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Differentiation of bee pollen samples according to the betaines and other quaternary ammonium related compounds content by using a canonical discriminant analysis

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ABSTRACT

In the last years, an increase has been observed in the adulteration of bee pollen. Consequently, different tools are required to authenticate the origin of this product, such as a study of the profile and composition of a specific family of compounds. The present study investigates the potential of betaines and related compounds as markers of the apiary of origin and harvest period of 71 bee pollen samples. These were collected from four apiaries (Pistacho, Tío Natalio, Monte and Fuentelahiguera), located in the same geographical area (Guadalajara, Spain) and sampled during three consecutive harvest periods in the same year (April-May, June, July-August). They were analyzed by means of a previously developed methodology, which involved solvent extraction, hydrophilic interaction liquid chromatography coupled to mass spectrometry, and a statistical analysis of the data (canonical discriminant analysis). Variable amounts of betaines and related compounds were found in the samples, with four of these being identified in all of them (betonicine, betaine, trigonelline and choline); betonicine was the predominant compound in a concentration range of 264 to 52384 mg/kg. It was possible to statistically assign over 50 % of the samples to the corresponding apiary of origin, the best results being obtained for the Tío Natalio apiary (75 %); this classification was even better in the case of the harvest period, as more than 75 % of the samples were correctly assigned, and in two periods (April-May and June) a 90 % rate was obtained.

1. Introduction

Bee pollen has formed part of the human diet for many centuries, and its ever-increasing consumption results from its nutritional value and health-promoting effects, such as those relating to its antioxidant, anti-inflammatory, anticarcinogenic, antibacterial or anti-fungal properties (Ares, Valverde, Bernal, Nozal, & Bernal, 2018; Chen, Zhao, Cheng, & Cao, 2019; Laaroussi, et al., 2020; Xu, Gao, & Sun, 2012). These properties are associated with its constituents, which include proteins, amino acids, lipids, carbohydrates, phenolic compounds vitamins, or minerals, among others (Campos et al., 2021; Thakur, & Nanda, 2021). However, its composition varies greatly depending on several factors, like botanical and geographical origins, climatic conditions, the type of soil, or the harvest and processing conditions (Ares et al., 2020a; Gardana, Del Bo, Quicazán, Corrrea, & Simonetti, 2018; Inacio et al., 2021; Thakur, & Nanda 2021). This is quite important in terms of preventing the fraudulent practice of adulteration with pollen from other sources (Wang, Ren, Wu, & He, 2021; Wang, Zhong, Hao, Wang, & Wang, 2022). As may

Abbreviations: **AEMET**, agencia estatal de meteorología; **AM**, April-May; **BET**, betaine; **BET-d9**, betaine-d9; **BETO**, betonicine; **BRCs**, betaines and related compounds; **CAR**, L-carnitine; **CDA**, canonical discriminant analysis; **CHO**, choline; **CIAPA**, Centro de Investigación Apícola y Agroambiental; **ESI**, electrospray; **FH**, Fuentelahiguera; **HILIC-MS**, hydrophilic interaction liquid chromatography-mass spectrometry; **JA**, July-August; **JN**, June; **LAU**, lauryl betaine; **LOD**, limit of detection; **LOQ**, limit of quantification; **MO**, Monte; **MYR**, myristyl betaine; **PI**, Pistacho; **PRO**, proline betaine; **TN**, Tío Natalio; **TRI**, trigonelline.

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be expected, it has been suggested that the profiles of a particular family of compounds (proteins, amino acids, lipids, phenolic compounds, glucosinolates or minerals) should be studied, in order to specify the botanical origin of the pollen and to evaluate the corresponding nutritional value or health-promoting effects (Ares et al., 2020a; Ares et al., 2022; Conte et al., 2017; Gardana et al., 2018; Gonçalves et al., 2018; Isopescu et al., 2020; Kaškonienė et al., 2015; Lv et al., 2015; Taha, Al-Kahtani, & Taha, 2019; Thakur, & Nanda, 2021; Xu et al., 2012; Zhou et al., 2015). Therefore, we consider it appropriate to search for new candidates, such as betaines and other quaternary ammonium related compounds (BRCs), as markers of bee pollen. This was decided as a result of a recent pioneer study, which investigated the potential presence of BRCs in bee pollen by developing and validating a new method based on hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS; Ares, Toribio, Nozal, Martín, & Bernal, 2020b). We observed that some of the compounds under study were present in all the samples, and, more importantly, their content varied between samples from different origins, as was the case with other bee pollen constituents. This finding suggests the potential of these compounds as markers of bee pollen. Betaines are zwitterionic quaternary ammonium compounds, produced by specific biosynthetic pathways involving nitrogen methylation of amino acids and imino acids or enzymatic oxidation of choline (see Supplementary Material, Fig. 1S; Ares et al., 2020; Rivoira et al., 2017); they perform physiological functions as an osmolyte and as a donor of methyl groups (Hefni, Schaller, & Witthöft, 2018). When they act as osmolytes, they accumulate in the cytoplasm and intracellular fluids, protecting cells and their components (proteins, nucleic acids, cell membranes, and enzymes) from environmental stress, such as that caused by lack of water (Servillo et al., 2016). These compounds have been investigated in foods owing to their potential effects on human health (Rivoira et al., 2017; Servillo et al., 2016); for example, a diet rich in betaine (BET) and choline (CHO) is recommended for protecting cells against hypertonic stress (Antonelo et al., 2020) or for improving cardiovascular health (Rivoira et al., 2017). However, the consumption of BET should be controlled, as a high intake could increase the risk of cardiovascular diseases (Hefni, Bergström, Lennqvist, Fargerström, & Witthöft, 2021; Rivoira et al., 2017). Scant attention has been paid determining these compounds in bee pollen, since, to the best of our knowledge, only our previous publication (Ares et al., 2020b) and a few more studies have been published on plant pollen. For example, a study has taken place of the accumulation of certain nutrients, amino acids and a BRC (glycine betaine) in tomato pollen through the action of a proline transporter (Schwacke et al., 1999). Results showed that although the proline transporter proved efficient for glycine betaine, it was not identified in tomato pollen. Despite the fact that this compound had been previously found in spinach and wild rye pollen, the study was not specifically focused on BRCs (Gorham, Wyn Jones, & McDonnell, 1985).

