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Phenolic Profile of *Castanea* Bee Pollen from the Northwest of the Iberian Peninsula

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Abstract: Bee pollen is a rich bee product, from the point of view of its nutritional and functional chemical characteristics. The chemical composition of bee pollen and its properties make this product an excellent food supplement for the human diet, due to its various functional bioactivities, such as having antioxidant, antibacterial, antifungal, and anti-inflammatory properties. These properties depend on the botanical origin of the bee pollen. *Castanea sativa* bee pollen is one of the most important types of pollen collected in the northwest of the Iberian Peninsula. Thus, the phenolic profile of *Castanea* bee pollen was featured in this study. For this, 11 samples of *Castanea* were selected through prior colorimetric separation using the CIELab* scale and verified with palynological analysis. Identification of the main phenol compounds was performed through LC/DAD/ESI-MS^n analysis. The phenols compounds were quantified using calibration curves for caffeic acid, quercetin, and naringenin. The main results showed a profile formed of 19 compounds for all samples, although quantitative differences were found. Most of these compounds were phenolamides, with N^1, N^5, and N^{10}-tricaffeoylspermidine being significantly ($p < 0.05$) the most abundant. Three isorhamnetin glycoside derivatives and one naringenin were also identified. The richness in phenolamides of *Castanea* bee pollen identified in this study suggests *Castanea* bee pollen as a functional food, owing to its healthy properties.

Keywords: *Castanea*; bee pollen; phenols; phenolamides; color

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1. Introduction

As in other social insect species, a honey bee colony functions as a single organism, with a division of labor. In this sense, the nutritional needs of honeybees depend on adult-brood interactions and trophallaxis; that is, by transferring food from an adult bee to another. Plant resources such as nectar and pollen are the basis for the nutrition of the colony. Nectar is converted into honey, being the main source of carbohydrates and providing the main energy source for the colony. On the other hand, pollen supplies protein and other nutrients, playing a crucial role in larval development and the growth of colonies. In fact, pollen reserves are fundamental for the survival of the colony, determining the quality and the number of adults in the next generation [1,2]. The role of pollen in the resistance threshold of honey bees to the stress caused by pathogens and pesticides has also been highlighted [3]. A colony requires between 10 and 26 kg of pollen per year, depending on the region [2], which is consumed during almost the entire season, maintaining a low reserve that may experience wide fluctuations [1]. Furthermore, within the colony, honey bees strictly regulate their collection rates in response to changes in pollen reserves. Foraging activity increases with decreasing levels of stored pollen. The

availability of flowering plants in the field is then necessary to maintain the development of the colony. However, not all pollens are collected equally. It is quite clear that honey bees prefer to collect some types of pollen over others [4]. In the case of chestnut trees (*Castanea sativa* Miller), they represent the most important plant resource for hives in the Northwest of the Iberian Peninsula, for both honey and pollen [5,6]. This species is very well distributed throughout the territory, either forming monospecific forests or in groups with other deciduous species. The species is monoecious, and the flowers are grouped in yellow-greenish catkins and are largely self-incompatible, so that honey bee activity favors cross-pollination. Furthermore, the flowering period from May to the end of July, depending mostly on the altitude above sea level, corresponds with the optimum population level of bee colonies. For this reason, the pollen and nectar collection from this species is generally excellent [5,7].

Bee pollen is a rich product, from the point of its nutritional and functional chemical characteristics. The main components are carbohydrates; lipids, especially fatty acids; proteins, in which there are many essential amino acids; phenolic compounds; enzymes and coenzymes; bioelements; and vitamins [8–10]. The chemical composition of bee pollen and its properties make this product an excellent food supplement, with high demand in the dietary market. A wide range of properties and bioactivities related to the composition of bee pollen and bee bread have been compiled in the literature (with antioxidant, anti-inflammatory, anticarcinogenic, antibacterial, antifungicidal, hepatoprotective, and anti-atherosclerotic activities) [11–15]. Despite the defined bee pollen composition, botanical and geographical origin influences the relative abundance of some phytochemicals [16,17]. In this sense, it is expected that a more detailed study of bee pollen would identify compounds profiles that can act as fingerprints, contributing to the authentication and value of this bee product.

