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# Liquid chromatographic–mass spectrometric analysis of anthocyanin composition of dark blue bee pollen from *Echium plantagineum*

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**Abstract**

Dark blue bee pollen samples from pollinic type *Echium plantegineum* were analysed in order to identify and quantify their anthocyanin pigments. Five samples were collected from different apicultural Spanish regions and the anthocyanin composition was determined by HPLC with diode array and MS detection. Eight different pigments were identified, the principal anthocyanin being petunidin-3-O-rutinoside. The other pigments found were delphinidin, cyanidin and petunidin-3-O-glucoside; delphinidin, cyanidin, peonidin and malvidin-3-O-rutinoside and cyanidin-3-(6″-malonylglucoside). The anthocyanin content ranged from 45 to 80 mg/100 g of blue pollen, which could represent a significant source of phytochemicals. Minor variations in the anthocyanin profiles were found, which could be explained by the geographical differences between collection regions.


*Keywords:* *Echium plantagineum*; Pollen; Anthocyanins

## 1. Introduction

In the last years, there has been an increased tendency to study natural products and nutritional supplementation, among them, the apicultural products have been widely used in diet complements as well as in phytotherapy. Bee pollen is a hive derived product of great commercial interest owing to its high nutritional quality and can be considered a potential source of energy and proteins for human consumption [1–3].

Bee pollen is collected from various selected flower species by the honeybee *Apis mellifera* and is the only natural source of proteins, lipids, vitamins, minerals and amino acids that are essential for bees' growth and development [4]. Pollens are present as a fine powder in the plants. Honeybees agglutinate the pollen with nectar and hypopharyngeal gland secretions and pack this into a compact load on each hind leg. A "pollen trap" must be installed in the hive, to remove the pollen carried when the bees return. The colour, size and morphology of each pollen pellet vary in relation to the species source [5]. Bee pollen is present as a mixture of different plant species' pollens. In the commercial designation "Spanish bee pollen" loads from *Cistus ladanifer* L. (autochthonous Iberian species) mainly appear with other species in minor proportions, such as *Echium plantagineum* L. and *Quercus rotundifolia* Lam., depending on the geographical origin [1].

Bee pollen is widely used for its therapeutic properties, thus extracts of bee pollen have been used in chronic prostatitis for their presumed anti-inflammatory and anti-androgenic effects [6,7]. Since rutin (quercetin rutinoside) is the most abundant component of pollen free flavonoids, bee pollen also reduces capillary fragility [8]. Furthermore, daily ingestion of bee pollen is recommended because it is able to regulate the intestinal functions and has beneficial effects on the cardiovascular system, skin, vision, etc. [9]. Moreover, bee pollen is used in the field of cosmetics for its contribution of vitamins to cold creams [10].

Among bee pollen components, the flavonoids profile and their biological activity have been the subject of several investigations in order to establish quality parameters of bee pollen [11], to characterise them in terms of botanical origin, recognise taxonomic markers [12,13] and to evaluate their nutritional and biological properties [3,4,14–16].

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Within the flavonoids family, anthocyanins are the most abundant group of plant pigments and they receive special attention because of their function as colourants and antioxidants [17,18]. Existing evidence indicates that the anthocyanins have positive therapeutic and nutritional properties, contributing to nutritional supply in human diet. Moreover, anthocyanins possess an antioxidant activity that prevents the oxidation of ascorbic acid, provides protection against free radicals, shows inhibitory activity against oxidative enzymes and have been considered important agents in the reduction of the risk of cancer [17,19]. Several researchers have demonstrated the effects of anthocyanins in the reduction of neurodegenerative and vascular diseases [20].

The recent studies of anthocyanins as antioxidant agents have increased the demand for new sources of these compounds [21,22]. Commercial bee pollen is usually composed of pollen species that the bees collect from various plants. Most of them are yellow or brown, owing to their production in higher amounts and to the consumer preference. Dark blue bee pollen is collected by the bees towards the end of the floral period and is not commercialised as "Spanish bee pollen", but is usually mixed with yellow pollens.

The dark blue colour is a feature that distinguishes it from other pollen types. In accordance with our knowledge, the anthocyanin profile of bee pollen loads that present a dark blue/purple colour, like those from pollinic type *E. plantagineum*, had not been well studied. Webby and Bloor [23] reported a comparison of the pigments in *Fuchsia excorticata* pollen with the anthocyanins extracted from "fuchsia bee pollen" in order to confirm the source of the bee pollen.