Therefore, the main goal of this paper is to investigate the potential of BRCs as bee pollen markers, determining (HILIC-MS) their content in 71 bee pollen samples from four different apiaries located within the same area (Guadalajara, Spain). These were collected in three consecutive foraging periods in the same year (April-May; June; July-August) and were chosen because of our previous study (Ares et al., 2020b). Accordingly, it is the first study of whether bee pollen samples can be classified, by means of canonical discriminant analysis (CDA) focused on BRC content, in terms of the corresponding apiary of origin or the harvest periods.

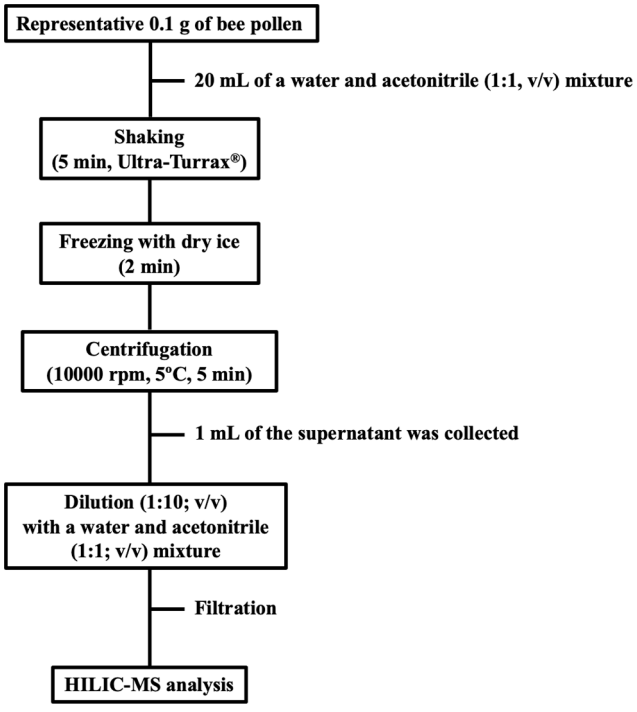

Fig. 1. Analytical procedure work-up flow chart.

2. Materials and methods

2.1. Chemicals, materials, and standards

Standards of BRCs, including an isotope labelled standard (BET-d9; see Table 1), and acetic acid were supplied by Sigma–Aldrich Chemie Gbmh (Steinheim, Germany). BRCs were chosen on basis on our previous study (Ares et al., 2020b). LC grade acetonitrile was obtained from Lab-Scan ltd. (Dublin, Ireland). Syringe filters (17 mm, Nylon 0.45 μm) were provided by Nalgene (Rochester, NY, USA), and ultrapure water was obtained from Millipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA). An Eppendorf Centrifuge 5810R (Hamburg, Germany), a Moulinette chopper device (Moulinex. Paris, France), IKA® Ultra-Turrax® T18 basic disperser (IKA®-Werke GmbH & Co. KG, Staufen, Germany), and a drying oven from J.P. Selecta S.A. (Barcelona, Spain) were used for the sample treatment. Individual standard stock (≈ 100 mg/L) solutions were prepared with ultrapure water and then further diluted with a mixture of acetonitrile and ultrapure water (1:1, v/v) to prepare

Table 1
Molecular weight, purity, and quantification/confirmation ions for each of the studied compounds. Adapted from Microchemical Journal, 157, Ares, A. M., Toribio, L., Nozal, M. J., Martín, M. T., Bernal, J., Simultaneous determination of betaines and other quaternary ammonium related compounds in bee pollen by hydrophilic interaction liquid chromatography-mass spectrometry, 105000, 2020, with permission from Elsevier.