In particular, the phenolic profile of bee pollen has been studied, due to its relationship with various functional bioactivities important for human health, such as antioxidant, antibacterial, antifungal, and anti-inflammatory properties [12,18,19]. Phenolic compounds are the largest secondary metabolites synthesized by plants and chemically are very heterogeneous, being grouped according to their chemical structure, mainly into the phenolic acids and flavonoids [20]. Structurally, they consist of an aromatic ring surrounded by one or more hydroxyl substituents and a range of simple and polymerized phenolic molecules [21]. Thousands of polyphenolic compounds have been found in plants, all of them synthesized via the shikimic acid-derived phenylpropanoid and/or polyketide pathways [22]. These compounds have different activities in plants, playing an important role in modulating plant–pathogen and plant–environmental interactions. Some of them are good antioxidants, and thus polyphenols are important compounds for this functional property of foods of plant origin [23]. In addition, many polyphenol compounds, such as flavonoids or anthocyanin, have been related to color, as well as the characteristic bitter taste of several honey types and bee pollen [22]. On the other hand, flavonoids are polyphenolic secondary metabolites composed of low molecular weight molecules, containing 15 carbon atoms distributed in three aromatic rings. These represent the major group of phenolic compounds present in plants, widely distributed in the seeds, leaves, flowers, and bark of plants and trees. In pollen, around 25 compounds have been identified, including flavonols and glycosidic acids, hydroxycinnamic acids and derivatives, flavones and chalcones, which together are responsible for the high antioxidant activity [21]. Flavonoids in bee pollen can be also used as chemo-taxonomic markers, because of the identical phenolic profiles of the same species of pollen from distant areas [24]. Within phenolic compounds, there are phenolic acids (principal chlorogenic acid) flavonoids (mainly kaempferol, quercetin, *p*-coumaric acid, and isorhamnetin), leukotrienes, and catechins [9], as well as many others are under study. Moreover, bee pollen is rich in polyamines conjugated with hydroxycinnamic acids, mainly spermidines and spermines. These compounds, named phenolamides or hydroxycinnamic acid amines, are an important group found in high concentrations mainly in pollen from the Asteraceae, Campanulaceae, Myrtaceae, Rosaceae, and Fabaceae [25,26]. Previous studies showed that multifloral bee pollen and beebread from Portugal have a pre-

dominance of phenolamides; in particular, in samples of beebread with a high composition of *Castanea sativa* pollens [25,27].

Spain is the European Union's leading producer of bee pollen. Bee pollen as a commercial product has achieved significant commercial returns in the beekeeping sector. [6]. This includes in the northwest of the Iberian Peninsula, where in recent years there has also been increasing pollen-producing activity. In addition, there is a growing demand from consumers for quality, safe, and healthy food. This makes it of great interest to study the qualities provided by bee pollen, as derived from the diversity of the flora, climate, and soil of the Spanish territory. However, research on the physicochemical and botanical characteristics of bee pollen in this region is scarce compared to other bee products such as honey. The current study aimed to identify the phenolic profile of bee pollen obtained from chestnut (*Castanea sativa*) in the northwest of the Iberian Peninsula. For this purpose, eleven samples with this origin were selected for further analysis.

2. Materials and Methods

2.1. Bee Pollen Samples

Samples were taken from eleven apiaries of the northwest of the Iberian Peninsula during the period of chestnut blooming. Seven samples were produced in Galicia and four in the North of Portugal. Front entrance pollen traps were installed in the hives for a week at the beginning of the flowering of chestnut trees. After that, the pollen loads collected were separated using colorimetry. Botanical origin was confirmed through melissopalynology.

2.2. Determination of the Botanical Origin of the Harvested Bee Pollen

First, the bee pollen sample was homogenized, and two grams was taken as the unit of study. This subsample underwent a visual colorimetric separation, considering the colors of the different pollen loads, using a white light and a black background. These subunits were weighed and ground, and after that, the botanical origin was determined through observation under an optical microscope. For this purpose, different slides were prepared using 0.1 g of each subunit dissolved in 10 mL of distilled water. When the pollen grains were completely dissolved and homogenized with the help of a Vortex, an aliquot of 100 µL was taken and deposited on a slide. The slides were allowed to dry on a hot plate at a temperature of 45 °C. Once the preparations were dry, the sediment was dyed by depositing a drop of fuchsin glycerin gelatin, and each sediment was covered with a coverslip. The selection of *Castanea* subsamples was performed according to the results of the palynological origin test. Two grams of *Castanea* pollen was separated from every bee pollen sample for phenolic profile analysis.

2.3. Bioactive Compound Extraction

Bioactive compounds were obtained according to a previously described procedure [28]. Phenolic compounds were extracted with 15 mL ethanol (70%) at 70 °C for 30 min under stirring. The obtained mixture was filtered (Watman n° 5 filter paper), and the extract was kept at −20 °C until analysis.

2.4. LC/DAD/ESI-MS^n Analysis

The identification of the main compounds was performed through LC/DAD/ESI-MSn analysis on a Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, San Jose, CA, USA) equipped with a diode-array detector and coupled to a mass detector. HPLC was run on a Macherey-Nagel Nucleosil C18 column (250 mm × 4 mm id; 5 mm particle diameter, end-capped), and its temperature was maintained at 30 °C. The mobile phase was composed of eluents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile), and both mixtures were degassed and filtered beforehand. The gradient elution of the mobile phase started with 80% A and 20% B until 10 min, 70% A and 30% B until 40 min, 60% A and 40% B until 45 min, and 10% A and 90% B until 50 min, then returning to the

initial conditions. The flow rate was 1 mL/min, with an injection volume of 10 µL. Spectral data for all peaks were accumulated in the range 190–600 nm.

