Evaluation of Polyphenolic and Flavonoid Compounds in Honeybee-Collected Pollen Produced in Spain

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Polyphenolic content, flavonoid content, and free flavonoid aglycon compounds were determined in 11 samples of Spanish honeybee-collected pollen. Adequate extraction was obtained with ethyl acetate in the determination of free flavonoid aglycon. Recovery (>83.6%), within-run repeatability (<6.67%), between-run reproducibility (<8.73%), and detection limits (1.4−1.9 mg/kg) were satisfactory. A total of 15 compounds were separated with gradient reversed phase HPLC, and 13 were identified and quantified using diode array detector. The most predominant compounds were flavonoid glycosides, mainly flavonols. Eighty-two percent of the samples contained at least 14 of the phenolic components, primarily rutin, quercetin, myricetin, and *trans*-cinnamic acid as free aglycons. Total phenols were present, at levels of >0.85 g/100 g in the form of non-tannins, and flavonoids of >0.35 g/100 g, using spectrophotometric procedures. Rutin is the best identifier of free flavonoid aglycon compounds. A minimum quantity of 200 mg/kg of rutin is suggested to guarantee the nutritional and biological properties required in the European market.

Keywords: *Honeybee-collected pollen; polyphenols; flavonoids; rutin; quality*

INTRODUCTION

Polyphenolic and flavonoid compounds are widely distributed in foods of plant origin and are regarded as effective antioxidants. The most current research on antioxidant action focuses on phenolic compounds such as flavonoids. Flavonoids consist mainly of anthocyanidins, flavonols, flavones, catechins, and flavanones (*1*). Several studies suggest that these compounds may be of importance in reducing the incidence of degenerative diseases such as cancer and arteriosclerosis. Food-derived flavonoids such as quercetin, rutin, and apigenin inhibited carcinogen-induced tumors in rats and in mice (*2*, *3*). Of special interest is the recent attention these compounds have attracted in the fields of nutrition, health, and medicine as a result of the quickly growing body of evidence suggesting they may act as potent antioxidants (*4*). On the other hand, a diet containing a large amount of lipids, which would generate ROO•, indeed enhanced colon carcinogenesis in rats treated with *N*-nitroso-*N*-methylurea (*5*). These findings suggest the cancer-promoting or carcinogenic potential of ROO•. Thus, a diet rich in these radical scavengers would reduce the cancer-promoting action of ROO• (*6*).

Another honeybee product, the propolis, exhibits a broad spectrum of activities such as antibiotic, antioxidative, antiinflammatory, antifungical, antitumorigenic, and antioxidative properties (*7*−*9*). Many natural products, vegetables,and fruits contain mutagenic compounds (e.g., chlorogenic acid) as well as antimutagens all together (*10*, *11*). For these reasons it is necessary to identify polyphenolic compounds in honeybee-collected pollen to evaluate its potential activity. This product can be considered a potential source of polyphenols and nutrients for human consumption.

Most pollen flavonoids exist as the glycoside; that is, a sugar is linked in a semiacetal bond at one or more hydroxy groups. The sugar-free moiety is called the aglycon and arises in vivo by the action of glucosidases. D-Glucose is the most common combined sugar, with D-galactose and L-rhamnose also frequently found. Some disaccharides and trisaccharides are ocassionally attached to the flavonoids (e.g., rutin). Free aglycons have also been found in various plants in increasing numbers. However, little attention has been paid as to whether they are genuinely present in the free state, and even less to the fact that their occurrence may be associated with secretory structures or that they are deposited externally (*12*). The honeybee-collected pollen pellets are cohered with honey and hypopharyngeal gland secretions of the honeybee, with the presence of the hydrolytic enzymes α- and β-glucosidase. Therefore, aglycons are encountered as such in the free state; the accumulation of free rutin in dried honeybee-collected pollen is evidently due to the activity of a glycosidase. Several theories have suggested that the flavonoids are involved in metabolism and growth; others have focused on a function in passive protection or as a flower attractant of a pollinating vector (*13*).