Compound name (Abbreviation)	Molecular weight	Purity of the standards	Ions
Myristyl betaine (MYR)	299	≥ 97 %	300.0[Q]; 230.1[C]; 322,0[C]
Lauryl betaine (LAU)	271	≥ 95 %	272.1[Q];149.9[C]; 294.0[C]
Betonicine (BETO)	159	≥ 98 %	160.3[Q]; 88.3[C]; 182.2[C]
Betaine (BET)	117	≥ 99 %	118.1[Q]; 58.1[C]; 140.1[C]
Betaine-d9 (BET-d9)	126	≥ 98 %	127.1[Q]; 67.1[C]; 149.1[C]
Trigonelline (TRI)	137	≥ 98.5 %	138.1[Q]; 94.1[C]; 160.0[C]
L-Carnitine (CAR)	161	≥ 98 %	162.3[Q];60.2[C]; 184.3[C]
Choline (CHO)	104	≥ 99 %	104.0[Q]; 60.2[C]; 58.1[C]
Proline betaine (PRO)	143	≥ 97 %	144.2[Q]; 102.2[C]; 166.1[C]

[C] confirmation ions; [Q]quantification ions.

the working solutions. Reference standard in solvent (matrix-free) calibration curves were used to measure the bee pollen compounds as there was no significant matrix effect (Ares et al., 2020b). The analytical range was between the limits of quantification (LOQs; see Supplementary Material, Table 1S) and 1 mg/L (LOQ, 0.05, 0.10, 0.20, 0.40 and 1.00 mg/L). The graphs obtained in all the calibration curves were straight lines with coefficient of the determination values (R^2) higher than 0.99 in all cases (see Supplementary Material, Table 1S). Stock solutions were stored in glass containers in darkness at −20 °C; working and calibration solutions were stored in glass containers and kept in the dark at 4 °C. All solutions remained stable for over two weeks.

2.2. Sample procurement and treatment

2.2.1. Samples

Bee pollen samples (n = 71) were collected during three consecutive foraging periods (initial, between April and May; intermediate, June; final, between July and August) in 2018, from four apiaries (Pistacho, PI; Tío Natalio, TN; Monte, MO; Fuentelahiguera, FH). The homogeneous colonies of *Apis mellifera iberiensis*, located in Marchamalo (PI, TN, and MO) and Fuentelahiguera de Albatages (FH) were all in the province of Guadalajara (Spain; see Supplementary Material, Fig. 2S). Bee pollen samples were collected using pollen catchers placed at the entrance of the hive. Every-two weeks, the pollen catcher grid was closed for a period of 24 h in the different hives. The pollen stored in the collection drawer during this period was collected, immediately sealed, identified (date of collection, apiary, and colony) and taken to the laboratory, where it was frozen until palynological analysis (Ares et al., 2022; see Supplementary Material, Table 2S). The results of the contents of corbicular pollens mostly collected in the samples corresponding to each period and colony are summarized in Table 3S (see Supplementary Material). The term multifloral (MF) has been used, which is based on specialized literature, and refers to bee pollen that include different taxa when a majority and specific taxon was not well defined (<80 %; Campos et al., 2008).

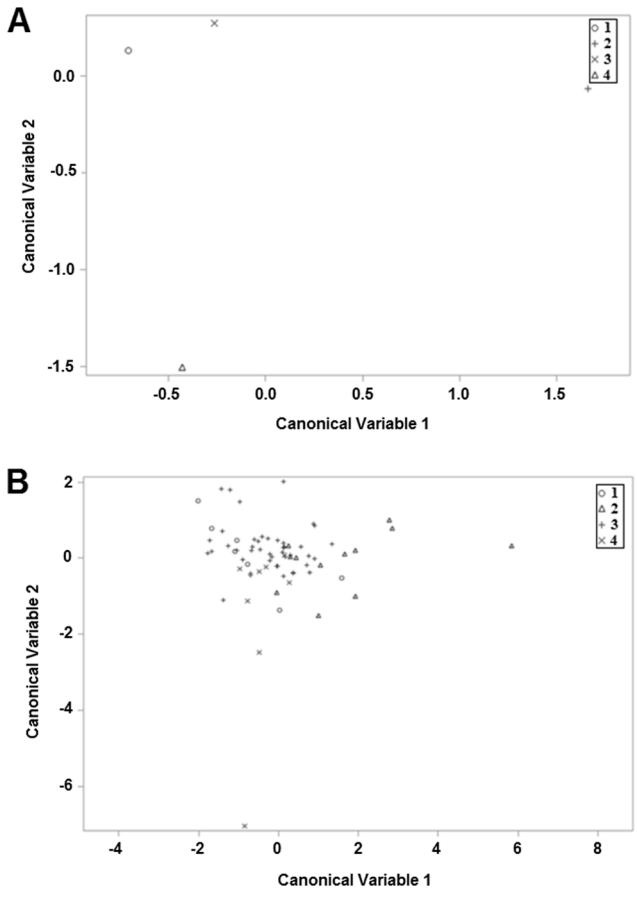

Fig. 2. **A**) Representation of the apiaries (FH, 1; MO, 2; PI, 3; TN, 4) as function of canonical variables 1 and 2; **B**) Representation of individual bee pollen samples (from the apiaries (FH, 1; MO, 2; PI, 3; TN, 4) as function of canonical variables 1 and 2.