Using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with an ESI source, the mass spectrometer was operated in negative ion mode. The ESI source parameters were the following: source voltage, 5 kV; capillary voltage, −20 V; tube lens voltage, −65 V; capillary temperature, 325 °C; and sheath and auxiliary gas flow (N_2) 50 and 10 (arbitrary units), respectively. Mass spectra were acquired with full range acquisition, covering 100–1000 m/z. To study fragmentation, a data-dependent scan was performed by displaying the collision-induced dissociation (CID). The normalized collision energy of the CID cell was set to 35 (arbitrary units). Data acquisition was carried out with the Xcalibur® data system (Thermo Scientific, San Jose, CA, USA).

Identification of the phenolic compounds was achieved through comparison of their chromatographic behavior, UV spectra, and MS information to those of reference compounds. Where standards were not available, the structural information was confirmed with UV data [29–31] combined with MS fragmentation patterns previously reported in the literature [30–32].

Quantification was carried out using calibration curves for caffeic acid (0.02–0.35 mg/mL; y = 2E + 07x − 352020; R^2 = 0.999; LOD (limit of detection, mg/mL) = 0.07; LOQ (limit of quantification, mg/mL) = 0.23), quercetin (0.04–2 mg/mL; y = 8E + 06x − 362356; R^2 = 0.998; LOD (mg/mL) = 0.04; LOQ (mg/mL) = 0.14), and naringenin (0.02–2 mg/mL; 2E + 07x − 264292; R^2 = 0.978; LOD (mg/mL) = 0.06; LOQ (mg/mL) = 0.21). When a standard was not available, the compound quantification was expressed in equivalents of the structurally closest compound. The assay was performed in triplicate, and the results were expressed as mg/g of fresh pollen.

*2.5. Color CIELab**

The color of bee pollen samples was determined visually and by measuring the CIELab coordinates. CIELab is a system defined by three colorimetric coordinates: L, a*, and b*, which are dimensionless magnitudes. This represents a color space consisting of three axes (coordinates), which enables accurate measurement and comparison of colors, and it is widely used for quality assurance. The L-axis defines the achromatic point, varying from white (L = 100) to black (L = 0). The a*-axis defines the chromatic color, going from green, when negative, to red if positive. Similarly, the b*-axis defines the deviation from yellow (+b*) and blue (−b*). The CIElab coordinates were measured using a Minolta Chroma Meter CR-210 colorimeter, on glass plates of 5 mL capacity, 3.5 cm diameter, and 0.5 cm high. All samples were first homogeneously ground.

2.6. Statistical Treatment

Data analysis was performed using STATGRAPHICS® Centurion XVI for Windows (Statgraphics Technologies, Inc., The Plains, VA, USA). Fisher's least significant difference (LSD) procedure was used to study the differences between each of the identified and quantified compounds. This allowed comparing the means of the compounds for each bee pollen sample, after having rejected the null hypothesis of equality of means using the ANOVA statistical technique.

3. Results

3.1. Main Phenolic Compounds and MS/MS Fragmentation Patterns

Identification of the phenolic profile was performed using the mass spectrum, mass-to-charge ratio (m/z), MS/MS fragmentation pattern, and characteristic retention time. The exact mass search and the MS/MS fragmentation studies described in the literature also allowed us to identify each of the compounds (Table 1).

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, and proposed phenolic and phenolamide compound identification in *Castanea* pollen loads.

Peak	Rt (min)	λ_{max} (nm)	[M-H]$^-$ *m/z*	MS^2 (% Base Peak)	Proposed Compound
1	12.3	255,354	623	315 (100)	Isorhamnetin-*O*-hexosyl-deoxyhexoside [a,c,d]
2	12.6	255, 354	609	315 (100)	Isorhamnetin-*O*-pentosyl-hexoside [a,c]
3	13.0	255, 355	609	315 (100)	Isorhamnetin-*O*-pentosyl-hexoside (isomer) [a,c]
4	13.6	254, 354	623	315(100)	Isorhamnetin-*O*-hexosyl-deoxyhexoside (isomer) [a,c,d]
5	14.3	255, 354	477	314 (100), 315 (51)	Isorhamnetin-*O*-hexoside [a,c]
6	15.1	295	630	494 (86), 468 (100), 358 (7)	N^1, N^5, N^{10}-tricaffeoylspermidine [a,e]
7	15.8	295, 315	630	494 (86), 468 (100), 358 (7)	N^1, N^5, N^{10}-tricaffeoylspermidine (isomer) [a,e]
8	16.2	298, 318	630	494 (86), 468 (100), 358 (7)	N^1, N^5, N^{10}-tricaffeoylspermidine (isomer) [a,e]
9	16.8	299, 319	630	494 (86), 468 (100), 358 (7)	N^1, N^5, N^{10}-tricaffeoylspermidine (isomer) [a,e]
10	18.0	296, 315	614	468 (21), 478 (100). 452 (78), 358 (18)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine [a,f]
11	19.3	297, 311	614	494 (24), 478 (100). 452 (78), 358 (18)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer) [a,f]
12	19.5	296, 319	644	508 (100), 482 (10), 468 (11)	N^1-feruloyl-N^5, N^{10}-dicaffeoylspermidine [a,g,h]
13	20.0	296, 319	644	508 (100), 482 (76), 468 (4)	N^1-feruloyl-N^5, N^{10}-dicaffeoylspermidine (isomer) [a,g,h]
14	22.2	294, 310	598	478 (41), 462 (100), 452 (39), 342 (13)	N^1, N^5-di-*p*-coumaroyl-N^{10}-caffeoylspermidine [a,e]
15	22.8	298, 309	598	478 (100), 436 (11), 358 (16)	N^1, N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine [a,e]
16	24.1	294, 308	582	462 (100), 436 (10), 342 (6)	N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine [a,e,f]
17	25.1	289	271	177 (15), 151 (100)	Naringenin [a,b]
18	25.5	293, 308	582	462 (100), 436 (10), 342 (6)	N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine (isomer) [a,e,f]
19	26.5	298, 308	582	462 (100), 436 (10), 342 (6)	N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine (isomer) [a,e,f]

