Nutrient Composition and Microbiological Quality of Honeybee-Collected Pollen in Spain

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The aim of this study was to determine the composition and microbiological quality of honeybee-collected pollen from various botanical and geographical origins of Spain and to define the following physicochemical and microbiological characteristics: water content, water activity (A_w), mean pollen pellet size, protein content, fat content, fatty acid composition, free amino acid distribution, sugar spectrum, mineral elements, dietary fiber, starch content, aflatoxins, and microbiological parameters. The free amino acid spectrum showed a high level of proline (63.1%) of the total free amino acid content ($x = 31.6 \pm 4$ mg/g). The main sugars identified by gas chromatography were fructose, glucose, and sucrose, with small amounts of di- and trisaccharides. A high level of dietary fiber was detected ($x = 13.7 \pm 1.3$ g/100 g). The main fatty acids detected corresponded to $C_{18:2}$, $C_{18:3}$, and $C_{18:1}$ acids. The mineral elements showed a predominance of potassium, phosphorus, calcium, and magnesium. The pollen of *Cistus ladaniferus* was detected as the dominant pollen. Among the microbiological parameters, a high number of molds, total aerobic counts, and the presence of coliforms and *Streptococci* "*D*" *of Lancefield* were found. Aflatoxins were not detected.

Keywords: *Honeybee-collected pollen; composition; quality; microbiology; aflatoxins*

INTRODUCTION

Among the European Union countries, Spain is one of the most important honeybee-collected pollen producer, with a production that is increasing constantly. Honeybees use pollen as their nutritional source of protein, fatty acids, lipids, sterols, vitamins, minerals, and certain carbohydrates. Honeybee-collected pollen can be considered as a potential source of energy and proteins for human consumption (Schmidt and Schmidt, 1984; Abreu, 1992; Block et al., 1994). Each pollen has specific characteristics linked to the floral species or cultivars (Serra Bonvehí, 1988), but the quality of the end product also depends on the cleaning, drying, and packaging processes currently applied by beekeepers to achieve longer storage life. The water content of the product determines microbiological and organoleptic qualities and also its shelf life (Serra Bonvehí, 1987; Serra Bonvehí et al., 1987a,b; Jodral et al., 1992; Collin et al., 1995). The water content ranges of 4−8 g/100 g allowed for the distribution of pollen as a processed food, and to achieve it, pollen is submitted to artificial drying processes (Vicente Pascual, 1988; Serra Bonvehí and López Alegret, 1986). This paper reports some of the studies carried out in order to improve pollen quality (Serra Bonvehí 1987, 1988; Serra Bonvehí and Martí Casanova, 1987; Simal et al., 1988; Muniategui et al., 1989, 1991a,b; Serra Bonvehí et al., 1985, 1986, 1991). The establishment of parameters of quality might facilitate the inclusion of pollen on the EU Codex Alimentarius. This paper contributes to the choice of criteria and setting of the legal limits necessary for a European normative [since 1976 it has only been quoted in the Spanish (BOE, 1976)].

MATERIALS AND METHODS

Samples. Twenty honeybee pollen samples were harvested by beekeepers from western Spain (Extremadura and Salamanca) during the two flower production months in 1993 and 1994. Pollen pellets were kept in a pollen trap for no more than 48 h. For commercial production pollen is dried by artificial heating. The initial water content of the pollen is low (14−18 g/100 g), and the drying process is not continuous. To assess the weight losses for each period of the process, a drying curve is plotted after the final water content has been determined (4−8 g/100 g) (Vincente Pascual, 1988). Using this curve, the producer carries out the industrial drying process: initial water content, 16.68 g/100 g; pollen spread in 2.5-cm layers; weight density, 20 kg/cm^2; air temperature, 40 °C; air flow value, 3586 kg$_{spread}$/h·m^2; total drying time, 105 min; final water content achieved, 7.65 g/100 g. This process is always done no more than 24 h after the pollen has been collected from the hive (Serra Bonvehí et al., 1987b). Since many samples were identical in pollen composition (80% *Cistus ladaniferus*), we selected 20 which included the total diversity of the honeybee-collected pollen in the country. For each parameter the mean of three replicates was taken as the variation limits.

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 described in Louveaux et al. (1978). 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 proceeding with pollen analysis (Serra Bonvehí, 1988). Pollen grain counts were done following the method suggested by Vergeron (1964) using a microscope.

Physicochemical and Microbiological Analyses. *Mean Pollen Pellet Size (MPS).* Approximately 50 g of honeybee pollen was sifted for 5 min through standard ASTM sieves for pollen pellet separation (4.76-, 4-, 3.36-, 2-, and 1.41-mm mesh; size 20.5-cm diameter) using an ASTM sonic sifter. The mechanical pulsing action was utilized (Laboratory Sieving Machines: Type Vibro, Retsch GmbH & Co., Haan, Germany). The amount of material retained on each sieve was weighed, and the mean pollen pellet size was determined as described by Enser (1970).

Water Content. The initial water content is low and reduced by the drying process. This parameter determines microbiological and organoleptic qualities and also the product shelf life. Karl Fisher titration (Mettler DL18) was preferred for its accuracy to any other method. The reagent used was

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Titrant U (Merck 9233) with a pyridine-free methanolic solvent (Merck 9241) according to Serra Bonvehí and Martí Casanova (1987).

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

Protein. The nitrogen content (protein + amino acids) was determined by conventional acid hydrolysis and Kjeldahl digestion using a copper catalyst in 2 g of pulverized and dried pollen. The ammonia was distilled and collected in a solution of boric acid which was then titrated against standard acid. Digestion and distillation were carried out using the Kjeltec apparatus (Model 1002, Tecator, Sweden). Protein content was calculated as total N × 5.60 (Rabie et al., 1983).