2.2.2. Sample treatment

Bee pollen samples were individually mixed, ground and pooled for optimum sample homogeneity. Next, bee pollen was dried until the mass stabilized (humidity was between 9 % and 12 %), and subsequently it was stored in the dark at −20 °C until analysis. The proposed procedure, which is described in Fig. 1, was optimized, and validated in a previous study (Ares et al., 2020b). As we mentioned in the previous article (Ares et al., 2020b), which as yet represents the only one in which BRCs have been analyzed in bee pollen, sample treatment proved to be simple, efficient (recoveries between 82 % and 95 %, and relatively fast (<20 min). Moreover, the matrix did not significantly affect the MS signals of the analytes (responses between 85 % and 101 %), which is of relevance when employing LC-MS (see Supplementary Material, Table 1S).

2.3. HILIC-MS conditions

An Agilent Technologies 1100 LC coupled to a single quadrupole MS detector equipped with an electrospray ionization (ESI) source, which was operated in positive mode, was employed in all experiments. A Kinetex® HILIC core–shell type column (50 × 2.1 mm, 2.6 μm, 100 Å) and a Kinetex® HILIC guard column (Phenomenex, Torrance, CA, USA) were used in this study. The HILIC-MS conditions were optimized in a previous study (Ares et al., 2020b). The mobile phase was composed of acetonitrile and 0.1 % (v/v) acetic acid in water. The gradient elution program and the final settings of the most relevant HILIC-MS parameters are summarized in Table 2. Under optimal chromatographic conditions, all compounds eluted in <11 min (see Supplementary Material, Fig. 3S). Full-scan direct infusion spectra were obtained by scanning from *m/z* 50 to 350. Compounds showing intense $[M + H]^+$ on their full scan spectra (see Table 1 and Supplementary Material, Fig. 4S) were used for quantification with the selected ion monitoring mode (SIM); meanwhile, two other ions displaying the highest signals (see Table 1) were used for confirmation. Method selectivity was demonstrated in our previous

Table 2
HILIC-ESI-MS parameters. Adapted from Microchemical Journal, 157, Ares, A. M., Toribio, L., Nozal, M. J., Martín, M. T., Bernal, J., Simultaneous determination of betaines and other quaternary ammonium related compounds in bee pollen by hydrophilic interaction liquid chromatography-mass spectrometry, 105000, 2020, with permission from Elsevier.

HILIC parameter	Final setting
Gradient elution program	Acetonitrile (solvent A) and 0.1 % (v/v) acetic acid in water (solvent B): **(i)** 0 min (A–B, 88:12, v/v); **(ii)** 3 min (A–B, 88:12, v/v); **(iii)** 5 min (A–B, 55:45, v/v); **(iv)** 9 min (A–B, 55:45, v/v); **(v)** 11 min (A–B, 88:12, v/v); (vi) 15 min (A–B, 88:12, v/v)
Flow-rate (mL/min)	0.5
Column temperature (°C)	25
Injection volume (μL)	3
ESI-MS parameter	**Final setting**
Capillary voltage (V)	2500
Drying gas (N_2) flow (L/min)	9
Drying gas (N_2) temperature (°C)	200
Fragmentor voltage (V)	140
Nebulizer gas (N_2) pressure (psi)	40
Gain	5

Table 3
Frequency and concentration range of each BRCs in the analyzed bee pollen samples.

Amino acid	FH		MO		TN		PI		OVERALL	
	FR	CR*	FR	CR*	FR	CR*	FR	CR*	FR	CR*
LAU	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD
MYR	0	< LOD	8	150	0	<LOD	0	<LOD	1	150
BETO	100	680–27458	100	920–52834	100	798–10518	100	264–34392	100	264–34392
BET	100	28–119	100	18–1898	100	14–1358	100	7–4910	100	7–4910
TRI	100	51–949	100	14–3628	100	29–187	100	12–3628	100	12–3628
CAR	0	<LOD	0	<LOD	0	<LOD	5	11–60	3	11–60
CHO	100	104–318	100	58–336	100	133–467	100	13–723	100	13–723
PRO	14	<LOQ	25	<LOQ-7	0	<LOD	7	<LOQ-17	11	<LOQ-17

FR, frequency (%). Number of samples in which a BRCs was identified (>LOD)/total number of samples (Fuentelahiguera (FH), n = 7; Monte (MO), n = 12; Tío Natalio (TN), n = 8; Pistacho (PI), n = 44; OVERALL, n = 71) *100; **CR**, concentration range (mg/kg, dry weight); * Content over LOD.