[a] Confirmed with MS^n fragmentation; [b] Confirmed with standard; Confirmed with references: [c] Sobral et al., 2017 [14]; [d] Falcão et al., 2013 [33]; [e] Aylanc et al., 2021 [25], [f] El Ghouizi et al., 2020 [34]; [g] Elejalde-Palmett et al., 2015 [30]; [h] Sobolev et al., 2008 [32].

The common phenolic profile of the *Castanea* bee pollen samples contained 19 peaks and can be seen in Figure 1. All the studied samples, independently of their geographical origin (Galicia or northern Portugal), presented the same number of compounds, suggesting a characteristic phenolic profile for this bee pollen.

The compounds included five isorhamnetin glycoside derivatives, one naringenin, and thirteen phenolamides (Figure 2). These spermidine derivatives consisted of molecular products chemically formed by covalent bonds between the carboxylic groups of hydroxycinnamic acids (e.g., coumaric acid, ferulic acid, and caffeic acid) and the amine groups of aliphatic polyamines, such as spermidines. This conjugation can occur with various degrees of saturation, as mono- or polysubstitutions with the same or different hydroxycinnamic acids [32].

The spermidine derivatives were tentatively identified as N^1, N^5, N^{10}-tricaffeoylspermidine (*m/z* 630) and its three isomers; N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (*m/z* 614) and its isomers; N^1-feruloyl-N^5, N^{10}-dicaffeoylspermidine (*m/z* 644) and its isomer; N^1, N^5-di-*p*-coumaroyl-N^{10}-caffeoylspermidine (*m/z* 598); N^1, N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine (*m/z* 598); and N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine (*m/z* 582) and its two isomers. Isorhamnetin glycoside derivatives appeared in the first five peaks, from the peak at 12.3 min to the peak at 14.3 min. These compounds were isorhamnetin-*O*-hexosyl-deoxyhexoside (*m/z* 623) and its isomer, isorhamnetin-*O*-pentosyl-hexoside (*m/z* 609) and its isomer, and

isorhamnetin-*O*-hexoside. Only one flavanone, naringenin (*m*/*z* 271; peak 17; RT: 24.2 min), was identified, as seen Figure 1.

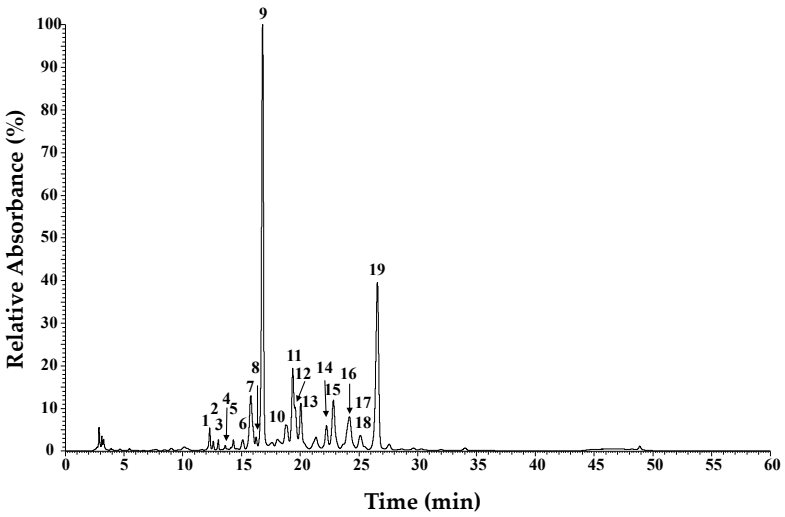


Figure 1. Chromatographic profile of *Castanea* pollen samples obtained at 280 nm using LC/DAD/ESI-MS^n.