Total Fat Content. Two grams of pulverized pollen was extracted with petroleum ether (40−60 °C) for 4 h using a Soxhlet apparatus. Fat was determined as the difference in weight of dried samples before and after extraction.

Fatty Acid Composition. Pollen oil was weighed for capillary chromatography (0.10 g). Transesterification was carried out in 10 mL of 0.02 N sodium methylate and heated under reflux for 5−10 min until a single phase was obtained. This phase was neutralized with 1.3 N HCl in methanol, and the acids were extracted with 3−4 mL of hexane and transferred into a flask containing a saturated solution of NaCl. Capillary chromatography was performed on a Hewlett-Packard 5680 GC system with an SP-2300 capillary column (30-m × 0.25-mm i.d. with 0.2-μm particle diameter), FID, and 3393A HP integrator. Injector temperature was 210 °C; detector temperature was 220 °C; column temperature was held at 180 °C; carrier gas (He) flow rate was 1−1.5 mL/min; carrier make-up gas (N_2) was 60 mL/min, and splitter vent flow was 60−70 mL/min; injection quantity was 1 μL (Serra Bonvehí and Ventura Coll, 1993). Identification of the fatty acids was accomplished by comparison of the retention times with standards chromatographed the same day. The data were expressed as percentages of each acid.

Sugar Spectrum. The extraction of sugars was carried out on a sample of 2 g of pulverized pollen using mechanical agitation in about 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 then obtained as follows: 1 mL of the extractant solution was mixed with 1 mL of 1,1,1,3,3,3-hexamethyldisilazane and 0.1 mL of trifluoroacetic acid in a Teflon tube with a screw-on top. It was placed in a glycerine bath at 60−70 °C for 1 h. It was left to cool and settle in order to proceed with gas chromatography in accordance with Serra Bonvehí and Bosch Callís (1989). The assessment of the technique repeatability has been previously described for mono-, di-, and trisaccharides.

Starch. The starch percentage was analyzed using the UV method (Boehringer Mannheim, cat. no. 207748). About 0.5 g of fat-free pollen was weighed. The starch was solubilized using 20 mL of dimethyl sulfoxide and 5 mL of 37% hydrochloric acid, incubated at 60 °C for 30 min. The pH was adjusted to between 4 and 5 in a 100-mL volumetric flask, made to volume with double-distilled water. We used 0.1 mL of the solution to carry out the analysis.

Dietary Fiber. The method used to determine total dietary fiber was adapted from the method proposed by Englyst and Hudson (1987). The Englyst procedure measures dietary fiber as non-starch polysaccharides (NSP). Starch is completely removed enzymatically, after solubilization, and NSP is measured as the sum of constituent sugars released by acid hydrolysis. Sugars were measured by colorimetry in 200−250 mg of pulverized (<200 μm) and fat-free pollen using Englyst Fiberzym Kit (Novo Nordisk Bioindustries, U.K. Ltd., cat. no. 736721). A value is obtained for total dietary fiber (TFD) and, if required, for soluble and insoluble dietary fiber (SDF and IDF). The actual values of repeatability and reproducibility obtained by the Englyst method are considered to be satisfactory for this particular determination (Englyst et al., 1987).

Free Amino Acid. Two grams of pulverized pollen was vortexed in 40 mL of water, distilled for 1 h, and centrifuged at 10 000 rpm (12 062 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 (Serra Bonvehí et al., 1991). HPLC was performed following these steps: Nucleosil C_{18} column (10 μm) (4.6-mm i.d. × 250 mm); photodiode array detector at 250 nm; solvents (a) buffer solution of 0.0185 M K_2HPO_4, pH 7 (with H_3PO_4), and (b) acetonitrile; flow rate, 2 mL/min; 10−42% acetonitrile in 40 min of linear gradient; loop, 20 μL. HPLC−UV was carried out on a HPLC system consisting of Model 590 Waters Associate LC pumping units, a Model 712 WISP valve loop injector fitted onto a 20-μL loop, and a Waters Associate Model 996 photodiode array detector. Chromatographic data from HPLC and UV were processed on NEC 486/66i computing integrators.

Mineral Elements. Mineral salt was determined by incineration of 2 g of pulverized pollen at 500−550 °C to constant weight. Metals were determined by dissolution with 2 mL of 2 N HCl, made up to 50 mL with distilled water at 40 °C, and analyzed using inductively coupled argon plasma−optical emission spectrometer (ICP−OES). The ICP−OES used for the study was a sequential multielement analyzer (Varian Model Liberty 220). The samples were diluted to provide concentration in the proper emission range (repeatability range, 2.2−10%).

Phosphorus. Phosphorus was analyzed colorimetrically using the Mission reaction in 1 g of incinerated pollen. The vanadium phosphomolybdate yellow-orange complex was read at 400 nm. The standards were prepared using KH_2PO_4 (AOAC, 1984) (repeatability = 0.726 + 0.067*x*; *x*, mean).

Aflatoxins. Aflatoxin was extracted from 50 g of pulverized honeybee pollen in 100 mL of 80% methanol, blended for 5 min, and filtered through Whatman no. 41 paper. A 100-μL sample from clear or yellow supernatant was taken to determine the aflatoxins by an enzyme-linked immunoassay (ELISA) using Alfa-10 Cup Test Kit (International Diagnostic Systems Corp., MI) (El-Nakib et al., 1981).