Quantitative determination of individual flavonoid glycosides is difficult because most standards are not commercially available. Hydrolysis of all glycosides to aglycons offers a practical procedure for the quantitative determination of flavonoids (*14*). In honeybee pollen this is not necessary because hypopharyngeal gland secretions of the honeybee hydrolyze flavonoid heterosides to free aglycons, increasing the possible biological activity of the product.

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The occurrence of other components (e.g., free amino acids) in honeybee-collected pollen has been established by measuring the levels of quality with respect to a fresh and/or processed product (*15*). For commercial production pollen is dried by artificial heating. Of all the numerous compounds identified in honeybee pollen, the better studied are protein content, free amino acid distribution, fat content, fatty acid and sterol composition, dietary fiber, vitamins, sugar spectrum, mineral elements, and microbiological quality (*15*, *16*). A minimum quantity of 2 g/100 g of the free amino acid content is suggested to standardize the commercial honeybee-collected pollen in the European market. The flavonoid patterns are a better indicator of the quality of the product than free amino acid content. The establishment of parameters and the development of a marker of quality might facilitate the inclusion of honeybee pollen on the European Union Codex.

The major objectives of our investigation were (1) to develop a simple and rapid method for the separation, characterization, and quantitation of free flavonoid aglycon compounds using HLPC and (2) to determine the total polyphenols and flavonoids using spectrophotometric procedures and to standardize the principal free flavonoid aglycon component as the best identifier of a quality parameter in honeybee-collected pollen produced in Spain.

MATERIALS AND METHODS

Samples. Eleven honeybee-collected pollen samples were harvested by beekeepers from western Spain (Extremadura and Salamanca) during the two flower production months in 1997 and 1998. The samples had been dried at 40 °C for 110 min (weight density = 20 kp/cm^2; pollen spread in 2.5 cm layers) according to the method of Serra Bonvehí and Escolà Jordà (*15*). This process is always done no later than 24 h after the pollen has been collected from the hive. Pollen pellets were kept in a pollen trap for no longer than 48 h. Because many samples were identical in pollen composition (80−85% *Cistus ladaniferus*), we selected 11 samples from the different batches that represented the main production of the honeybee pollen in the country. For each parameter the mean of two replicates was taken as the variation limit.

Reagents and Reference Standards. Solvents were of analytical (Panreac, Barcelona, Spain) and HPLC (Merck, Darmstadt, Germany) grade. Ultrapure water (Milli-Q, Millipore Corp., Bedford, MA) was prepared for chromatographic use. Syringic, vanillic, and coumaric acids were obtained from Sigma Chemical Co. (St. Louis, MO). Kaempferol, quercetin, rutin, protocatechuic acid, myricetin, and 4-hydroxybenzoic acid ethyl ester were obtained from Carl Roth GmbH and Co. (Karlsruhe, Germany). *trans*-Cinnamic acid and chrysin were found at Fluka Chemika (Buchs, Switzerland). Isorhamnetin, 3,4-dihydroxybenzoic acid, was obtained from Extrasynthèse (Genay, France).

Instrumentation. A Hitachi model UV-2000 double-beam spectrophotometer with 1 cm quartz absorption cells was used for all measurements. HPLC-UV was carried out on an HPLC system consisting of a Waters 600 pump, a 715 autoinjector, a 996 photodiode array detector (DAD), and a 712 WISP rheodyne valve loop injector fitted onto a 20 μL loop (all from Waters Chromatography Division, Milford, MA).

The system was equipped with a Compaq Deskpro Pentium II computer and Waters Millennium software, version 2.2 for data processing, and was operated at ambient temperature.