The main objective of this work was to identify and quantify the anthocyanin pigments of bee pollen samples from pollinic type *E. plantegineum*. The characterisation of these pigments is interesting because an additional value can be given to the bee pollen due to its phytochemical content. What is more, it can be considered as a new commercial product for the international market.

## 2. Experimental

### 2.1. Bee pollen samples

Five bee pollen samples (A, B, C, D and E) were provided by Spanish bee-keepers, harvested during the two periods of floral production in 2002 and 2003. They were collected in the province of Cáceres (Community of Extremadura) and in the province of Avila (Community of Castilla y León) (Fig. 1). Bee pollen samples (50 g) were manually sorted on the basis of colour, and the botanical origin of blue pollen pellets was confirmed by microscopic examination after acetolysis [24]. The percentage of blue pollen loads belonging to pollinic type *E. plantagineum* was calculated for each bee pollen sample and separated for the subsequent analysis.


Fig. 1. Map of Spain. Location of collection regions of bee pollen in the Communities of Extremadura and Castilla y León. A: Valencia de Alcántara, B: Coria, C: Candeleda, D: Arenas de San Pedro, E: Navaluenga.

### 2.2. Extraction and purification

The blue pollen samples (5 g) were broken down in a methanol–1 M HCl (95:5, v/v) solution, using a Polytron homogeniser (Kinematica, Littau, Switzerland) and macerated overnight at 4 °C. Subsequently, they were centrifuged for 20 min at 10 000 rpm and 10–15 °C in a refrigerated superspeed centrifuge (Sorvall RC 5B). The supernatant was separated and the solid residue was re-extracted until all the colour had been removed from the pollen material. The extracts were combined and concentrated under vacuum at 30 °C until all the methanol was removed. The aqueous extract obtained was defatted with *n*-hexane and then an aqueous HCl solution (pH 0.5) was added to final volume of 10 mL. One millilitre of this solution was purified through a $C_{18}$ Sep-Pak cartridge (Waters, Milford, MA, USA), previously activated with methanol followed by water. The cartridge was then rinsed with two volumes of ultra-pure water to remove sugars and acids and then with methanol–1 M HCl (95:5, v/v) to elute the anthocyanins. Afterwards, the elute was concentrated under vacuum (30 °C) to remove the methanol and then dissolved in aqueous HCl (pH 0.5), with a final volume of 2 mL. All samples were extracted in duplicate.

### 2.3. Quantification of anthocyanins by HPLC

The anthocyanin extracts were analysed using a Hewlett-Packard 1050 Series liquid chromatograph, equipped with an AQUA (Phenomenex, Torrance, CA, USA) reversed-phase column (150 mm × 4.60 mm, 5 μm, $C_{18}$). The column temperature was thermostatted at 35 °C using a column heater module (Waters, USA). The HPLC conditions were in accordance with the method employed by Lopes da Silva et al. [25], with minor modifications. The solvents were (A)

aqueous 0.1% trifluoracetic acid (TFA of analytical-reagent grade, Riedel-de Haën) and (B) 100% HPLC-grade acetonitrile (Merck, Darmstadt, Germany). The gradient employed was: isocratic 10% B for 5 min, increasing from 10 to 13% B over 15 min, from 13 to 15% B over 15 min, and from 15 to 35% over 20 min and returned to initial conditions over 10 min. The flow-rate was 0.5 mL/min and aliquots of 100 μL were injected into the HPLC column. A photodiode array detector (SPD M10A VP, Shimadzu, Japan) set at 520 nm as the preferred wavelength was employed. Solvents and samples were filtered through an 0.45 μm Millipore filter type HA (Millipore, Bedford, MA, USA).

Quantification of anthocyanins was performed from the peak areas by reference to calibration curves obtained using 3-O-β-glucopyranosides of delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin standards (HPLC purity grade, Polyphenols Labs., Sandnes, Norway) at concentrations between 0.001 and 0.1 mg/mL. For the anthocyanin-rutinosides, the concentrations were calculated as the corresponding monoglucoside. Each extract was analysed in duplicate.

### 2.4. Identification of anthocyanins

The anthocyanin identification was determined by HPLC using dual detection by diode array spectrophotometry and mass spectrometry (HPLC–DAD–MS). Identifications were based on retention times and UV–Vis spectra as compared with anthocyanins standards as well as those available in an in-house library. For the complete identification, individual sugars were detected by HPTLC analysis, after carrying out an acid hydrolysis of the extracts.