Aerobic Colony Counts. Ten grams of pollen was dissolved in 90 mL of 0.1% sterile peptone water (Difco-1807, Difco Laboratories, Detroit, MI); 0.1 mL of the solution was poured into each of two Petri plates, and 10−15 mL of plate count agar (Difco-0479), prepared as directed by the manufacturer, was then added. When solidified, plates were incubated at 30 °C for 72 h, and then bacterial colonies were recorded.

Coliforms. One milliliter of the solution used in the aerobic colony count determination was poured into each of two Petri plates, and 8−10 mL of violet red bile agar (Difco-0012), prepared as directed by the manufacturer, was then added. When solidified, a second layer of culture media (8−10 mL) was added. When solidified, plates were incubated at 37 °C for 48 h, and then coliform colonies were recorded.

Escherichia coli. Ten grams of pollen was dissolved in 90 mL of 0.1% sterile peptone water (Difco-1807); 1 mL of the solution was poured into each of two Petri plates, and 10−15 mL of plate count agar (Difco-0479), prepared as directed by the manufacturer, was then added. When solidified, plates were incubated at 44.5 °C for 24 h. Colonies were recorded and identified according to biochemical criteria (API System).

Streptococcus "D" of Lancefield. One milliliter of the solution used in the aerobic colony count determination was poured into each of two Petri Plates, and 10−15 mL of KF-*Streptococcus* agar (Difco-0496), prepared as directed by the manufacturer, was then added. When solidified, plates were incubated at 37 °C for 48 h (Kenner et al., 1961). Colonies were recorded and identified.

Clostridia. Ten milliliters of the solution used in the aerobic colony count was heated at 80 °C for 10 min. Then, 1 mL of the solution was poured into each of two Petri plates, and 10−15 mL of SPS-agar (Difco-0845), prepared as directed by the manufacturer, was then added. When solidified, plates were incubated in anaerobic conditions at 37 °C for 48 h. Colonies were recorded and identified.

Table 1. Physicochemical Characterization and Composition[a]

sample no.	W	A_w	pollen pellet size (mm)(%) 3−4.76	2−3.6	1.41−2	powder	N × 5.6	F	ash	DF	IDF	SDF	starch
1	5.44	0.267	14.85	76.27	5.76	2.93	15.2	5.85	1.8	14.8	12.1	2.66	2.21
2	4.95	0.271	14.44	79.50	3.98	1.89	13.6	6.40	1.97	13	10.8	2.21	1.92
3	4.84	0.263	24.92	67.75	3.97	2.98	16.2	5.40	1.63	12.4	9.8	2.60	2.43
4	4.83	0.269	33.76	59.16	3.09	3.49	17.5	5.30	1.98	15.9	12.2	3.66	1.90
5	4.56	0.278	27.34	66.65	3.65	2.17	14.7	5.69	1.88	14.2	11.5	2.70	1.79
6	5.05	0.261	31.62	64.05	3.55	0.59	15.4	5.94	1.90	14.7	12.3	2.35	2.12
7	4.83	0.264	25.31	67.95	4.30	2.44	18.2	5.72	1.97	13.2	10.8	2.38	2.36
8	4.70	0.269	17.37	73.50	4.43	4.52	14.3	6.22	1.88	13.8	12.1	1.79	1.87
9	4.70	0.274	35.43	56.92	3.44	3.96	13.5	5.97	1.69	12.9	10.8	2.06	2.12
10	4.90	0.270	11.78	76.06	5.02	7.14	14.3	7.08	2.03	15.2	13.1	2.13	2
11	5.87	0.263	17.21	71.58	4.64	6.57	16.9	5.34	2.20	14.2	11.6	2.56	1.83
12	4.70	0.264	37.46	54.70	2.83	4.35	14.1	5.44	1.89	13.3	10.6	2.66	2.40
13	4.85	0.278	42.47	49.70	2.70	5.04	15.8	5.90	2.05	10.6	9	1.59	1.88
14	4.68	0.267	32.35	62.27	2.94	2.35	14.7	7.18	1.85	12.6	10.2	2.39	2.01
15	5.05	0.280	31.79	58.80	3.47	5.69	17.2	4.80	1.87	13.8	11.6	2.21	2.37
16	4.98	0.273	28.18	66.59	2.89	2.25	13.7	5.90	2.14	15.1	12.8	2.27	1.99
17	4.78	0.265	31.79	62.17	3.52	2.52	12.6	5.40	2	15.5	11.9	3.57	2.38
18	5.05	0.261	37.91	58.22	2.98	0.59	17.8	6.16	2.13	12.4	10.7	1.74	2.41
19	4.86	0.264	42.11	54.25	2.21	1.34	15	5.80	1.77	14.4	11.8	2.59	2.63
20	4.91	0.265	35.83	61.35	2.05	0.68	15.7	6.65	2.05	12.9	10.6	2.32	1.98
x	4.93	0.268	28.70	64.37	3.57	3.17	15.3	5.91	1.93	13.7	11.3	2.42	2.13
SD	0.29	0.006	9.36	8.14	0.93	1.91	1.6	0.59	0.15	1.3	1	0.51	0.25
V_{max}	5.87	0.280	42.47	79.50	5.76	7.14	18.2	7.18	2.20	15.9	13.1	3.66	2.63
V_{min}	4.68	0.261	11.78	49.70	2.05	0.59	12.6	4.80	1.63	10.6	9	1.59	1.79

[a] Key: W, water content (g/100 g); A_w, water activity; F, fat content (g/100 g); DF, dietary fiber (g/100 g); IDF, insoluble dietary fiber (g/100 g); SDF, soluble dietary fiber (g/100 g); N × 5.6, ash, and starch (g/100 g).