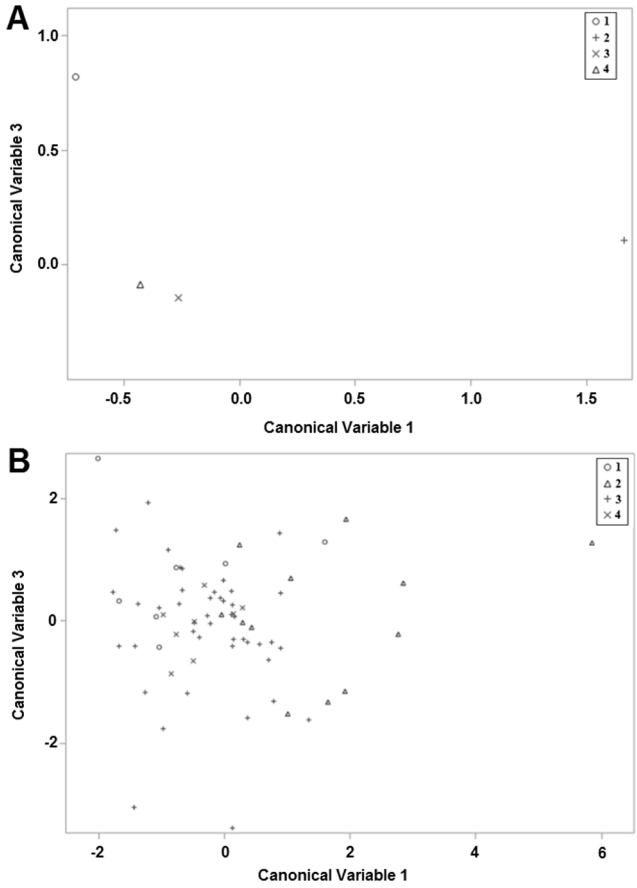

Fig. 3. **A**) Representation of the apiaries (FH, 1; MO, 2; PI, 3; TN, 4) as function of canonical variables 1 and 3; **B**) Representation of individual bee pollen samples from the apiaries (FH, 1; MO, 2; PI, 3; TN, 4) as function of canonical variables 1 and 3.

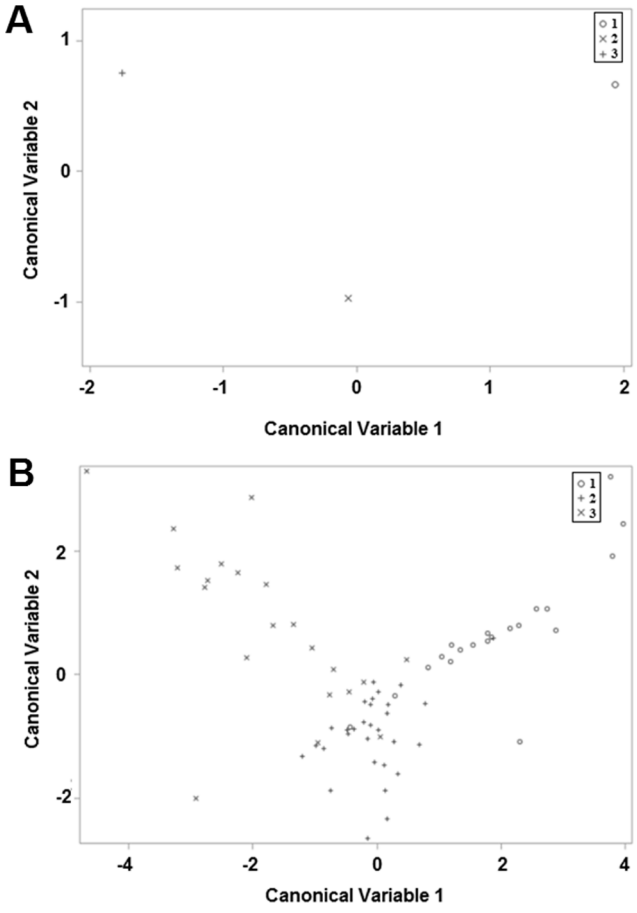

Fig. 4. **A**) Representation of the harvest periods (April-May, 1; June, 2; July-August, 3) as function of the first two canonical variables; **B**) Representation of individual bee pollen samples from the different harvest periods (April-May, 1; June, 2; July-August, 3) as function of the first two canonical variables.

study (Ares et al., 2020b). No matrix interferences co-eluted with the analytes, whilst MS spectra in matrix-free and matrix-matched standards were quite similar (see Supplementary Material, Fig. 4S).

2.4. Canonical discriminant analysis

The calculations for CDA required in this paper were performed using SAS PROC CANDISC (version 9.4; SAS Institute Inc., Cary, NC, USA). CDA obtains linear combinations of the quantitative variables that emphasize the differences among the groups (Ares et al., 2022; Jobson, 1991). The data base used in the present study comprised the response of each sample to the qualitative variable (apiary of origin or harvest period), together with the three measurements of each individual sample for the BRCs under study (quantitative variables). If the mean (average value) of the three measurements of each sample were considered rather than the three different values, the variability of the original data would be greatly affected; this is not advisable when performing a CDA.