#### 2.4.1. LC–MS analysis

Hewlett-Packard 1100 HPLC equipment was used, provided with a quaternary pump and a photodiode array detector. The same HPLC conditions already described in Section 2.3 were used. MS analysis was performed using a Finnigan LCQ (Thermoquest, San José, USA) equipped with an API source using an ESI interface. Both the auxiliary and the sheath gas were a mixture of nitrogen and helium at flow rates of 6 and 1.2 L/min, respectively. The capillary voltage and temperature were 4 V and 195 °C, respectively. Spectra were recorded in positive ion mode between *m/z* 150 and 1500. The MS detector was programmed to perform a series of three consecutive scans: a full scan, an MS–MS scan of the most abundant ion in the first scan and an $MS^3$ of the most abundant ion in the MS–MS scan, using a normalised collision energy of 45%. The HPLC system was connected to the probe of the mass spectrometer via the UV cell outlet, using polyether ether ketone (PEEK) tubing.

#### 2.4.2. Acid hydrolysis of the anthocyanins

The defatted extract obtained in Section 2.2 was used to carry out the acid hydrolysis. Before the hydrolysis, aliquots of 2.5 mL of each sample were purified through a $C_{18}$ Sep-Pak cartridge in the conditions described previously. The purified extract of anthocyanins was evaporated to dryness under vacuum. The residue was dissolved in 6 M HCl, and heated to 100 °C in a screw-cap test tube for 40 min. Afterwards, the extract was cooled and concentrated under vacuum. The solution obtained was diluted up to 2 mL and was again purified through a $C_{18}$ Sep-Pak cartridge. The $C_{18}$ cartridge was eluted with water and the sugar fraction was collected, concentrated and diluted up to 1 mL for the subsequent HPTLC analysis.

#### 2.4.3. HPTLC procedure

The analysis was carried out on 5 cm × 5 cm Silica Gel 60 HPTLC plates (Merck). The chromatoplate was impregnated with 0.02 M boric acid and activated at 100 °C for 30 min before use. An aliquot (5 μL) of each sample was spotted on the plate and was horizontally developed using *n*-propanol–water (7:1, v/v) as solvent. After drying, the sugar spots were located by spraying with diphenylamine–aniline reagent (4 g diphenylamine, 4 mL aniline, 20 mL phosphoric acid 85% and 200 mL acetone) and the sprayed plate was maintained at 85 °C for 10 min. Standards of D(+)-glucose, D(+)-galactose, L(+)-arabinose, α-L-rhamnose (Sigma–Aldrich Chemie, Steinhein, Germany) and D(+)-xylose (Panreac Química, Barcelona, Spain) at 1 mg/mL were run simultaneously for identification of individual sugars.

## 3. Results and discussion

The pigment identifications of five dark blue bee pollen samples was carried out by HPLC–DAD–MS. The retention times, UV–Vis and mass spectra of the peaks obtained were compared with those of the anthocyanin standards as well as those found in the available literature and in an in-house library. We can indicate that, in this work, the thin-layer chromatography (HPTLC analysis) provided a rapid and sensitive method that allowed us to identify the sugars associated with the anthocyanidins.

Fig. 2 shows an HPLC chromatogram recorded at 520 nm, obtained from the purified blue pollen extract corresponding to sample A, in which eight major peaks can be detected, the compound corresponding to peak 6 being the main pigment. In the MS spectrum obtained for this compound, a molecular ion at *m/z* 625 was observed, yielding signals at *m/z* 479 and 317 in the MS–MS analysis (Fig. 3), corresponding to the monoglucoside and aglycon of petunidin, respectively. In accordance with previous observations [26,27], the fragmentation of C3 substituted anthocyanins results in cleavage of glycosidic bonds only between the flavylium ring and the sugars directly attached to it, producing only one fragment in the MS–MS that corresponds to aglycon. The only exception to this pattern was observed in the case of anthocyanins glycosilated with rutinose, owing to the α(1 → 6) linkage between rhamnose and glucose that allows for free