Bacillus. Ten milliliters of the solution used in the aerobic colony count was heated at 80 °C for 10 min. Then, 1 mL of the solution was poured into each of two Petri plates, and 10−15 mL of plate count agar (Difco-1807), prepared as directed by the manufacturer, was then added. When solidified, plates were incubated at 37 °C for 48 h. Colonies were recorded and identified.

Yeast and Molds. A 0.1-mL aliquot of the solution used in the aerobic colony count determination was poured into each of three Petri plates, and 10−12 mL of Sabouraud agar modified (Difco-0747), prepared as directed by the manufacturer, was then added. When solidified, plates were incubated at 22 °C for 5 days. Colonies were recorded and identified.

Statistical Analysis. Data were processed by analysis of variance (one-way ANOVA, using the LSD test with 95% confidence limit) using Statgraphics (Statistical Graphics System Co., Portland, OR, 1992).

RESULTS AND DISCUSSION

In the 20 samples, 52 different pollen types were identified. Approximately 15% of the identified taxons appear as predominant pollen (D) (>45%), secondary pollen (S) (16−45%), and important minor (s) (3−15%). Among the most frequent pollens, *Cistus ladaniferus* (80%), *Echium plantagineum*, *Quercus* sp., *Erica lusitanica*, *Helianthemum* sp., and *Cistus* sp. were predominant (>95%) depending on the geographical origin and the presence of *Compositae*, *Eucalyptus* sp., *Plantago* sp., and *Papaveraceae*. The results obtained coincide with those of Serra Bonvehí (1988). On the basis of the melissopalynological analyses, the samples were classified as monofloral *Cistus* pollen. The results of the physicochemical analysis of the samples are reported in Table 1. The size range of pollen pellets oscillates between 2 and 4.76 mm, with a mean range of 2−3.36 mm. The water content and water activity are in the acceptable range for a good storage stability of the honeybee pollen.

Table 2 shows data from fatty acid composition. The ratio of unsaturated/saturated fatty acid was 1.96. Diet proposals tend to reduce the total amount of fats and cholesterol and to reach a relation of unsaturated/saturated fatty acids greater than 1. Due to the ratio unsaturated/saturated fatty acids found in pollen, its addition to processed food can improve the nutritional quality of the manufactured food. These results agree with the hypothesis that bees select those pollens with a high level of unsaturated fatty acids, which are more adequate for bee metabolism (Battaglini and Bosi, 1968). The lipids in the extracts consist mainly of $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ acids which cover the pollen grain surface as a thin layer (Table 2). Discrepancies appear with values found by other researchers (Battaglini and Bosi, 1968; Loper et al., 1980), particularly in the contents of $C_{18:1}$ and $C_{18:2}$. These differences are due to the floral origin of the pollen samples. In contrast, the total fat weight average ($x = 5.91 \pm 0.59$ g/100 g) was similar to that of pollens from different floral and geographical origins (Muniategui et al., 1989). The oil extract from the fat showed a yellow-brown color, which corresponds with the predominance of *C. ladaniferus* (Serra Bonvehí, 1988). Besides fats and fatty acids, ether extracts may contain certain vitamins, pigments, higher alcohols, waxes, sterols, and saturated hydrocarbons (Serra Bonvehí et al., 1986; Loper et al., 1980; Muniategui et al., 1991a,b).

The analytical data for sugars are reported in Table 3. In terms of the sugar fraction, the most revelant result is the high concentration of reducing sugars. In most pollen, carbohydrates constitute a major dry matter fraction and vary with species. The carbohydrate group includes such substances as sugars, starch, and dietary fiber. Differences have been found between pure hand-collected pollen (1−37 g/100 g) and honeybee-collected pollen (20−48 g/100 g) (Maurizio, 1954). Angiosperm pollen collected from bees is richer in reducing sugars and generally poorer in nonreducing sugars than pollen isolated directly from the plant. The mean reducing sugar content was 32.9 g/100 g, and that of sucrose was 6.12 g/100 g (Table 3). This agrees with the results of Solberg and Remedios (1980). Thus honeybee pollen always contains large amounts of reducing sugars, due to the presence of honey or nectar

Table 2. Fatty Acid Composition (%)

