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Chemical, Nutritional and Bioactive Characterization of Colombian Bee-Bread

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"Bee bread" is a product of the hive obtained from pollen collected by bees, to which they added honey and digestive enzymes and subsequently stored in the combs, starting a lactic fermentation which gives it greater power conservation. A proper hive management promotes bee-bread collection, aimed at marketing it for human consumption since it can be considered as food supplement due to its content of a wide range of nutrients. One of the contributions to their high nutritional value is the presence of significant amounts of proteins, vitamins and phenolic compounds as natural antioxidants. The potential application of "bee bread" as a food and as a nutraceutical supplement depends in large part on its chemical composition which varies directly with the flora of the region and the time of collection by the bees. In this work, 15 samples of "bee bread" from the Colombian central region known as Cundiboyacense Highland were analysed. Physical-chemical analyses were moisture, ash, lipids, proteins and *in vitro* digestibility. On the other hand, the content of total flavonoids, phenolic compounds and antioxidant activity (ABTS and FRAP) were also measured. Bee-bread from this region had 15.7 ±3.6 g/100 g moisture content, and a following centesimal composition based on dry matter: ashes 2.4±0.2 g, lipids 3.4 ±1.1 g and proteins 23.1 ±2.9 g. *In vitro* digestibility had values of 79.1 ±16.0 g hydrolyzed protein/ 100 g total protein. The total content of flavonoids and phenolics showed values of 3.2 ±1.0 mg Quercetin/g bee-bread and 8.9±3.1 mg Gallic acid/g bee-bread, respectively. Antioxidant activity by FRAP and ABTS reported values of 46.1±13.0 and 61.5±10.2 µmol TROLOX/ g bee-bread. The antioxidant activity as measured by both techniques suggests a linear correlation between the levels of phenolic compounds. According to the results found, the "bee bread" is a product with high potential for use as a food supplement.

1. Introduction

There is currently a favourable market for foodstuff considered as natural by consumers which, in addition, could supply beneficial health effects. Particularly, beekeeping products such as honey or bee-pollen have been used in popular medicine and food diets due to their nutritional and physiological properties (Kroyer and Hegedus, 2001). In the hive, the nutrients needed to grow bees' colony populations and maintain their health come from nectar and pollen. Nectar provides carbohydrates and pollen supplies the remaining dietary requirements such as protein, lipids, vitamins, and minerals (DeGrandi-Hoffman et al., 2013). Nevertheless, bees do not consume either nectar or pollen directly; in both cases they induce biochemical processes, so nectar is transformed into honey and pollen into bee-bread (Krell, 1996).
In the case of bee-bread, until some years ago, beekeepers used to not collect it since the inherent difficulty of extraction that obligated the beekeeper to partially destroy the hive (Fuenmayor, 2009), and preferably they collected pollen through a well-designed systems of traps and containers (Almeida-Muradian et al., 2005), being this product intended to human consumption. However, several reports have shown how bee-bread has and increased availability of nutrients and bioactive components with respect to pollen (Fuenmayor, 2009; Del Risco et al., 2012). Currently, specialized materials and devices have been designed to extract bee-bread without any damage to the hive (Wilara, 2014).

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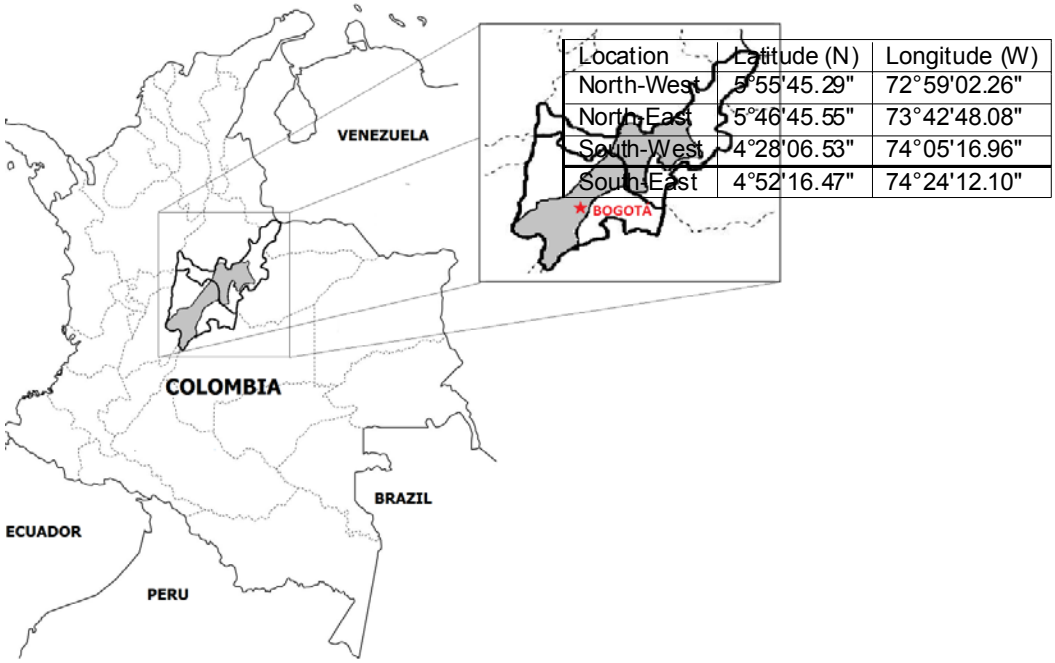
The process of obtaining bee-bread inside the hive occurs in a progression of appearance/disappearance of colonizing microorganisms, especially acid-lactic bacteria, according to the conditions of both the cell of the hive and the substrate (Del Risco et al., 2012). In addition, enzymes are segregated by bees through their saliva, inducing both fermentation and enzymatic processes. Previous investigations have shown that these biochemical transformations are needed to break the outer layer overlying the pollen known as exine, made of sporopollenin, a compound that provides chemical resistance to pollen and preserves the compounds which are within it, and responsible for the limited capacity for absorbing nutrients and bioactive substances that are inside the pollen grain (Atkin et al., 2011). Different simulations in vitro of human digestion suggest that the bee-pollen is partially digested -between 48 % and 59 %- (Campos et al., 2010), which has been used by some researchers as evidence of the strength of the outer wall of pollen, even to gastric acid (Rimpler, 2003).
On the other hand, in Colombia beekeepers have recognized the geographical advantages of the region known as the Cundiboyacense Highland (Figure 1), where about 90 % of the bee-pollen domestic production is concentrated (Martínez, 2006). This area presents yields per hive of near 40 kg of product / year (Martínez, 2006), compared to other countries that dominate most of the market such Spain, Portugal, China, Brazil or Argentina, which do not reach 15 kg/hive/y (Bogdanov, 2011). Bee-pollen nutritional and bioactive composition from Colombia has been reported, and results found show an extraordinary content of proteins and lipids (23.8 g/100 g bee-pollen and 6.9 g/ 100 g bee-pollen, respectively, in a dry basis), and also a great bioactive composition and antioxidant capacity, in comparison to bee-pollen worldwide (Fuenmayor et al., 2014). Given these conditions, this region can be also considered a potentially top bee-bread producer.
In this study, the objective was to characterize Colombian bee-bread, coming from the Colombian Cundiboyacense Highland, from a nutritional and functional point of view. As nutritional parameters, the content of moisture, ash, lipids and proteins were measured. On the other hand, the content of total flavonoids, phenolic compounds and antioxidant activity (ABTS and FRAP) were also quantified. Finally, an *in vitro* digestibility assay was performed in order to compare the availability of both bee-pollen and bee-bread proteins.