Melissopalynological Analysis. Analyses were carried out in accordance with the International Commission of Bee Botany (ICBB) of the International Union of Biological Sciences (IUBS); methods are described in Louveaux et al. (*17*). Ten grams of pollen was mixed in 50 mL of water for 15 min. A 10 mL fraction of the resulting solution was further diluted with 30 mL of water before pollen analysis was begun (*18*). Pollen grain counts were done following the method suggested by Vergeron (*19*) using a microscope.

Physicochemical Analyses. *Total Polyphenols and Tannins*. The amounts of total polyphenols and tannins were determined using a modification of the method described by Marigo (*20*), which is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. The sample (5 g) of finely ground pollen was extracted by shaking with 75 mL of 70% aqueous methanol (v/v) for 45 min. After filtration through a Büchner funnel, the residue was washed and the final volume was diluted to 100 mL with 70% methanol. Phenols in the extract were determined with Folin−Ciocalteu reagent (RFC). A blank was prepared by dissolving polyvinylpolypyrrolidone (PVPP) (Polyclar AT, Carlo Erba, Milano, Italy) in 10 mL of this solution at pH 3.5. PVPP was introduced as an adsorbent for phenolic compounds (*21*). Absorbance was read at 760 nm, and phenols were determined using a calibration curve for 5, 25, 50, 100, 150, and 200 mg/kg of gallic acid (Fluka). Tannin contents were determined by the difference of absorbances, by precipitating an aliquot of the extract (10 mL) at pH 4 using 1% NaCl in 10% gelatin solution.

Flavonoids. The total flavonoid content was estimated in 5 g of finely gound pollen. One milliliter of 0.5% hexamethyltetramine (w/v), 20 mL of acetone, and 2 mL of 0.10 N HCl were added to the sample and set to boil with reflux for 30 min. The resulting solution was filtered, the volume was leveled to 100 mL with acetone, and the residue was washed with 20 mL of acetone. Ten milliliters of the extract was put into a separation funnel, along with 20 mL of H_2O and 25 mL of ethyl acetate. Extraction with ethyl acetate was carried out three times. The extract was washed twice, using 50 mL of H_2O each time, and was subsequently made up to 100 mL with ethyl acetate. The total flavonoid content was determined in 10 mL of the extract using 1 mL of 2% $AlCl_3$ in methanol solution (5% acetic acid in methanol) according to the method of the *German Pharmacopoeia* (*22*). Absorbance was read at 425 nm, and flavonoid percentage was estimated using calibration curves at 8, 16, 24, and 32 mg/kg of rutin.

Free Flavonoid Aglycon Spectrum. HPLC analysis of free aglycon was performed according to the method of Amiot et al. (*23*). The sample (5 g) of finely ground pollen was dissolved in 25 mL of ethyl acetate; then 12.5 mL of 40% $(NH_4)_2SO_4$ and 2.50 mL of 20% HPO_3 were added, and the flask was stirred for 20 min. The extract was filtered through a Büchner funnel. The solution was poured into a separation funnel, the top phase was collected, and the extraction process was repeated. The organic phases were collected into a 100 mL flask and then vacuum-dried at a maximum of 40 °C. The sample was redissolved in 4 mL of methanol, filtered through 0.45 μm mesh nylon (Lida Manufacture Corp., Kenosha, WI), and leveled to 5 mL. HPLC was performed following these steps: Nucleosil C_{18} column (10 μm) (4.6 mm i.d. × 250 mm) (Machery-Nagel, Duren, Germany); DAD at 278−282 and 278−350 nm; mobile phase a, bidistilled water, pH 2.6 (with H_3PO_4); and mobile phase b, methanol; flow rate, 2 mL/min; 0% methanol to 100% methanol in 33 min of linear gradient; loop, 20 μL. The column was reequilibrated for 10 min. Phenolic compound quantitation was achieved by the absorbance relative to external standards.