sample no.	C_8	C_{10}	C_{12}	C_{14}	$C_{14:1}$	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	C_{20}	C_{22}
1	0.50	1.09	2.92	2.73	1.24	24.5	3.32	0.67	14.9	26.5	19.4	0.25	1.39
2	0.65	2.03	3.38	3.35	0.39	26.2	1.12	1.27	10.3	28.1	19.2	0.78	2.55
3	1.59	0.89	2.73	3.54	0.30	22.1	2.07	1.40	9.1	33.3	19.7	1.04	1.87
4	1.81	0.71	2.16	2.84	0.21	21.5	3.20	1.82	9.3	28.7	24.6	0.78	1.49
5	1.61	0.72	2.12	2.56	0.25	23.1	4.40	2.18	9.9	26.3	24.1	0.85	1.24
6	0.35	2.86	1.78	2.01	1.11	18.7	3.17	1.68	9.9	29.8	25.3	0.8	1.90
7	0.34	2.92	1.85	1.53	1.01	19.1	4.20	2.11	9.6	30.6	24.3	0.67	0.95
8	0.67	0.48	0.87	0.56	0.33	18.5	1.03	1.92	16.1	41.6	16.3	0.18	0.91
9	0.47	3.06	1.42	1.46	0.67	25	4.11	2.99	12	32.8	14.8	0.14	0.34
10	0.30	0.96	3.47	2.60	0.10	21.1	2.30	1.37	19	29.8	17.5	0.41	0.11
11	0.51	0.76	2.30	3.00	0.58	22.8	3.11	1.48	11.4	32.7	19.8	0.48	0.74
12	0.61	1.24	1.50	2.55	0.53	25.7	2.70	1.56	12.1	33.7	16.9	0.15	0.33
13	0.23	2.13	1.38	2.15	0.42	18.7	4.45	1.38	10.8	35.5	19.9	0.70	1.72
14	0.20	5.31	2.74	3.13	0.18	33.7	3.44	2.21	18.6	10.6	16.6	0.58	2.19
15	0.38	0.95	2.05	1.32	1.30	21.5	3.58	1.30	10.1	36.1	19.5	0.14	1.08
16	0.52	2.97	3.52	3.06	0.18	25.3	0.27	0.35	8.7	35.7	18.1	0.15	0.92
17	0.31	0.51	1.86	1.92	0.40	25.5	0.41	2.4	8.9	37.1	18.7	0.21	0.96
18	0.14	1.76	2.52	4.05	0.37	21.3	0.10	2.43	18.6	35.4	11.7	0.14	0.61
19	0.27	2.95	2.18	1.83	1.71	18.8	5.05	1.99	15.7	30.3	17.2	0.68	0.75
20	0.40	0.89	1.68	2.37	1.45	21.1	4.1	4	12.1	31.6	18.3	0.54	0.76
x	0.59	1.76	2.21	2.43	0.64	22.7	2.81	1.83	12.4	31.3	19.1	0.48	1.14
SD	0.49	1.25	0.74	0.85	0.49	3.6	1.51	0.80	3.5	6.2	3.4	0.30	0.65
V_{max}	1.81	5.31	3.52	4.05	1.71	33.7	5.05	4	19	41.6	25.3	1.04	2.55
V_{min}	0.14	0.48	0.87	0.56	0.10	18.5	0.10	0.35	8.7	10.6	11.7	0.14	0.11

Table 3. Carbohydrates (g/100 g)[a]

sample no.	Fru	Glc	S	Tre	Iso	Mal	Raf	Erl	Mel	total
1	15.20	10.86	7.80	0.28	0.22	1.36	0.21	0.30	0.27	36.5
2	15.90	12.00	4.46	0.26	0.15	1.32	0.12	0.19	0.22	34.6
3	15.89	12.33	7.42	0.18	0.10	3.20	0.15	0.13	0.16	39.6
4	16.25	11.92	5.34	0.23	0.10	1.04	0.24	0.11	0.12	35.4
5	15.74	12.47	6.21	0.25	0.11	2.88	0.13	0.12	0.25	38.2
6	15.80	12.05	5.40	0.22	0.10	0.79	0.11	0.10	0.14	34.7
7	16.10	12.21	4.92	0.13	0.11	0.87	0.21	0.14	0.11	34.8
8	17.20	13.00	5.34	0.29	0.11	1.52	0.15	0.11	0.10	37.8
9	21.10	17.90	4.90	0.10	0.32	2.20	0.12	0.13	0.10	46.9
10	18.10	13.80	9.40	0.17	0.26	2.04	0.10	0.11	0.12	44.2
11	16.40	11.70	4.90	0.16	0.13	1.10	0.11	0.12	0.13	34.8
12	15.80	12.10	5.40	0.22	0.19	0.80	0.11	0.12	0.11	34.9
13	20.90	17.10	5.00	0.29	0.32	2.08	0.23	0.10	0.24	46.3
14	16.60	12.60	7.10	0.13	0.18	1.41	0.10	0.11	0.17	38.4
15	18.30	13.90	9.40	0.17	0.26	1.90	0.16	0.18	0.15	44.4
16	22.40	17.90	5.10	0.31	0.30	2.10	0.18	0.23	0.29	48.8
17	17.90	15.80	6.70	0.39	0.60	2.80	0.13	0.14	0.11	44.6
18	17.20	13.60	7.40	0.25	0.51	2.50	0.13	0.21	0.21	42
19	20.20	13.20	4.20	0.17	0.12	1.00	0.11	0.16	0.18	39.3
20	17.90	13.10	6.00	0.13	0.21	0.98	0.16	0.19	0.21	38.9
x	17.54	13.48	6.12	0.22	0.22	1.69	0.15	0.15	0.17	39.8
SD	2.08	2.07	1.53	0.07	0.14	0.75	0.04	0.05	0.06	4.6
V_{max}	22.40	17.90	9.40	0.39	0.60	3.20	0.24	0.30	0.29	48.8
V_{min}	15.20	10.86	4.20	0.10	0.10	0.79	0.10	0.10	0.10	34.6

[a] Key: Fru, fructose; Glc, glucose; S, sucrose; Tre, trehalose; Iso, isomaltose; Mal, maltose; Raf, raffinose; Erl, erlose; Mel, melezitose.

in the fluid cementing the pollen pellets together (Stanley and Linskens, 1974). As Table 3 shows, fructose, glucose, and sucrose are the free sugars found in the highest concentration in honeybee pollen, and lower percentages of the disaccharides trehalose, isomaltose, and maltose and the trisaccharides raffinose, erlose, and melezitose are present. A high level of fructose is found in the sugar spectrum, with the fructose/glucose (F/G) index varying from 1.13 to 1.53. Starch content of pollen is highly variable (Solberg and Remedios, 1980) and was found to be 2.13 g/100 g (Table 3).