3. Results and discussion

3.1. Determination of BRCs content

content was determined in 71 samples of bee pollen from four apiaries, located in Marchamalo (PI, n = 44; MO, n = 12; TN, n = 8; FH, n =

7). All the samples were analyzed in triplicate, and occasionally it was necessary to dilute the extracts (1:5 or 1:100; v/v) with an acetonitrile and water (1:1, v/v) mixture, as the high concentrations were outside the linear range for some of the compounds (see Supplementary Material, Figure 5S). The results are listed in Tables 3, 4, and 4S-7S (see Supplementary Material); the frequency (the percentage of the number of samples in which a compound was identified/the total number of samples) and concentration intervals (mg/kg; dry weight) are shown. Given the data obtained from the concentrations for each BRC, it can be concluded that the largest number of compounds was found in the PI apiary, followed by MO, FH and TN. However, if the mean content per sample is compared (see Table 5), then the highest values were found in samples from the FH apiary, these being almost twice the content of the TN samples. In addition, the highest total concentration of BRCs was observed for the samples harvested in June, followed by the period April-May, and lastly, July-August; however, as with a comparison of the different apiaries, the order changed if the mean values are considered. Thus, the highest values corresponded to the samples harvested in July-August, with those for the other two periods being slightly lower. This could be related to the tendency of these compounds to accumulate in plants in response to abiotic stresses, such as a reduction in the availability of water (Rivoira, Studzińska, Szultka-Młyńska, Bruzzoniti, & Buszewski, 2017; Servillo et al., 2016), the average rainfall for the July-August period in this area and year (1.2 L/m^2) being much lower than in the case of the other two harvest periods (April-May, 67.4 L/m^2; June, 32.0 L/m^2; AEMET, 2022). Betonicine (BETO) was by far the predominant BRC in all the apiaries and harvest periods, ranging between 264 and 52384 mg/kg. This result differs entirely from that of the previous study of BRCs in bee pollen (Ares et al., 2020b), the latter not being identified in any of the samples analyzed. BET, trigonelline (TRI) and choline (CHO) were also identified in all the samples in our previous study, although in this case concentration values were much higher. A tentative explanation for these differences may relate to the different origin of the samples. As can be seen in our previous work (Ares et al., 2020b), bee pollen samples were from different monofloral origins (maize, rapeseed, sunflower, radish, rock rose) in most cases, except for three samples from wild *Brassica* plants and one multifloral commercial sample. These botanical origins were different than those from the samples of the present study (see Supplementary Material, Table 3S), and this could explain some of differences observed in the BRC content. Moreover, bee pollen samples from Marchamalo were not obtained from the same apiaries in both studies, and consequently the geographical origin was not the same, which also influenced the BRC content. In addition, lauryl betaine (LAU) was not identified in any of the samples, while L-carnitine (CAR) and myristyl betaine (MYR) residues were seen to be present in only two samples from the PI apiary, and one from the MO apiary; proline betaine (PRO), on the other hand, was identified in 8 samples from four apiaries. Concentration values for these compounds were quite low in comparison with those of other BRCs; it is worth noting that none of these compounds were identified in the only related study (Ares et al., 2020b). As previously mentioned, there is only one study in which BRCs were determined in bee pollen, albeit with far fewer samples. We have already mentioned the similarities and differences with regard to this study. With respect to analyzing BRCs in plant pollen, glycine betaine was identified in spinach and wild rye pollens at concentrations ranging from 13,000 to 21000 mg/kg (Gorham et al., 1985), which are comparable to the concentration observed in the bee pollen samples analyzed here for other BRCs. In the case, however, of other food matrices like pasta and cereals (Ross, Zangger, & Guiraud, 2014) or chestnut (Servillo et al., 2016), concentrations are usually below 1000 mg/kg, whilst the number of compounds identified is very much dependent on food type and origin. Finally, it has not been possible to correlate BRCs with their botanical source, as the bee pollen samples had not been previously separated by colour or taxa; as a result, most were of multifloral origin of unspecified plant source. In cases where they were specified, there is not a large amount of detailed information available regarding BRC composition.

Table 4
Frequency and concentration range of each BRCs depending on the harvest period.

Amino acid	AM		JN		JA	
	FR	CR*	FR	CR*	FR	CR*
LAU	0	<LOD	0	<LOD	0	<LOD
MYR	0	<LOD	3	150	0	<LOD
BETO	100	264–27458	100	448–52834	100	798–27235
BET	100	7–243	100	15–463	100	49–4910
TRI	100	15–3628	100	12–701	100	14–621
CAR	0	<LOD	0	<LOD	10	11–60
CHO	100	13–262	100	15–723	100	25–508
PRO	10	<LOQ-9	13	<LOQ-17	10	<LOQ

FR, frequency (%). Number of samples in which a BRCs was identified (>LOD)/total number of samples (April-May (AM), n = 20; June (JN), n = 30; July-August (JA), n = 21) *100; **CR**, concentration range (mg/kg, dry weight); * Content over LOD.

Table 5
Total and mean content per sample (mg/kg) of BRCs in the bee pollen samples grouped by apiary (Monte, MO; Pistacho, PI; Tío Natalio, TN; Fuentelahiguera, FH) and harvest period (April-May, AM; June, JN; July-August, JA).