Calibration curves were constructed by linear regression of the peak area against the concentration of the calibration standard (vanillic acid, syringic acid, and isorhamnetin, 5−20 mg/kg; *p*-coumaric, *o*-coumaric, 4-hydroxybenzoic acid ethyl ester, *trans*-cinnamic acid, protocatechuic acid, myricetin, and kaempferol, 5−100 mg/kg; quercetin, 25−250 mg/kg; rutin, 100−1000 mg/kg). All calibration curves were linear when forced through the origin, with correlation coefficients close to 1: 0.988−0.999. Solutions containing the target standards or extracts were always protected from light to avoid degradation. Every week the stability of the components in methanol was checked spectrophotometrically at 375 nm (flavonols) and 340 nm (flavones). Standard solutions proved to be stable between 1 and 3 months at 4 °C.

Table 1. Absolute Recoveries of Rutin and Quercetin from Spiked Samples

	rutin			quercetin		
product	added (mg/kg)	found (mg/kg)	recovery (%)	added (mg/kg)	found (mg/kg)	recovery (%)
honeybee-collected pollen	200.7	171.4	85.4	30.2	26	86.1
	259.3	223	86	59.6	50.7	85.1
	298.7	249.7	83.6	90.3	77.1	85.4

Table 2. Analytical Relative Standard Deviation (RSD) of Rutin and Quercetin

rutin			quercetin		
expected (mg/kg)	mg/kg	RSD (%)	expected (mg/kg)	mg/kg	RSD (%)
Intraday					
200.6	174.8 ± 11.3	6.47	100.3	87.8 ± 5.56	6.33
199.7	173.4 ± 10.8	6.23	99.5	86.5 ± 5.36	6.20
200.1	173.8 ± 11.6	6.67	99.3	86.6 ± 5.53	6.39
Interday					
200.6	170.3 ± 13.7	8.05	100.3	84.5 ± 6.89	8.15
199.7	170.6 ± 13.4	7.86	99.5	83.4 ± 7.02	8.42
200.1	169.4 ± 13.8	8.15	99.3	82.9 ± 7.24	8.73

Limits of detection were determined by injecting 20 μL of a standard mixture. The limit of detection was defined as the amount of rutin that resulted in peak height 3 times the standard deviation of the baseline noise.

Recovery Study. To verify the accuracy and relative standard deviation of the analytical procedures, recovery studies were carried out by spiking selected samples of honeybee pollen with the addition of various standards. The spiked samples as well as the unspiked controls were analyzed in duplicate. Recoveries were calculated from the differences in the total amount of standard between the spiked and unspiked samples, respectively (Table 1).

Repeatability and Reproducibility in Total Phenol and Flavonoid Contents. Within-run repeatability of the methods was assessed by analyzing five samples in 1 day. Five duplicate analyses were carried out on separate days within a period of 2 months to determine between-run reproducibility (Table 2).

Phenolic Compound Identification. The different phenolic compounds were identified by their UV spectra, which had been recorded with a DAD coupled to the HPLC, bathochromic movement of band I (320−380 nm) and band II (240−280 nm) using hydroxylation, methylation, and metallic complexes in accordance with the method of Marham (*24*), as well as cochromatography with pertinent markers.The compound confirmation procedure requires peak purity testing and spectrum matching. The Millenium PDA software uses the spectral contrast technique to compare spectra. Combining spectrum matching and purity testing results in compound confirmation. Peaks were considered to be pure when there was exact correspondence among the spectra (peak purity match >99). Peaks were identified by comparing their retention times (time window of 0.5%) with the retention times of pure standards. A DAD was used to confirm peak identity and to check peak purity. Peak identity was confirmed by superimposing the spectrum of each peak with the corresponding standard (peak identity match >99) (Table 3).

Water Content. A Karl Fisher titration model DL178 (Mettler-Toledo GmbH, Greifensee, Switzerland) was preferred for its accuracy above any other method. The reagent used was Titrant U (Merck 9233) with a pyridine-free methanolic solvent (Merck 9241) according to the method of Serra Bonvehí and Martí Casanova (*25*).

Water Activity (A_w). This parameter was determined at 25 ± 0.02 °C with a Thermoconstanter instrument (Humidat-TH2, Novasina, Switzerland).