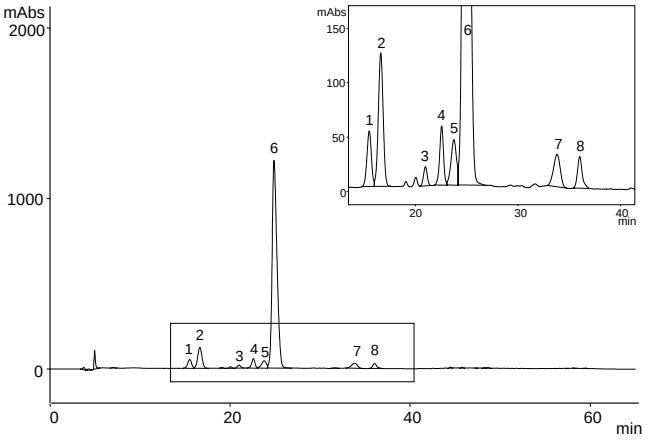


Fig. 2. HPLC chromatogram recorded at 520 nm corresponding to bee pollen sample A from type *E. plantagineum*. Peaks: 1 = delphinidin-3-O-glucoside; 2 = delphinidin-3-O-rutinoside; 3 = cyanidin-3-O-glucoside; 4 = cyanidin-3-O-rutinoside; 5 = petunidin-3-O-glucoside; 6 = petunidin-3-O-rutinoside; 7 = malvidin-3-O-rutinoside; 8 = cyanidin-3-(6″-malonylglucoside).

rotation and more accessibility of the gas used to produce the fragmentation. This fact allowed us to confirm that the molecular ion with *m/z* 479 was originated by the loss of one rhamnose residue (−146 u). The same results were confirmed by HPTLC analysis, where both glucose and rhamnose could be identified. In relation to anthocyanin spectral features, Harborne (1958) (cited by Ribéreau-Gayon [28]), demonstrated that if an anthocyanin lacks C5 substitution, an increase of absorbance at 440 nm is produced, forming a shoulder in this spectrum region, which is characteristic of C3 substituted compounds. Based on these considerations, we could identify peak 6 as petunidin-3-O-rutinoside.

Similarly, peaks 2, 4 and 7 were identified as 3-rutinosides of delphinidin, cyanidin and malvidin, respectively. In all these cases, the same fragmentation pattern described by Giusti et al. [26] and Alcalde-Eón et al. [27] (Table 1) and the shoulder on 440 nm region were observed, which allowed us to confirm the identity of the disaccharide and substitution pattern of the anthocyanin.

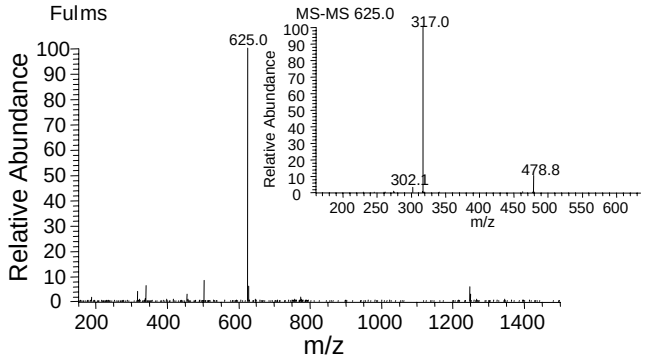


Fig. 3. Full mass spectra and MS–MS spectra corresponding to peak 6 (petunidin-3-O-rutinoside).

Table 1
Chromatographic characteristics, mass spectra and identities of anthocyanins detected in pollinic type *E. plantagineum* bee pollen samples

| Peak | $t_R$ (min) | Molecular ion [M$^+$], *m/z* | MS–MS of [M$^+$], *m/z*[a] | Anthocyanin |
|---|---|---|---|---|
| 1 | 15.4 | 465 | 303 | Delphinidin-3-O-glucoside |
| 2 | 16.5 | 611 | 303, 465 | Delphinidin-3-O-rutinoside |
| 3 | 21.5 | 449 | 287 | Cyanidin-3-O-glucoside |
| 4 | 23.0 | 595 | 287, 449 | Cyanidin-3-O-rutinoside |
| 5 | 23.5 | 479 | 317 | Petunidin-3-O-glucoside |
| 6 | 24.4 | 625 | 317, 479 | Petunidin-3-O-rutinoside |
| *x* | 32.4 | 609 | 301, 463 | Peonidin-3-O-rutinoside |
| 7 | 33.5 | 639 | 331, 493 | Malvidin-3-O-rutinoside |
| 8 | 36.9 | 535 | 287, 449 | Cyanidin-3-(6″-malonylglucoside) |

[a] The fragment ions are shown in order of their relative abundance.