The dietary fiber is an important parameter to be analyzed in any food, especially when considering that its deficiency constitutes a health hazard for developed societies (Periago et al., 1993; Stamler, 1994). The method used in this study (Englyst) is cheap, easy to use, and fast. The analysis of dietary fiber is limited to the determination of non-starch polysaccharides (NSP) and does not include lignin and sporopollenin. The results of the determination of total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) are shown in Table 1. The total amount of TDF is higher for honeybee pollen. The average TDF values range from 10.6 to 15.9 g/100 g, in agreement with the findings of Abreu (1992). The results of the determination of the IDF show that polysaccharides (cellulose and callose) constitute the most important fraction ($x = 11.3 \pm 1$ g/100 g) of the dietary fiber. These results show that the pollen fiber is in the correct amount and variety (soluble fiber/insoluble fiber) needed for good health and has suitable properties that could complement the soluble dietary fiber fraction (usually deficient) present in manufactured foods. The honeybee pollen is characterized by a high protein content. The N $\times$ 5.6 content oscillates between 12.6 and 18.2 g/100 g, with an average content of 15.3 g/100 g (Table 1). The main bulk of pollen nitrogen is in the protein fraction,

Table 4. Free Amino Acid Composition (mg/g)[a]

sample no.	Asp	Glu	Aspa	Glut	Ser	Thr	Gly	Ala	Arg	Pro	Val	Met	Ile	Leu	Phe	Lys	His	Tyr	total	pro/total
1	0.31	0.26	0.43	0.14	0.29	0.14	0.12	1.06	3.89	20.58	0.27	0.49	0.48	0.59	0.43	0.40	0.61	0.45	30.9	66.5
2	0.53	0.33	0.57	0.26	0.55	0.35	0.29	1.38	5.37	18.56	0.81	0.22	0.77	1.54	1.00	1.01	0.69	1.07	35.3	52.6
3	0.54	0.35	0.56	0.26	0.53	0.36	0.28	1.38	5.37	18.55	0.80	0.21	0.76	1.57	1.03	1.01	0.71	1.03	35.3	52.6
4	0.53	0.33	0.57	0.27	0.53	0.35	0.29	1.34	5.28	18.67	0.79	0.22	0.68	1.50	0.98	0.97	0.64	0.97	34.9	53.5
5	0.58	0.55	0.64	0.31	0.61	0.40	0.33	1.52	4.89	19.89	0.95	0.25	0.78	1.78	1.02	1.15	0.72	1.25	37.6	52.9
6	0.58	0.48	0.59	0.28	0.57	0.38	0.28	1.48	4.97	20.05	0.79	0.31	0.56	0.86	0.78	0.85	0.58	0.86	35.3	56.9
7	0.54	0.40	0.61	0.27	0.56	0.37	0.29	1.39	5.24	19.10	0.82	0.25	0.70	1.54	0.92	1.04	0.69	1.02	35.8	53.4
8	0.28	0.30	0.69	0.19	0.43	0.21	0.70	0.70	4.30	19.94	0.46	0.17	0.43	0.78	0.60	0.59	0.83	0.59	32.2	61.9
9	0.20	0.14	0.31	0.09	0.24	0.11	0.08	0.48	1.00	21.96	0.30	0.22	0.18	0.48	0.46	0.37	0.76	0.37	27.8	79.1
10	0.25	0.13	0.30	0.10	0.25	0.10	0.09	0.49	1.14	23.60	0.34	0.21	0.21	0.54	0.50	0.48	0.69	0.44	29.9	79
11	0.27	0.17	0.39	0.11	0.29	0.13	0.09	0.53	1.16	22.58	0.36	0.25	0.23	0.59	0.56	0.49	0.91	0.43	29.5	76.4
12	0.17	0.12	0.53	0.40	0.46	0.40	0.28	0.84	3.78	18.79	0.37	0.21	0.77	1.29	1.10	0.16	0.16	0.62	30.5	61.7
13	0.13	0.09	0.72	0.20	0.25	0.14	0.09	0.44	1.42	16.31	0.42	0.18	0.35	0.40	0.49	0.37	0.63	0.32	23	71.1
14	0.11	0.08	0.55	0.25	0.34	0.16	0.12	0.49	2.21	17.30	0.52	0.16	0.32	0.53	0.55	0.42	0.56	0.40	25	69.3
15	0.09	0.07	0.32	0.25	0.29	0.15	0.11	0.41	3.86	16.56	0.23	0.28	0.27	0.44	0.43	0.35	0.49	0.47	25.1	66.1
16	0.26	0.20	0.50	0.15	0.30	0.15	0.14	1.07	3.89	19.85	0.34	0.11	0.35	0.78	0.44	0.49	0.59	0.51	30.1	65.9
17	0.30	0.31	0.48	0.15	0.30	0.15	0.14	1.06	4.89	20.85	0.32	0.15	0.39	0.82	0.58	0.62	0.72	0.61	32.8	63.5
18	0.30	0.21	0.51	0.14	0.34	0.12	0.11	0.99	5.10	18.56	0.28	0.21	0.71	1.54	1.00	1.06	0.69	1.07	32.9	56.3
19	0.23	0.33	0.48	0.16	0.43	0.18	0.21	1.21	3.89	21.10	0.32	0.28	0.48	1.45	1.08	0.96	0.54	1.05	34.4	61.4
20	0.36	0.31	0.48	0.18	0.37	0.21	0.18	1.18	4.16	20.56	0.45	0.23	0.65	1.37	0.87	0.79	0.47	0.49	33.3	61.7
x	0.33	0.26	0.51	0.21	0.40	0.23	0.21	0.97	3.79	19.67	0.50	0.23	0.50	1.02	0.74	0.68	0.63	0.68	31.6	63.1
SD	0.16	0.13	0.12	0.08	0.12	0.11	0.14	0.39	1.54	1.88	0.23	0.08	0.21	0.48	0.26	0.30	0.15	0.30	4	8.6
V_{max}	0.58	0.55	0.72	0.40	0.61	0.40	0.70	1.52	5.37	23.60	0.95	0.49	0.78	1.78	1.10	1.15	0.91	1.25	37.6	79.1
V_{min}	0.09	0.07	0.30	0.09	0.24	0.10	0.08	0.41	1.00	16.31	0.23	0.11	0.18	0.40	0.43	0.16	0.16	0.32	23	52.6