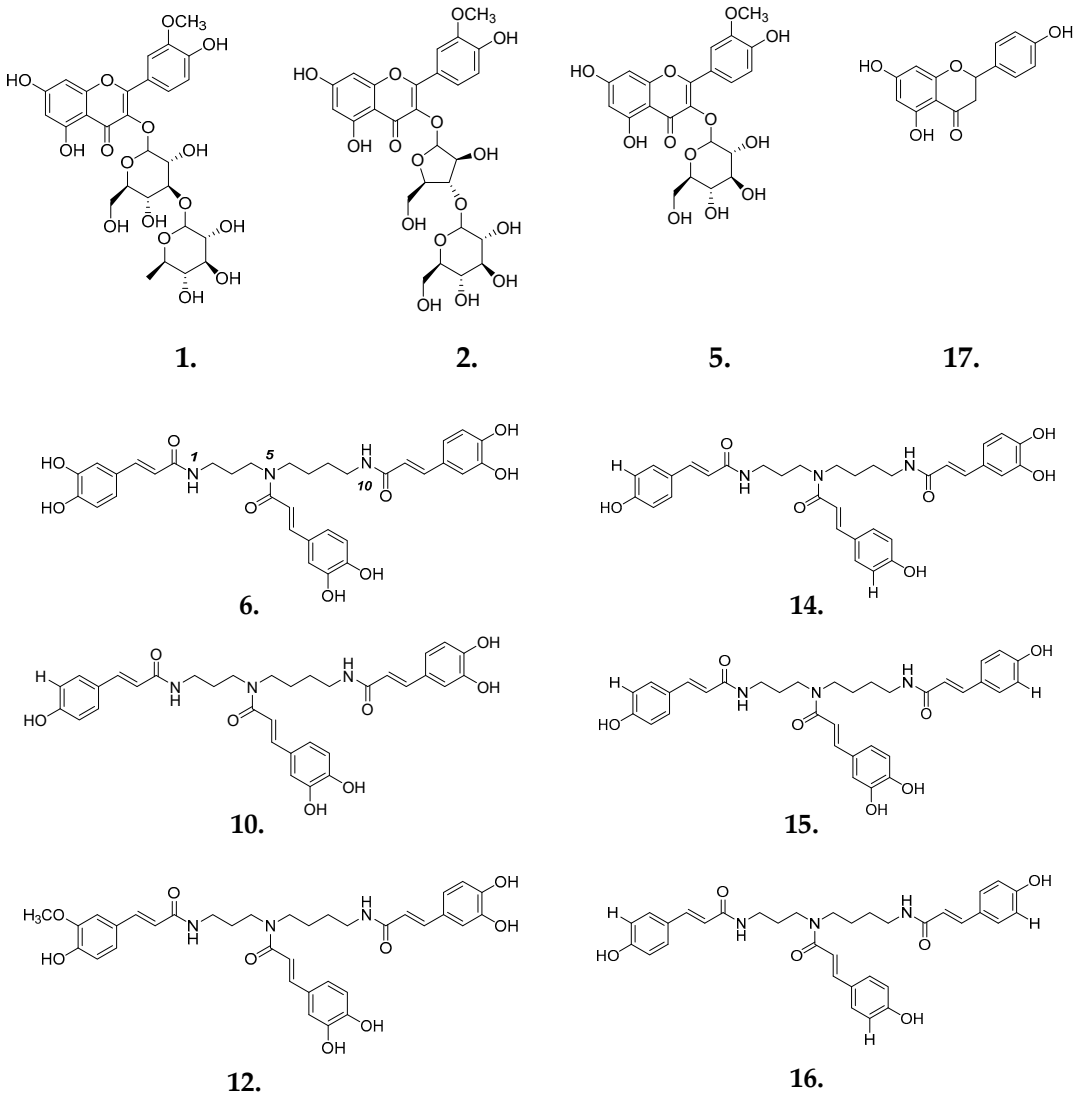


Figure 2. Proposed structure of the flavonoids and phenolamides found in *Castanea* pollen.

Spermidine–phenolic acid conjugates showed a UV spectrum similar to caffeic acid, with a UV specific spectrum with UVmax at around 298 nm [28]. Peaks 6 to 9 presented the same pseudomolecular ion $[M\text{-}H]^-$ at m/z 630, with a release of MS^2 fragment at m/z 468, attributed to the loss of the caffeic acid residue from the spermidine moiety, suggesting a trisubstituted spermidine with caffeic acid, most likely N^1, N^5, N^{10}-tricaffeoylspermidine [25]. The compounds of peaks 7, 8, and 9 represented different *cis*/*trans* isomers.

The evaluation of the MS/MS fragmentation pattern of peaks 10 and 11 showed a pseudomolecular ion $[M\text{-}H]^-$ at m/z 614, with the MS^2 fragment at m/z 478 indicating the loss of 136 Da from the caffeic acid fragment linked in the position N^{10} of the spermidine moiety. The second major ion at m/z 452 was the result of the release of another caffeic acid residue from the N^5 position. Finally, the fragment at m/z 468 was attributed to the release of a *p*-coumaric residue from the position N^1 of the spermidine moiety. Compounds 10 and 11 were tentatively identified as N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine and its isomer.