When peaks 1, 3 and 5 were analysed by mass spectrometry, they showed molecular ions at *m/z* 465, 449 and 479, respectively. The subsequent MS–MS spectrum gave signals corresponding to delphinidin, cyanidin and petunidin aglycons originated by loss of 162 u (Table 1). With regard to spectral and chromatographic features, the UV–Vis spectra and retention times were consistent with those from available standards. Furthermore, the HPTLC analysis revealed the presence of glucose, rhamnose and the rutinose, glucose being the only existing hexose. On the basis of these considerations, it was possible to affirm that all pigments are substituted on the C3 position and the peaks were identified as delphinidin-3-O-glucoside, cyanidin-3-O-glucoside and petunidin-3-O-glucoside, respectively.

Another important factor to emphasise is that the anthocyanin reversed-phase HPLC elution profile in our chromatograms is consistent with that reported by Strack and Wray [18]. In general, diglycosides elute earlier than monoglycosides, but this is not a strict rule, since the nature of the sugars can affect pigment retention. This is the case of rutinosides that elute after the monoglucosides (see $t_R$ in Table 1).

Finally, the identification of peak 8 was carried out. Its mass spectra presented a molecular ion at *m/z* 535, yielding two molecular fragments at *m/z* 287 and 449 in the MS–MS analysis. The fragment corresponding to the aglycon was the most abundant, and its loss of 248 u could be associated to malonyl-glycoside (162 + 86 u) residue. This fragmentation pattern was consistent with that of a pigment found in a commercial anthocyanin-rich powder from purple corn extract, which was associated with cyanidin-3-(6″-malonylglucoside), previously identified by de Pascual-Teresa et al. in our laboratory [29]. In order to determine the identity of this peak, the purple corn extract was analysed in the same chromatographic conditions described in Section 2.3, confirming that the UV–Vis spectrum and the retention time (36.9 min) coincided with that of peak 8 pigment. Acylation of the compound decrease its polarity, thus, the retention time was longer than those of non-acylated pigments. Taking into account all of these considerations,

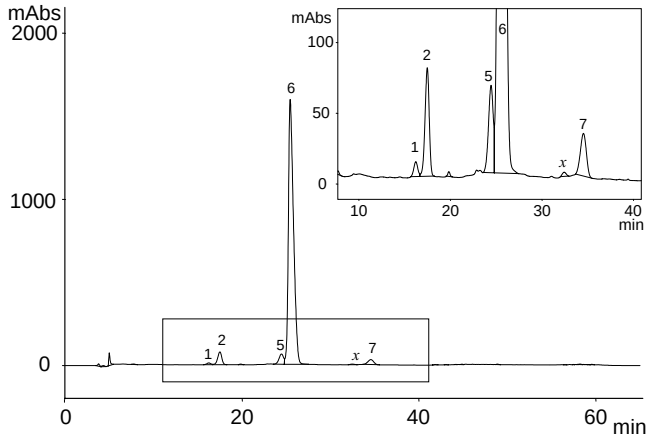


Fig. 4. HPLC chromatogram recorded at 520 nm corresponding to bee pollen sample B from type *E. plantagineum*. Peaks: 1 = delphinidin-3-O-glucoside, 2 = delphinidin-3-O-rutinoside, 5 = petunidin-3-O-glucoside, 6 = petunidin-3-O-rutinoside, *x* = peonidin-3-O-rutinoside, 7 = malvidin-3-O-rutinoside.

the compound was, thus, associated with cyanidin-3-(6″-malonylglucoside).

Sample B presented six different peaks, five of them corresponding to peaks found in sample A (peaks 1, 2, 5, 6, and 7) and a new peak, which eluted between peaks 6 and 7, designated with *x* in Fig. 4. This new compound was identified by its mass spectrum, taking into account the same considerations for rutinoside derivatives described before, such as peonidin-3-O-rutinoside. However, it was not possible to determine the individual pigment concentration because it was below the quantification limit of the method.

The rest of the bee pollen samples, C, D and E, showed different anthocyanin profiles when compared to samples A and B. In these cases, the chromatograms registered at 520 nm revealed only five pigments corresponding to peaks 1, 2, 5, 6 and 7 described above. The structures and substitution patterns of anthocyanins were confirmed in all pigments, on the basis of their fragmentation patterns (mass spectrometry), absorption spectra and chromatographic features and taking into account the same considerations used for sample A.