[a] Key: Asp, aspartic acid; Glu, glutamic acid; Aspa, asparagine; Glut, glutamine; Ser, serine; Thr, threonine; Gly, glycine; Ala, alanine; Arg, arginie; Pro, proline; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Lys, lysine; His, histidine; Tyr, tyrosine.

the second most abundant group of compounds after the carbohydrates. The analysis of both hand- and bee-collected pollens shows that in general the total amount of crude protein from various plant species varies considerably (Stanley and Linskens, 1974).

Free amino acid composition appears in Table 4, with values expressed in mg/g of honeybee pollen. Eighteen amino acids were present, the most abundant being proline. *S*-Methylmethionine was not identified with this procedure (Dethier et al., 1991). Since pollen is dried at low temperature for a short period of time, Maillard compounds were not produced. Only when the drying process is carried out at 60 °C/2 h or at 40 °C/1 week does free degradation of *S*-methylmethionine result in dimethyl sulfide (undesirable flavor) (Collin et al., 1995). We observed high amounts of various essential amino acids (methionine, phenylalanine, arginine, and histidine), with cystine in low quantities. A comparison of the free amino acid profiles between the samples showed that levels of many of the individual amino acids were similar. However we detected significant variations of the total free amino acid content among the samples (Table 4). The basic amino acids (i.e., histidine, lysine, and arginine) constituted 5.74–19.76% of the total amino acid content. Honeybee pollen is a source of free amino acids, and for this reason its use in the human diet should be considered. The free amino acid distribution shows a high predominance of proline (above 19.7 mg/g, reaching a level as high as 63.1% of the total free amino acid content), already reported by Dillon and Louveaux (1987) and Serra Bonvehí et al. (1991). The composition of the free amino acid profile in honeybee pollen is influenced by external and internal factors. Among the former there are the drying processes (heating conditions) and the pollen storage. The quality of honeybee pollen is strongly dependent on its preservation. On the other hand, the floral composition is the most important internal factor. Published reports provide no evidence for any significant addition of proline to pollen by bees when they pack it into comb cells (Standifer et al., 1980). As Table 4 shows, Spanish honeybee pollen presented percentages of free amino acid significantly different ($P \leq 0.05$) from other floral honeybee pollen (Bosi and Ricciardelli d'Albore, 1975; Standifer et al., 1980; Collin et al., 1995), indicating that the nutritional properties can very according to the floral origin. Proline is the predominant free amino acid in pollen which has been well dried and stored, and glutamic acid is the predominant in freshly collected pollen. The level of proline formed from glutamic acid during the drying process is influenced by the presence and activity of the glutamate dehydrogenase (Britikov and Musatova, 1964; Pálfi et al., 1974; Stanley and Linskens, 1974; Gilliam et al., 1980; Serra Bonvehí et al., 1986). When the temperature and the drying process period are excessively high or long, the free amino acid content decreases (<2 g/100 g) and consequently the average of proline against the

Table 5. Mineral Elements (mg/kg)

sample no.	Fe	Ca	Zn	K	Na	Cu	Mg	P	Mn
1	57.5	448.6	19.5	4187	1714	9.6	475.8	672.5	10.3
2	41.9	268	18.8	2512	1908	7.2	358.7	829	8.6
3	63.2	317.4	22.8	3065	825	7.1	355.5	488.6	7.9
4	48.3	556.7	21.9	2485	1467	5.9	325.6	835	14.5
5	59.9	353.4	26.7	3191	898	7.8	425.5	448.7	18.3
6	11.1	850	44.8	5777	1603	14.6	500.5	390.9	10.5
7	41.6	372.1	47.2	4587	1502	9.5	375.6	539.9	9.1
8	25.1	540.2	57.4	4179	1113	10.7	380.1	548.2	8.7
9	53.4	510.5	24.6	4473	901	12.4	340.5	501.8	10.9
10	14.4	524.2	81.1	4878	1285	9.3	325	376.9	11.1
11	41.6	412.8	29.2	5304	1194	8.9	273	294.7	12.7
12	53.6	516.9	33.3	4630	702	5.4	285.6	447.4	15.3
13	37.4	608.7	35.2	4370	1883	5.8	375.6	537	9.1
14	20.6	750.9	43.1	6411	1258	5.9	415.5	584	8.6
15	80.3	252.9	41.1	4087	1895	10.4	635.2	688.3	10.2
16	56.1	317.1	24.8	3715	866	4.2	485	617.6	10.9
17	22.1	618.8	25.6	4721	2375	5.5	615.8	610.8	14.5
18	26.2	749.2	26.1	3699	718	4.1	458.3	753.3	13.7
19	11.2	373.6	27.7	3838	614	15.7	815.7	854.4	7.4
20	18.8	483.2	27.2	2953	1299	13.2	410.7	612.5	8.7
x	39.2	491.3	33.9	4153	1301	8.7	432.2	581.6	11.1
SD	19.8	166	15.2	1026	487	3.4	132	157	2.9
V_{max}	80.3	850	81.1	6411	2375	15.7	815.7	854.4	18.3
V_{min}	11.1	252.9	18.8	2485	614	4.1	273	294.7	7.4