Distribution of Free Amino Acids. Two grams of pulverized pollen was vortexed in 40 mL of water distilled for 1 h and centrifuged at 10000 rpm (12062*g*) for 15 min at 4 °C. The supernatant volume was poured into a 50-mL volumetric flask and made up to volume with distilled water; 0.5 mL of a mixture including 0.2 mL of norleucine solution (39 mg in 100 mL of 0.01 N HCl), 0.5 mL of 0.1 M Na_2CO_3 (pH 10.5), 0.2 mL of dansyl chloride solution (2 g/100 mL in acetone), and 1 mL of acetone was placed in a glycerine bath at 100 °C for 2 min. It was left to cool and settle in order to proceed with HPLC in accordance with the method of Serra Bonvehí and Escolà Jordà (*15*).

Table 3. Identification of Flavonoids: Relative (RRT) and Absolute (RT) Retention Times (Minutes)

component	RT	V_{max}	V_{min}	RRT	V_{max}	V_{min}
1 3,4-dihydroxy-benzoic acid	11.32	12.58	11.18	0.45	0.49	0.44
2 vanillic acid	19.87	19.91	19.53	0.78	0.78	0.77
3 syringic acid	20.53	20.63	20.45	0.80	0.81	0.80
4 *p*-coumaric acid	23.01	23.17	22.81	0.90	0.91	0.89
5 *o*-coumaric acid	24.71	24.76	24.63	0.97	0.98	0.96
6 rutin	25.50	25.55	25.40	1.00		
7 unknown 1	26.55	26.65	26.50	1.04	1.05	1.03
8 unknown 2	26.89	26.99	26.79	1.05	1.06	1.04
9 4-hydroxybenzoic acid ethyl ester	27.25	27.29	27.16	1.07	1.08	1.07
10 *trans*-cinnamic acid	27.90	27.96	27.77	1.09	1.10	1.09
11 quercetin	28.52	28.56	28.44	1.12	1.12	1.12
12 protocatechuic acid	28.76	28.83	28.70	1.13	1.13	1.12
13 myricetin	29.23	29.27	29.18	1.15	1.15	1.14
14 kaempferol	30.37	30.90	30.24	1.19	1.19	1.18
15 isorhamnetin	36.56	36.56	33.73	1.33	1.43	1.32

Sugar Spectrum. The extraction of sugars was carried out on a sample of 2 g of pulverized pollen using mechanical shaking in ~40 mL of standard solution plus an oximation reagent (1 g of xylose and 15 g of hydroxylamine hydrochloride in 250 mL of pyridine) and warming the solution slightly. Trimethylsilyloxime derivatives were obtained in order to proceed with gas chromatography in accordance with the method of Serra Bonvehí and Bosch Callís (*26*). The assessment of the procedure repeatibility has been previoulsy described for mono-, di-, and trisaccharides.

Statistical Analyses. Analysis of variance was performed using an ANOVA program of Statgraphics Statistical packet, version 6.0 (*27*), on the data set to determine the polyphenol content, flavonoid levels, and flavonoid components that varied significantly with the samples analyzed. Significant differences among and by analytical procedural means were determined by Duncan's multiple-range test. Group differences were considered statistically significant at a level of $p \leq 0.05$.

RESULTS AND DISCUSSION

On the basis of the melissopalynological analyses, the samples were classified as monofloral pollen (*Cistus ladaniferus*), with presence of *Echium plantagineum*, *Quercus* sp., Compositae, Papaveraceae, *Plantago* sp., and *Erica lusitanica* such as is frequent in pollens, which are in agreement with the findings of Serra Bonvehí and Escolà Jordà (*15*). The water content and water activity are at an adequate level for good storage stability of the honeybee pollen. The water content determines microbiological and sensorial qualities and also the shelf life of this product. Except for sample 4 a minimum free amino acid content of 2 g/100 g was obtained, indicating an acceptable degree of freshness and correct dryness of these samples (*15*). A deficient amount of free amino acid in honeybee pollen was considered as evidence of overheating during the arti-