Results obtained clearly show that anthocyanin profiles were similar in all bee pollen samples from type *E. plantagineum*, with minor differences. Table 2 compiles the content percentages of each pigment in all the bee pollen samples analysed, where it can be seen that sample A presents the highest amount of delphinidin-3-O-glucoside and delphinidin-3-O-rutinoside. In all cases the major pigment was petunidin-3-O-rutinoside (peak 6), although it accounts for 80.1% in sample A, while in the rest of samples this amount is about 90%. These differences could be explained by the climate, ground and altitude characteristics of each collection region. When the Spanish map is observed [30], we can see that samples C, D and E belong to a zone where the geographical characteristics are similar,

Table 2
Comparison of anthocyanin profiles of each bee pollen sample from type *E. plantagineum*

| Anthocyanin | Anthocyanin content (area %) of each bee pollen samples[a] | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | E |
| Delphinidin-3-O-glucoside | 2.72 | 0.48 | 0.48 | 0.58 | 0.70 |
| Delphinidin-3-O-rutinoside | 7.14 | 3.53 | 3.30 | 3.75 | 4.03 |
| Cyanidin-3-O-glucoside | 0.79 | – | – | – | – |
| Cyanidin-3-O-rutinoside | 2.51 | – | – | – | – |
| Petunidin-3-O-glucoside | 2.70 | 3.18 | 3.56 | 3.29 | 3.94 |
| Petunidin-3-O-rutinoside | 80.09 | 90.69 | 90.18 | 89.90 | 88.70 |
| Peonidin-3-O-rutinoside | – | 0.13 | – | – | – |
| Malvidin-3-O-rutinoside | 2.39 | 1.98 | 2.48 | 2.48 | 2.63 |
| Cyanidin-3-(6″-malonylglucoside) | 1.66 | – | – | – | – |

[a] For the location of each sample see Fig. 1.

whereas samples A and B belong to zones with different geographical features, sample B belonging to a lightly alluvial region. Based on these slight differences between collection regions, it could be supposed that the anthocyanin composition is related to the geographical origin of the pollen. To our knowledge, there are no bibliographic data that permit us to compare our results with other studies.

Moreover, if the anthocyanin profile of *E. plantagineum* is compared to that of *F. excorticata* [23], it can be seen that they are clearly different. In our study, glucoside and rutinoside derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin, and cyanidin-3-(6″malonylglucoside) were found, petunidin-3-O-rutinoside being the major pigment. The *Fuchsia* compounds were *p*-coumaroylated derivatives of delphinidin, petunidin and malvidin-3-O-glucosides. The differences found between *Echium* and *Fuchsia* allowed us to suppose that anthocyanin composition could be a useful tool for the botanical characterisation of pollen.

The total anthocyanin content from dark blue bee pollen, calculated as the sum of individual pigment concentrations, ranged between 45 and 80 mg/100 g of fresh sample. As previously explained, bee pollens are present as a mixture of different plant species pollens, therefore the dark blue bee pollen contained in each sample can vary in accordance with the collection period. The content of dark blue bee pollen of the five samples studied varied between 24.26 and 47.31%, excepting sample A, which contained only 6.39% of blue pollen.

In this work, we have only characterised dark blue bee pollen from type *E. plantagineum*. However, it is important to emphasise that there are more floral species that produce coloured pollen that the bees can use as a source of nutrients. Therefore, this study can be extended to the characterisation of pigment profiles from other plant species of apicultural interest, in order to evaluate their nutritional contribution and their usefulness in the botanical and/or geographical characterisation.

## 4. Conclusions

The HPLC–DAD–MS analysis allowed us to identify the structure of the pigments that give colour to dark blue bee pollen. Bee pollen from type *E. plantagineum* showed a characteristic anthocyanin profile, where petunidin-3-O-glucoside is the major pigment in all of the five samples analysed. The slight variations in the profiles could be explained by the geographical differences between each collection region. Similarly, the differences found when the *Echium* anthocyanin profile is compared with that of *Fuchsia* anthocyanins, permits the hypothesis that the anthocyanin profile can be used as a botanical source marker of bee pollen.

Furthermore, the anthocyanin content (45–80 mg/100 g) of blue pollen suggests that, although it is not a product appreciated by the consumer yet, it could represent an important source of phytochemicals for human diet, giving an additional value to already recognised nutritional properties of bee pollen.

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