Table 6. Microbiological Quality[a]

sample no.	aerobic	coliform	*E. coli*	*Streptoc.*	*Clostrid.*	*Bacillus*	yeast	fungi	aflatoxins
1	9000			15		5	500	5000	<5
2	3000	3		85	2	7	500	1500	<5
3	2000			110		5	1500	2000	<5
4	4000			30		2	200	1450	<5
5	5000	3		50		7	200	2000	<5
6	2000			15		2	500	3350	<5
7	1900			35		2	400	2000	<5
8	910	7		5		5		3000	<5
9	400			30		7		600	<5
10	200			10		2		100	<5
11	3550	9		200	10	5	3000	3000	<5
12	2850			15		2	400	2250	<5
13	3750	23		290		12		5500	<5
14	30000	4		550	2	10	1000	4500	<5
15	2800	9		5		5	500	1600	<5
16	3400	3		30		2	500	3000	<5
17	540					5	150	550	<5
18	1650	4				2	1000	5000	<5
19	1350	9		85		10	200	2450	<5
20	7250	43		60		5		2000	<5

[a] Key: cfu/g; aflatoxins, μg/kg; *Streptoc.*, *Streptococcus "D" of Lancefield.*

total free amino acid increases (>80%). In contrast, when the drying process is correctly done to achieve a final water content of 4−8 g/100 g, the free amino acid content remains high (>2.5 g/100 g) (Table 4) and the average of proline against the total free amino acid is less than 65%. The conjunction of the process of biosynthesis of proline from the glutamic acid, the loss of 0.15 g/100 g/month of free amino acid content at room temperature (18−22 °C) in the dark (Serra Bonvehí et al., 1986), and the progressive loss of minor free amino acid identified in the pollen (Table 4) determine the progressive increase of the ratio of proline (>65%) in the total free amino acid content (>2 g/100 g) in old pollen (Serra Bonvehí et al., 1986). A minimum quantity of 2 g/100 g of free amino acid content is suggested to standardize the commercial honeybee-collected pollen in the European market. The ratio of proline/total free amino acid can be used as an indicator of the age of the pollen. The average proline index value is 64.6% and oscillates from 52.6% to 79.1% (Table 4). The index values obtained are typical of correctly processed honeybee pollens.

The main mineral component contents are reported in Table 5. The analyzed samples have a homogeneous composition, with low variation coefficients that only exceed 10% in a few elements like sodium and potassium. The mineral composition of pollen is dependent not only on the floral origin but also on the growing conditions (soil, geographical origin). The average mineral content of ashes is 1.93 g/100 g (Table 1). The mineral elements found are similar to those reported by Loper et al. (1980). The presence of nine chemical elements was determined, and potassium was predominant [(59% of the total) followed by P, Ca, Na, and Mg (39.9%)]. There were differences in the mineral content of bee- and hand-collected pollen. Only P, Ca, and Mg have similar values in both types of pollen (Stanley and Linskens, 1974). Zn was found in higher proportion in pollen that in other bee products. Both Fe and Zn suprass the 15% established for Recommended Dietary Allowance (RDA) (National Research Council, 1989). The presence of zinc, copper, and iron and a high potassium/sodium ratio make honeybee pollen interesting for diets with a defined electrolytic balance (Wesh and Marston, 1983; Stamler, 1994; Block et al., 1994). The sources of microorganisms present in honeybee pollen samples are both the natural habitat and human manipulation (Table 6) (Gilliam, 1979a,b; Serra Bonvehí and López Alegret, 1986). The natural habitat is characterized by low levels of water content, and as a result of that, the predominant microflora is constituted by yeast, molds, spore-forming bacteria and cocci (Table 6). The samples which have been dried quickly (samples 9, 10, and 17) show a low level of microorganisms because the low water availability suppresses microorganisms. Human manipulation increases the microorganism counts. A fast drying process also reduces the number of manipulation steps, thus maintaining a low level of streptococci and the absence of coliforms (Table 6). A high water activity increases the number of microorganisms in honeybee pollen. As a result there is a health hazard due not only to the microorganisms themselves but also to their metabolites, especially aflatoxins (Mossel, 1974; Jodral et al., 1992). To avoid this hazard the following steps must be followed: (a) pollen has to be collected no later than 48 h after the pollen trap has been installed in the hive (samples 9, 10, and 17); (b) the collected pollen must be dried until water activity (A_w) is reduced under 0.60 no later than 24 h after collection (no later than 72 h after installing the pollen trap in the hive). Therefore the traditional predrying process by the sun must be avoided, and the drying process must be done only indoors. If these instructions are observed and the manipulations are done with care, especially in terms of hygiene, no human pathogen will reach the product, and a natural food with its own microflora can be commercialized without health hazards. The natural microflora will remain constant in a number lower than 0.5×10^3 cfu/g in the case of molds and yeasts and inferior to 10 cfu/g in the case of streptococci and aerobic spore forms, coliforms and human pathogens must be absent, and the level of aflatoxins must be <5 μg/kg. Any circumstances, especially a high water activity (A_w) level, which increases the amount of microorganisms and/or their metabolites would involve a health hazard in the honeybee pollen itself or in the honeybee pollen-based manufactured food.

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