Table 4. Polyphenols, Flavonoids, and Free Amino Acid Content (Grams per 100 g)[a]

	sample												
compound	1	2	3	4	5	6	7	8	9	10	11	mean	SD
total phenols	1.25	1.00	1.16	0.87	1.43	1.29	1.35	1.21	1.29	1.05	1.46	1.24	0.20
non-tannins	1.25	1.00	1.16	0.87	1.43	1.29	1.35	1.21	1.29	1.05	1.46	1.24	0.20
tannins													
total flavonoids[a]	0.38	0.78	0.64	0.35	0.53	0.51	0.40	0.35	0.76	0.40	0.48	0.51	0.16
flavonoids/phenols	30.4	78	55.2	40.2	37.1	39.5	29.6	28.9	58.9	38.1	32.9	42.6	15.3
total free amino acid	3.42	3.02	2.56	3.12	1.79	3.61	2.08	2.24	2.91	2.95	2.08	2.71	0.60
proline	2.23	2.03	1.78	2	1.56	2.30	1.63	1.66	2.01	2.02	1.72	1.90	0.25
proline/total (%)	65.2	67.1	69.4	64.2	86.9	63.8	78.3	74.3	69.2	68.4	82.7	71.8	7.79

[a] Spectrophotometric method.

Table 5. Free Flavonoid Aglycons (HPLC) (Milligrams per 100 g)[a]

	sample												
component	1	2	3	4	5	6	7	8	9	10	11	mean	SD
3,4-dihydroxybenzoic[a]	5.66	0.11	0.52	0.22	3.63	3.07	3.51	0.25	3.49	2.10	0.38	2.09	1.90
vanillic acid				0.08		0.10							
syringic acid	0.67	0.48	0.79	0.57	0.36	0.25	0.30	0.51	0.27	0.50	0.43	0.47	0.17
p-coumaric acid	1.41	0.94				12.42	4.59	0.95	5.26	1.15	0.85	2.51	3.74
o-coumaric acid	1.83	1.02	0.81	0.67	2.61	1.88	1.66	2.94	1.50	1.29	2.00	1.66	0.71
rutin	39.03	30.76	26.24	32.77	18.35	44.45	21.16	23.48	30.47	30.87	21.16	29	7.98
unknown 1	6.39	3.90	2.96	4.94	2.85	2.80	2.76	2.92	2.77	3.46	4.11	3.62	1.16
unknown 2	8.36	6.19	4.36	5.51	3.73	4.48	4.62	10.32	4.78	5.57	11.36	6.30	2.57
4-hydroxybenzoic acid[b]	4.15	2.52	2.14	2.29	2.45	2.47	2.31	2.87	2.65	2.63	3.37	2.71	0.58
trans-cinnamic acid	2.79	1.11	1.28	1.59	2.53	4.16	3.98	2.59	6.77	2.02	4.33	3.01	1.68
quercetin	6.50	4.69	4.87	3.63	7.13	9.75	6.23	6.26	7.25	6.25	10.04	6.6	1.96
protocatechuic acid	2.08	1.06	0.27	0.34	2.29	1.72	1.85	2.88	1.83	1.54	5.74	1.96	1.48
myricetin	1.96			13.64	3.01	4.92	4.00	2.87	3.35	2.08	0.87	3.34	3.76
kaempferol	0.91	0.82	0.71	0.83	0.92	1.68	1.14	1.28	0.93	1.57	1.49	1.12	0.34
isorhamnetin	0.67	0.67	0.78	0.72	0.68	0.67	0.68	0.67	0.67	0.67	0.64	0.68	0.04
total	82.41	54.27	45.73	67.80	50.54	94.82	58.79	60.79	71.99	61.70	66.77	65.05	14.2

[a] 3,4-Dihydroxybenzoic acid. [b] 4-Hydroxybenzoic acid ethyl ester.

ficial drying or packaging process and implies loss of nutritional quality. The ratio of proline to total free amino acid can be used as an indicator of the age of the pollen. Except for samples 5, 7, and 11 the index values obtained are typical of both correctly processed and fresh honeybee pollens (<75%) (Table 4).