Apiary of origin		
Apiary	Total content	Mean content
FH	70,730	10,104
MO	105,562	8797
TN	43,458	5432
PI	349,537	7944
Harvest period		
Period	**Total content**	**Mean content**
AM	132,576	6629
JN	283,667	6447
JA	153,044	7289

3.2. Canonical discriminant analysis

As was mentioned in the Introduction, the main objective of this study was to demonstrate whether BRCs can be considered bee pollen markers. Therefore, two different statistical examinations (canonical discriminant analysis) were performed in line with the apiary of origin (PI, n = 44; MO, n = 12; TN, n = 8; FH, n = 7) or the harvest period (April-May, n = 20; June, n = 30; July-August; n = 21). Three of the compounds were not included in the CDA, as LAU was not identified in any of the samples, while MYR and CAR were found in one and two samples, respectively.

3.2.1. Apiary of origin

In this study, the sample size was seventy-one, distributed among four classes (apiaries: FH, MO, PI, and TN), with five variables (each variable was analyzed in triplicate, which represented fifteen measures for apiary). Our statistical approach reduces analysis to three dimensions instead of fifteen without losing any type of information. Preliminary results showed that the first three canonical variables accounted for 100 % of original data variability. Next, the weights of the first three canonical variables were obtained; these are linear combinations of quantitative variables, summarized in Table 8S (see Supplementary Material). BETO_1, BETO_2, BETO_3 refer to the three replicates made when analyzing each of the pollen samples; it is important to consider the signs of the coefficients (positive and negative) as well as the absolute values. Weights close to zero signify that this variable has little relevance in relation to the corresponding variable, as was the case of betonicine, whilst higher values, such as those obtained for proline betaine, represent a great influence on the behaviour of the canonical variable. The signs indicate relevance in each of the canonical

variables selected. Next, the averages of the first three canonical variables for the four apiaries were obtained (see Supplementary Material, Table 9S) and represented graphically (Fig. 2A and 3A). The location of the points in the graphic representation is the result of the positive or negative weight of the canonical variables of the different compounds. As can be seen in Fig. 2A, two of the apiaries (MO and TN) were clearly distinguishable from the others when the mean values of the first two canonical variables were applied. MO was the only apiary with positive values for both variables, with its location in the upper right-hand corner of the graph; meanwhile, TN, with negative values, is situated in the lower left-hand corner. The other two apiaries (FH and PI) had the same signs for both variables, although their absolute values differed. However, a clearer separation between the latter was observed by a comparison of canonical variables 1 and 3 (see Fig. 3A), FH having a positive value for canonical variable 3 and PI a negative one. An individual study of each sample showed that in many cases it was not possible to observe a clear differentiation when using only the first two canonical variables (see Fig. 2B), and that representation of canonical variables 1 and 3 was required for a better separation of the samples (see Fig. 3B). Moreover, results of the CDA also showed that over 50 % of the bee pollen samples could be correctly assigned to their corresponding apiary of origin (see Table 6). In some cases, these values were lower than those obtained when evaluating glucosinolates as bee pollen markers (greater than75 %; Ares et al., 2022); it should be considered, however, that glucosinolates were especially abundant in plants from the Brassicaceae (cruciferous) family, and consequently their presence in bee pollen is much more specific than in the case of BRCs of botanical origin. The reason for certain samples not being classified correctly and for several of them not being clearly differentiated, as seen in Fig. 2B and 3B, may to a large extent be due to the multifloral nature of most of the samples; as a result, there was a considerable difference in terms of plant origin and composition. In addition, as can be seen in Table 6, the lowest classification rates corresponded to the FH (57.14 %) and PI (52.27) apiaries. Two of the FH samples were confused with PI, this corresponded to 28.57 %, while seven of the PI samples were assigned to the FH apiary, this was a 15.91 % of total. Therefore, the discrimination between these two was not better because several of their samples have close measures in the observed variables. Notwithstanding, these findings have demonstrated the potential of BRCs as bee pollen markers in one of the worst-case scenarios for differentiating the origin of a bee pollen sample, as is the similarity of plant sources and the environment.

3.2.2. Harvest period

The sample size and number of variables were the same as in the above-mentioned study, seventy-one and fifteen, respectively, but in this case, samples were categorized in three classes (April-May, June, July-August). The weights of the canonical variables were obtained as previously described, and these are listed in Table 10S (see Supplementary Material); in this case, only the first two canonical variables were necessary to account for 100 % variability of the original data. Next, the averages of canonical variables 1 and 2 were obtained (see Supplementary Material, Table 11S) and graphically represented (see Fig. 4A). It can be appreciated that the harvest periods were clearly distinguishable by means of these two variables. The April-May period displays positive values for both canonical variables, these being in the top right-hand corner; this is the opposite situation in the case of June, when both values are negative, and they are situated in the lower central part of the graph. The last period (July-August) has a positive value for canonical variable 2 and a negative value for canonical variable 1, its location (upper left corner) being clearly distinguishable from the other two periods. This clear differentiation of the harvest periods based on the average values of the first two canonical variables could be also extrapolated to the individual samples (see Fig. 4B), as few samples were mixed in the representation. This satisfactory outcome was confirmed when examining the results of sample classification according to their corresponding harvest period (see Table 7); more than 75 % of the samples were correctly grouped, results which are similar to those obtained when investigating glucosinolates (Ares et al., 2022). Indeed, samples corresponding to the harvest periods of April-May and June presented classification rates higher or equal than 90 %. Meanwhile, this value was lower for July-August period (76.19 %), which from our point of view, is very reasonable, as five samples (23.81 %) were classified in June (none in April-May). This can be justified by the proximity between the harvest periods of late June and early July, and because the measures taken in these harvest periods were similar. Nevertheless, this is a quite relevant finding, as, to the best of our knowledge, it is the first time that a distinction has been made between bee pollen samples with respect to the harvest period by determining the individual content of BRCs. Nevertheless, it should be considered that climatic conditions, and in particular a lack of rain, affect BRC content in plants (Servillo et al., 2016), these in turn being characteristic of the geographical and botanical origin of bee pollen. Therefore, the differences observed in BRC content depending on the harvest period serve as a complement to ensure the geographical origin of the pollen samples. Indeed, the improvement observed when classifying the samples in line with their harvest period, compared with the apiary of origin, could be tentatively attributed to the fact that climatic conditions, and specifically a lack of water, have more influence on BRC content in the bee pollen samples analyzed than the apiary or plant origin. All the same, for BRCs to be used successfully as markers of pollen origin, the geographical area of origin of the pollen should maintain its flora over the years, which, fortunately, is usually the case. The climatic conditions during the harvest periods should be similar, which, due to climate change, is not so predictable, especially as regards average rainfall; also, it would be appropriate for more data to be obtained on the content of betaines in various plants, in order to create a database allowing an immediate comparison of samples.