Peaks 12 and 13, with a $[M\text{-}H]^-$ at m/z 644, showed a similar fragmentation pattern as the last peaks, releasing fragments at m/z 508 and m/z 482, corresponding to caffeic acid residues in the position N^1 and N^5 of the spermidine moiety. A fragment at m/z 468 was present, corresponding to the loss of a ferulic acid (176 Da), most probably in position N^1. These compounds were tentatively identified as N^1-feruloyl-N^5, N^{10}-dicaffeoylspermidine and its isomer.

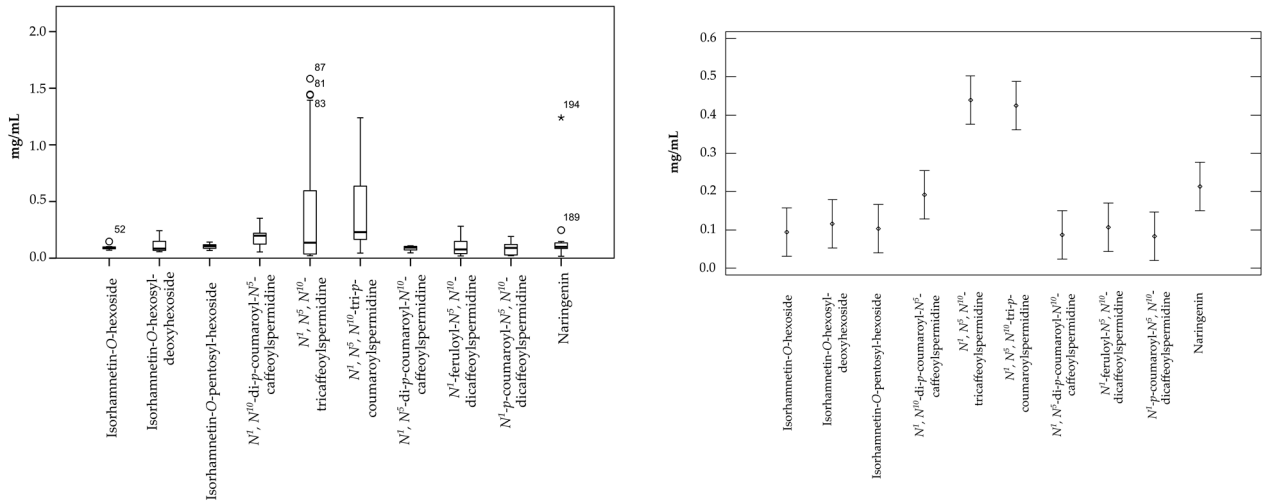
Although peaks 14 and 15 presented the same pseudomolecular ion $[M\text{-}H]^-$ at m/z 598, the two compounds showed different MS^2 fragments, indicating different hydroxycinnamic acid residues linked to the spermidine moiety. Having the same fragmentation pattern observed for the previous compounds, they were tentatively identified as N^1, N^5-di-*p*-coumaroyl-N^{10}-caffeoylspermidine and N^1, N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine, respectively.

Finally, peaks 16, 18, and 19 were identified as N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine and its isomers owing to the product ion at m/z 582 and the MS^2 fragment at m/z 462, attributed to the loss of the 120 Da fragment from the *p*-coumaric residue from the N^1, N^5 and N^{10} spermidine position.

Regarding the glycosylated isorhamnetins, the UV data (255, 354) were similar to those published in the literature (Table 2). Peaks 1 and 4 presented a pseudomolecular ion $[M\text{-}H]^-$ at m/z 623, with a MS^2 fragmentation showing an ion at m/z 315 (−308 Da), compatible with the loss of a deoxyhexosyl-hexoside from the isorhamnetin moiety. The compound was tentatively identified as isorhamnetin-*O*-hexosyl-deoxyhexoside and its isomer, respectively. Concerning the sugar moiety, this most probably was a rutinoside linked in the C3 position of the flavonol. The peaks 2 and 3 ($[M\text{-}H]^-$ at m/z 609) showed loss of the pentosyl-hexoside residue (m/z 315; −294 Da), allowing identification as isorhamnetin-*O*-pentosyl-hexoside and its isomer. Finally, peak 5 with a pseudomolecular ion $[M\text{-}H]^-$ at m/z 477 showed an ion at m/z 315 (-162 Da), corresponding to the loss of a hexoside residue from the isorhamnetin moiety, most probably glucose, and being identified as isorhamnetin-*O*-hexoside.

3.2. Quantification of Phenolic Compounds

The phenolamide group showed the widest mean values, ranging from 0.02 to 1.58 mg/mL (0.15–11.88 mg/g). N^1, N^5, N^{10}-tricaffeoylspermidine and N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine were the phenolamides with the highest concentration in isolated *Castanea* bee pollen (Figure 3a), both with a mean value of 0.4 mg/mL (3.00 mg/g). N^1, N^5, N^{10}-tricaffeoylspermidine presented the highest value in peak 9, with a mean value of 1.1 mg/mL (8.25 mg/g), see Figure 1, ranging between 0.02 and 1.58 mg/mL (0.98–11.88 mg/g); followed by N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine, which ranged from 0.04 to 1.24 mg/mL (0.33–9.30 mg/g), where the isomer present at peak 19 showed the highest value. Both compounds presented significant differences ($p < 0.05$) with the other peaks (Figure 3b). It can be observed that the mean and 95% intervals based on Fisher's least significant difference (LSD) are quite distant.