To compare the compositions of the products, the polyphenol (1.24 ± 0.20 g/100 g) and flavonoid contents (0.51 ± 0.16 g/100 g) were determined using spectrophotometric procedures. The polyphenols were not identified as tannins in the samples. Sixty-four percent of the samples showed a minimum content of polyphenolic, not lower than 1.20 g/100 g. The honeybee segregates α/β-glucosidase during pollen collecting, causing partial enzymatic hydrolysis of glycosides to free aglycons. Free flavonoid aglycons were identified and quantified by HPLC, showing an average of 65.05 ± 14.2 mg/100 g (Table 5). Without chemical hydrolysis the following free sugars can be identified by gas chromatography: glucose, fructose, and sucrose; lower percentages of the disaccharides trehalose, isomaltose, and maltose and of the trisaccharides raffinose, erlose, and melezitose are present (*15*). Once honeybee pollen has been hydrolyzed [1.2 M HCl, 90 °C in 50% aqueous methanol (v/v), and a reaction period of 60 min] (*14*), other sugars are detected (e.g., rhamnose) and the percentage of flavonoid compounds is much greater (>600 mg/100 g) in comparison with natural free aglycons (<95 mg/100 g) (Table 5).

Plant pollen contains an important amount of flavonol glycosides, which are located on the surface of the exine (*28*). It is supposed that the higher percentage of flavonoid glycosides and the proportion of flavones with vicinal hydroxyl groups detected in the honeybee pollen will be the difference between the chromatographic and spectrophotometric values obtained in natural free aglycons and total flavonoid content. On the other hand, the HPLC profile of the phenolic compounds did not permit discrimination between hydrolyzed honeybee pollen samples and those without chemical hydrolysis. We cannot distinguish the specific plant origin of honeybee pollen. According to the results obtained (Tables 4 and 5), the free flavonoid aglycon analysis can also be used for defining the quality of honeybee pollen. These results are consistent with previously published data on the flavonoid composition of honeybee-collected pollen in Spain (*29*).

Detection at wavelengths of 275 and 350 nm is a specific and sensitive method to determine the majority of flavonoid aglycons and glycosides. Detection limits of standards oscillate between 1.4 and 1.9 mg/kg in honeybee pollen. Comparable detection limits were reported by Hertog et al. (*14*). The recovery of flavonoids was quantified as rutin and quercetin. An acceptable recovery range for the levels determined is 83−86% (Table 1), meeting the requirements of the AOAC *Peer-Verified Methods Program* (*30*). Analytical relative standard deviations were <6.67% (intraday) and <8.15% (interday) for rutin and were <6.39% (intraday) and <8.73% (interday) for quercetin (Table 2).

However, the purpose of this study was not to maximize the sensitivity of the HPLC method, which is sufficient in routine analyses. Polyphenol oxidase is an enzyme that is widely distributed in plants and which catalyzes the oxidation and polymerization of phenol components to brown pigments. Extraction with ethyl acetate and methanol reduces these enzymatic activities. Prolonging the extraction time from 30 to 45

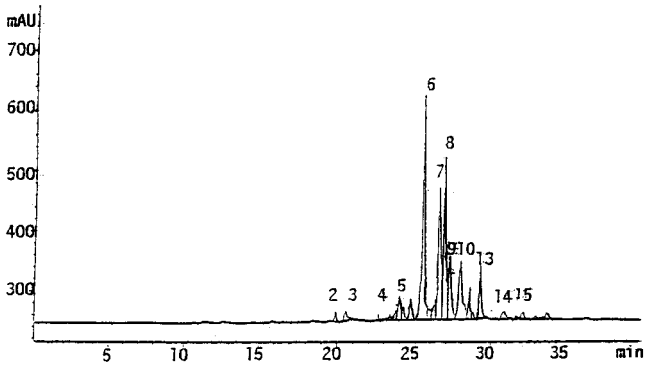

Figure 1. HPLC free flavonoid aglycon profiles of honeybee pollen.