4. Conclusions

An HILIC-MS analytical study of the content of betaine and other quaternary ammonium-related compounds was carried out on seventy-one samples of bee pollen, originating from four different apiaries

Table 6
Number of observations and percentage classified in each group (apiary of origin) using canonical discriminant analysis and the first three canonical variables.

Origin	FH	MO	PI	TN	Total
FH	4	1	2	0	7
%	57.14	14.29	28.57	0.00	100.00
MO	1	8	2	1	12
%	8.33	66.67	16.67	8.33	100.00
PI	7	6	23	8	44
%	15.91	13.64	52.27	18.18	100.00
TN	0	0	2	6	8
%	0.00	0.00	25.00	75.00	100.00
Total	12	15	29	15	71
	16.90	21.13	40.85	21.13	100.00

Table 7
Number of observations and percentage classified in each group (harvest period) using canonical discriminant analysis and the first two canonical variables.

Origin	April-May	June	July-August	Total
April-May	18	2	0	20
%	90.00	10.00	0.00	100.00
June	1	29	0	30
%	3.33	96.67	0.00	100.00
July-August	0	5	16	21
%	0.00	23.81	76.19	100.00
Total	19	36	16	71
	26.76	50.70	22.54	100.00

located in Marchamalo (Guadalajara, Spain). Variable amounts of compounds were found in the samples analyzed. All of them contained BETO, BET, TRI and CHO, while LAU was not identified in any sample. BETO was the predominant compound encountered regardless of the apiary of origin or harvest period. The largest number of BRCs was found in samples from the PI apiary and in those harvested in June; this is to be expected, as the largest number of samples belonged to these groups. Regarding mean content per sample, the highest concentrations of BRCs were observed in those collected in July-August from the FH apiary. The fact that BRC content per sample was at its highest in the driest period (July-August), confirms the existing knowledge that the presence of these compounds is favored by lack of water. It was not possible to correlate BRC content with a specific plant source, since most of the samples were of multifloral origin, and because there was a lack of information regarding specific content in the plants of origin. Results of the CDA demonstrated for the first time that these compounds have a potential as markers of the origin of bee pollen. By means of the first three canonical variables it was possible to differentiate between the four apiaries, because of the BRC content of their corresponding samples. Moreover, it was possible to assign over 50 % of the samples to the corresponding apiary, a hitherto unreported noteworthy finding. Even better results were obtained when correlating BRC content in the bee pollen samples with the corresponding harvest period. Differentiation was achieved by means of only the first two canonical variables, with more than 75 % of the samples being correctly classified. These results demonstrate the potential of these compounds as bee pollen markers, especially of the harvest period. This is quite a relevant finding, as, to our knowledge, such studies have never been previously conducted by focusing attention on BRCs. This study may serve as a starting point for ascertaining whether this potential of BRCs as bee pollen markers can be extended to longer periods, perhaps by analyzing samples for two or more consecutive years to account for variation in climatic conditions that affects BRCs content, and to samples from different regions and countries.

CRediT authorship contribution statement

Ana M. Ares: Conceptualization, Methodology, Investigation, Supervision, Validation, Visualization, Writing – original draft. **María T. Martín:** Methodology, Investigation, Validation, Visualization, Writing – original draft. **Jesús A. Tapia:** Conceptualization, Formal analysis, Software, Visualization. **Amelia V. González-Porto:** Conceptualization, Methodology, Investigation, Resources, Visualization, Writing – original draft. **Mariano Higes:** Conceptualization, Resources, Visualization. **Raquel Martín-Hernández:** Conceptualization, Funding acquisition, Project administration, Resources, Visualization, Writing – original draft. **José Bernal:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The datasets generated during the current study are included in this published article and the Supplementary Information, or they are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111698.

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