(**a**) (**b**)

Figure 3. Graphical representation of the quantitative values of each compound: (**a**) representation of the range values of each compound by means of a boxplot; (**b**) mean and 95% intervals based on Fisher's least significant difference (LSD) for each compound. The asterisk (*) indicates extreme outliers in the data.

The next outstanding compound was naringenin, with a mean value of 0.2 mg/mL (1.5 mg/g) and with values ranging between 0.02 and 1.24 mg/mL (0.12–9.30 mg/g) corresponding to peak 17. Finally, the isorhamnetin glycoside derivatives showed values between 0.06 and 0.24 mg/mL (0.45–1.80 mg/g), with peak 1 presenting the highest mean value (0.2 mg/mL). These compounds did not show significant differences between them or with the rest of the compounds.

3.3. CIELab Color of Castanea Bee Pollen

The lightness (L∗), redness (a∗), and yellowness (b∗) colorimetric parameters were used to characterize the color of the pollen load. The chestnut bee pollen had an average luminosity of 59.7, varying from 52.5 to 64.2 (Table 2). The a* values of the samples varied between positive values of +4.8 and +6.8, indicating that the hues of the samples were closer to red than green. The b* values of the bee pollen samples ranged from +26.7 to +49.3, having a mean value of +41.9, thus yellow dominated the blue–yellow axis in every case. Therefore, in the samples there was a predominance of yellow. Regarding the chroma value (C*ab), chestnut pollen showed an average value of 42.3, ranging from 49.6 to 27.4, and the hue (hab*) values obtained varied from 77.0 to 84.2, with a mean value of 81.3. The chroma represents the color intensity or saturation, indicating the distance from the lightness L-axis = 0, and the hue is the angle between axis a* and b*, varying from 0 to 360°. In summary, considering that a color can be specified by its hue, brightness, and saturation, the values of the CIELab* coordinates positioned the chestnut pollen with a yellow color and a medium brightness and saturation.

Table 2. Description of the color variables in the CIELab* scale.

CIELab Coordinates	Mean	Maximum	Minimum	SD
Lightness (L*)	59.7	64.2	52.5	3.5
Redness (a*)	6.1	6.8	4.8	0.7
Yellowness (b*)	41.9	49.3	26.7	7.3
Chroma (Cab*)	42.3	49.6	27.4	7.2
Hue angle (hab*)	81.3	84.2	77.0	2.3

4. Discussion

Bee pollen contains a considerable number of biologically active substances of plant origin. Among them, the polyphenolic compounds, carotenoids, anthocyanins, phospholipids, and proteins stand out [10,25,31,33–35]. Moreover, multiple studies have demonstrated certain therapeutic benefits of this beehive product, due to its anti-radical activity [36–39], inhibition of lipid peroxidation [40], and even suppression of cellular and tumor responses [15,41]. These properties of bee pollen are strongly dependent on the botanical origin, so it is relevant to further characterize the phenolic profile of bee pollen according to its origin.

In the scientific literature, chestnut bee pollen has been described as having a high total phenolic content [19,27,42–45]. However, there have been limited studies describing the phenolic profile of this bee pollen [19,25,42,44]. For the first time, this study identified and quantified the main phenolic compounds of chestnut bee pollen samples from the northwest of the Iberian Peninsula. The most representative were the phenolamides, particularly N^1, N^5, N^{10}-tricaffeoylspermidine *and* N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine, with a composition of 0.98–11.88 mg/g (peak 9) and 0.33–9.30 mg/g (peak 19), respectively. Although the phenolic profile was the same, some variations were found between *Castanea* bee pollen samples in the concentration of compounds, due to the different geographical origins of the samples, which influenced the edaphoclimatic conditions that the plants were exposed to. A similar phenolamide profile was found in bee bread, which is bee pollen that has been stored in the beehive and undergone lactic fermentation, with a percentage of 48% of *Castanea* pollen, and where a rich composition in N^1, N^5, N^{10}-tricaffeoylspermidine (0.50–4.15 mg/g) and N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine (1.02–15.23 mg/g) was found [25], while the flavonoid profile presented herbacetin and kaempferol derivatives, rather than the isorhamnetin derivatives shown by the *Castanea* pollen.

