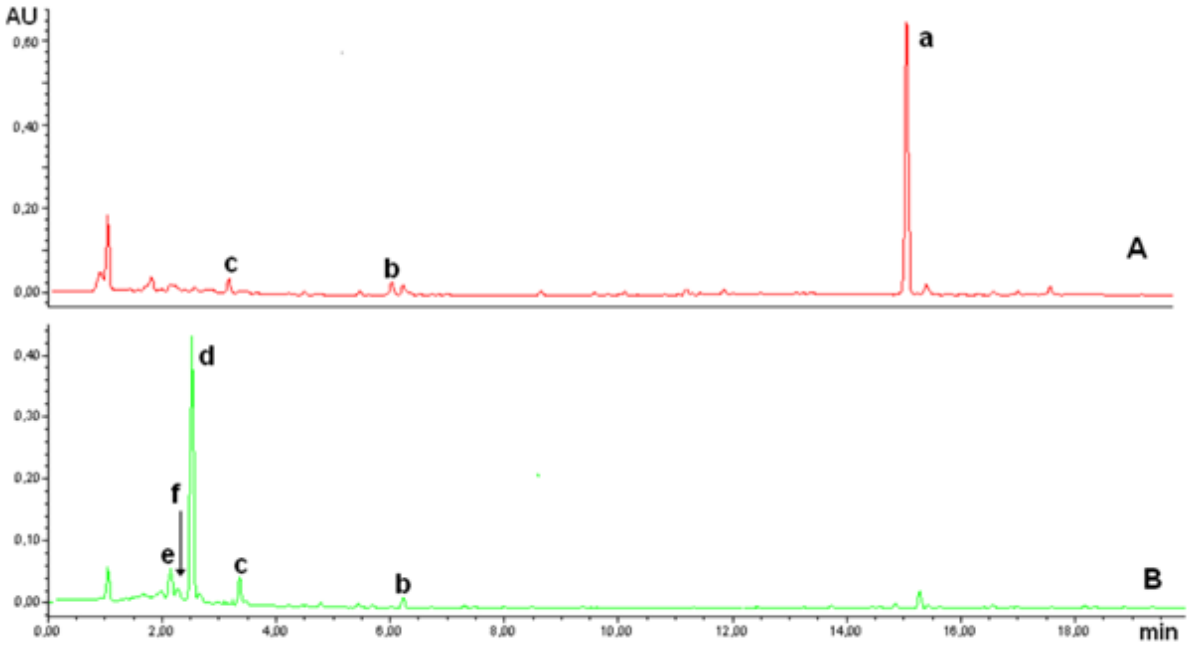
NOT FOUND

736 **Figure 4**

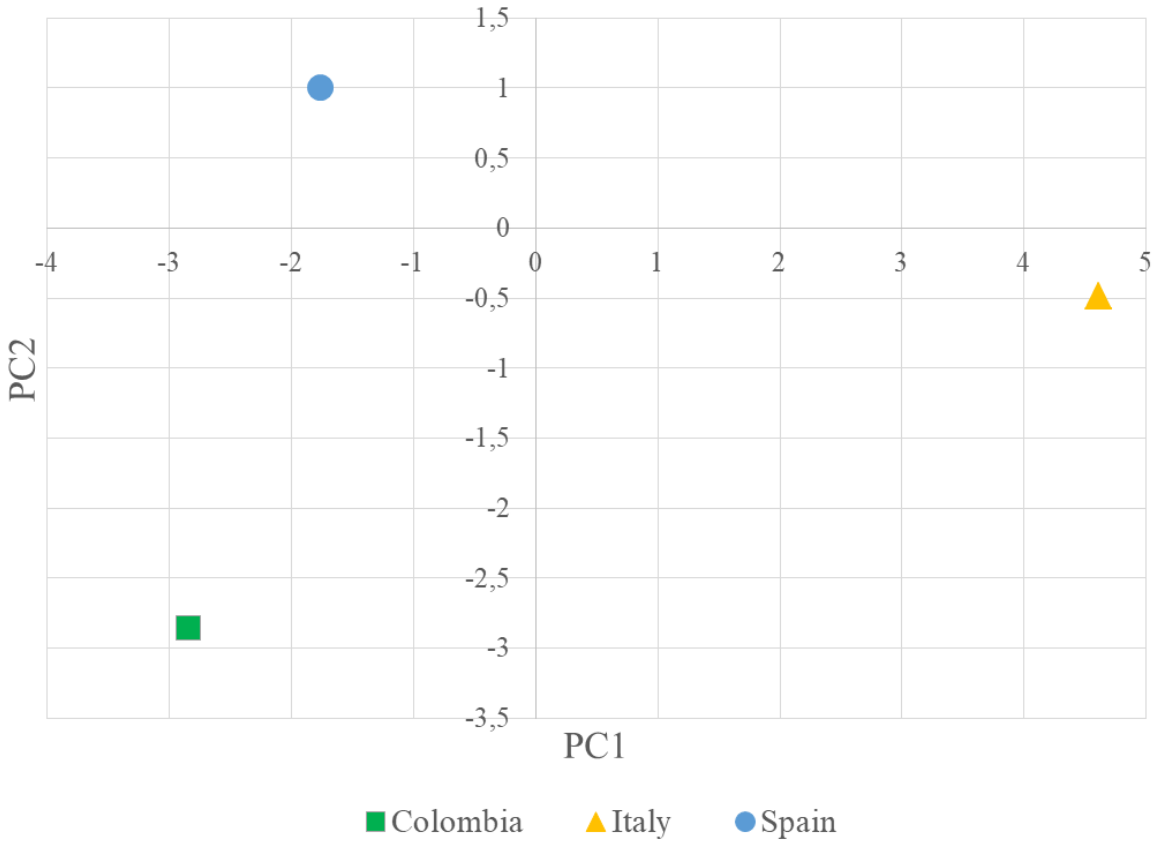
737



738

739 **Figure 5**

740



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