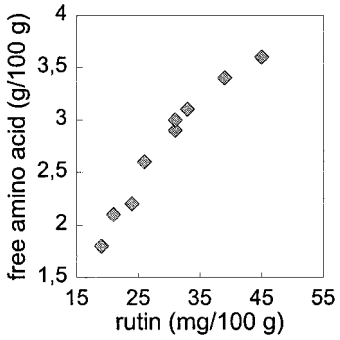

Figure 2. Correlation between total free amino acid and rutin contents.

min or increasing the amount of sample from 3 to 5 g did not affect extraction efficiency and did not degrade the components. Analyses of total polyphenolics are high in comparison with other foods (*31*).

On the basis of these results, free flavonoid aglycons were related to the quality of honeybee, and the minimum values of these compounds could be used as objective specifications for acceptance of the product. In recent studies, polyphenols, tannins, flavonoids, catechins, and (−)-epicatechin were critical factors in defining the quality of cocoa used in chocolate manufacture, fruit, vegetables, and processed foods (*32*−*34*). The HPLC profile of the natural free aglycons present in honeybee pollen indicated the presence of 15 components with similar qualitative compositions. Table 3 shows the detected chromatographic peaks in elution order, average relative retention (RRT), absolute retention time (RT), and name attributed to each identified compound. Of those 15, we were able to identify 13 using the described methods. The following compounds were identified: (1) benzoic acid derivatives (C_6-C_1) (3,4-dihydroxybenzoic acid, protocatechuic acid, 4-hydroxybenzoic acid ethyl ester, vanillic acid, syringic acid); (2) hydroxycinnamic acids (C_6-C_3) (*p*-coumaric acid, *o*-coumaric acid, *trans*-cinnamic acid); (3) flavonols and flavones ($C_6-C_3-C_6$) (rutin, quercetin, kaempferol, myricetin, isorhamnetin) (Figure 1). Rutin (quercetin rutinoside) was the most abundant component of free flavonoid aglycons. The qualitative compositions of the 11 samples were similar, containing mainly flavonols. Variance analysis showed significant difference ($p \leq 0.05$) among polyphenol, flavonoid, and compound contents. Among the phenolic compounds identified, rutin showed the highest $ROO^{\bullet}$-scavenging activity on a molar basis and protective effect on photosensitized lysis of human erythrocytes (*8*, *35*, *36*).

In general, the flavonols, commonly found flavonoids from leaves, seeds, and fruits, were good antioxidants. Phenolic acids (hydroxycinnamic and hydroxybenzoic) present in honeybee pollen, with the exception of *p*-coumaric acid, are also very good antioxidants. Their activity increases when the number of *o*-dihydroxy groups increases (*37*). Determination of rutin can be a means of determining the quality of honeybee pollen. Unusually low values may indicate long periods of storage or excessive heating applied in the drying process. The amount of rutin detected in Spanish honeybee pollen is high (29 ± 7.98 mg/100 g), varying from 18.35 to 44.45 mg/100 g. From a correlation of the results of total free amino acid with rutin content, a coefficient of $r^2 = 0.96$ was obtained (Figure 2). Regression analysis showed that there was a lineal correlation between these measurements, indicating that honeybee pollen quality can be adequately evaluated from biological and nutritional points of view. As a consequence, a minimum quantity of 20 mg/100 g rutin is suggested to standardize the commercial Spanish honeybee-collected pollen in the European market. The quality of honeybee pollen is strongly dependent on its preservation, revealing that the nutritional and biological properties can vary according to the quality of the product.

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