Phenolamides, a group of widely distributed secondary plant metabolites, were the most representative compounds in the chestnut bee pollen. These compounds are biogenic *N*-acylated amines conjugated with phenolic acids, mainly hydroxycinnamoyl acids (such as ferulic, *p*-coumaric, caffeic, and sinapic acids), which is why they are also known as hydroxycinnamic acid amides (HCAA). They can also be derived from aromatic monoamines, such as tyramine, dopamine, tryptamine, and anthranilic acid, or aliphatic polyamines, such as putrescine, spermidine, spermine, and agmatine. There is a great diversity in these compounds. This variety arises from various degrees of saturation of the amino groups of the polyamine with mono- or poly-substitution, carrying the same or different hydroxycinnamic acids [30]. These compounds are involved in development and reproduction and serve as defense compounds in biotic interactions [30,46,47]. Some of these compounds, such as spermidine derivatives, have already been shown to form part of the pollen composition. Spermidines are attributed to many diverse functions, such as sporopollenin formation and pollen protection against UV or pollination [30]. Spermidine derivatives such as N^1, N^5, N^{10}-tricoumaroyl spermidine and N^1, N^5-dicoumaroyl-N^{10}-caffeoyl spermidine, were shown to accumulate specifically in the pollen grain coat of apple trees [30]. These compounds are associated with functional properties in bee pollen, such as a high antioxidant activity, with protective effects against oxidative stress [34,38]. Previous works reported polyamines as a possible "anti-aging vitamin" for humans, as the tissue levels of spermidine decrease with ageing in humans. [17,36]. The antifungal activity of these compounds has also been proven, exhibiting a role in the microbial protection of pollen grains [18]. In addition, other functional properties dependent on the number and type of phenolic residues, such as the inhibitory activity of the tyrosinase enzyme that regulates the speed of melanin synthesis [48,49] or diuretic effects, have been attributed; therefore, bee pollen could be a beneficial product in the diet [34]. The spermidine derivatives present in bee pollen have also been associated with a hypouricemic effect, so this product could have potential clinical application value as a bioactive drug [50]. These properties give bee pollen value as a functional food with commercial use and potential value in applications for the development of cosmetic materials or for clinical applications. This study

showed high levels of phenolic acid-spermidine conjugates, especially N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine and N^1, N^5, N^{10}-tricaffeoylspermidine, in chestnut bee pollen when compared with specific monofloral bee pollens such as buckwheat, phellodendron, tea, and melon [17].

Two isorhamnetin glycoside derivatives were identified as flavonoids in the chestnut bee pollen samples. Isorhamnetins are methylflavonols, basically quercetin in which the hydroxy group at position 3' has been replaced by a methoxy group, commonly found in vegetables and fruits [51]. They have a role as a tyrosinase inhibitor, an anticoagulant, and a metabolite, and have anti-inflammatory, antioxidant, and anticancer activities. This type of flavonoid has been identified in the bee pollen of *Cistus incanus* [52], *C. ladanifer* [53], and other species of *Cistus* [54]. Recently, isorhamnetin-*O*-pentosyl-hexoside was identified in multifloral bee pollen, with a high percentage of *Crepis capillaris* (60%) and *Plantago* sp. (47%). Moreover, it was present in beebread with high amount of *Castanea* pollen [25].

Moreover, isorhamnetin glucoside derivatives have been widely identified in bee pollen, such as isorhamnetin-3-*O*-rutinoside in *Echium plantagineum* [55] and *Helianthus annuus* [56] unifloral bee pollen. These types of compounds are the main flavonoids located in the exine membrane. Therefore, they have been found as part of the flavonoid composition of bee pollen of diverse botanical origins [33,42,56,57]. It should be noted that these flavonoids have been detected in various extracts of chestnut shell, inner shell, bur leaves, and products, as well as in the flowers, relating their presence to a botanical origin [58]. Finally, chestnut bee pollen samples presented the flavanone naringenin. Naringenin is one of the main bioactive polyphenols found in citrus fruits, the consumption of which has been shown to be beneficial for human health. Its antioxidant and antiandrogenic properties have been reported, as well as its ability to protect against inflammation and cancer [59]. This compound was identified as part of the phenolic profile of chestnut bee pollen from Turkey [19,44].

This class of compounds provide chestnut bee pollen with a high antioxidant capacity and other bioactive properties, which make this product an ideal supplement for the human diet and could replace the consumption of drugs with harmful side effects. It is noteworthy that the composition of bee pollen can depend on the conditions during processing and storage, as well as on processes such as fermentation [60]. Color is another quality parameter affected by the conservation of the products, and it is one of the qualities most appreciated by consumers. Pollen loads can have a very wide range of colors, from light colors such as grayish white or yellow, to dark colors such as purple or even black. All these colors can be seen in the same sample of bee pollen, indicating the variety of plant species from which the pollen was collected. In the case of chestnut pollen, a yellow color was identified, corresponding to a medium luminosity (L = 59.7), a* mean value of 6.1, and b* mean value of 41.9.

5. Conclusions

Castanea sativa bee pollen is the one of the most important types of pollen collected in the northwest of the Iberian Peninsula. It is characterized by a yellow-greenish color and its phenolic profile is characterized by the presence of nineteen compounds, including thirteen phenolic acid-spermidine conjugates, five isorhamnetin glycoside derivatives, and one naringenin. The most representative were phenolamides, concretely N^1, N^5, N^{10}-tricaffeoylspermidine and N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine, with variable concentrations of 0.98–11.88 mg/g and 0.33–9.30 mg/g, respectively. The variation observed was probably due to the different geographical origins of the samples. Considering the health properties attributed to spermidines, further studies are necessary to evaluate these properties in chestnut bee pollen.

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