Location	Latitude (N)	Longitude (W)
North-West	5°55'45.29"	72°59'02.26"
North-East	5°46'45.55"	73°42'48.08"
South-West	4°28'06.53"	74°05'16.96"
South-East	4°52'16.47"	74°24'12.10"

Figure 1: Localization of the Colombian Cundiboyacense Highland (area shaded in gray). Source of coordinates: (IGAC-ORSTOM, 1984)

2. Materials and Methods

2.1 Samples

In this study, 15 bee-bread samples (bee species: Apis mellifera) were collected. These samples were removed from the cells of the hive, packed in polypropylene bags and stored in refrigeration at 4 °C, until physical-chemical analyses were performed. The samples were collected during years 2012 and 2013.

2.2 Nutritional compounds

Moisture. 3 g of sample was weighed and heated at 65 °C for 24 h. Moisture content was obtained by difference (Fuenmayor et al., 2014).
Ash. Ash determination was made through gravimetry after incineration in an oven at 600 °C until constant weight (AOAC 968.08) (AOAC, 2005).
Lipids. Lipids were determined by the Soxhlet method in which they were extracted from the food matrix by solvent drag; this latter is then separated from lipids by heating (AOAC 920.39) (AOAC, 2005).
Protein. It was determined following the Kjehldahl method with the Winkler variation. Boric acid was used for quantifying nitrogen content in the sample, applying an N x 6.25 conversion factor (AOAC 984.13) (AOAC, 2005).

2.3 Bioactive compounds and antioxidant activity

Preparation of ethanol extracts. About 1 g of sample was weighed into a 100 mL beaker and then 30 mL of ethanol (96% v/v) were added; beaker was covered and stirred at low speed for 24 h in darkness. Solution was filtered using 3hw filter paper and completed quantitatively to 100 mL with ethanol (96 % v/v).
Total flavonoids. 4 mL of distilled water, 0.3 mL of a 5 % $NaNO_2$ solution and 1 mL of extract were mixed. After five minutes 0.3 mL of a 10 % $AlCl_3$ solution was added, and, one minute later 2 mL of NaOH 1 M and 2,4 mL of distilled water are mixed. Final solution absorbance was read at 510 nm. Results are expressed as quercetin equivalents: mg eq-quercetin/ g bee-bread (Almaraz-Abarca et al., 2004).
Total phenolics (Folin-Ciocalteu). The total content of phenols was estimated according to Folin-Ciocalteu method with some modifications (Singleton et al., 1999), using gallic acid as standard. Absorbance was measured with a spectrophotometer JASCO Model V-530 UV / VIS, by using Spectra Manager software (Jasco, Italy), at 765 nm using water as the blank. The curve of gallic acid was plotted in a range of 0.2 to 1.0 mg / ml. The results were expressed as gallic acid equivalents: mg eq-gallic acid/g bee-bread (dry basis).
Trolox equivalent antioxidant activity (TEAC). The determination of the antioxidant activity towards the radical ABTS reaction was performed (Erel, 2004). The stock solution of ABTS radical cation was prepared by reacting a solution of the ABTS diammonium salt and a potassium persulfate solution. 1 mL of assay solution and 10 µL of extract of sample were mixed and the absorbance was read at 734 nm after 6 min. The degree of discoloration was calculated as the percentage reduction in absorbance, which was calculated in relation to the trolox equivalent concentration (0.2 - 2 mM). The results were expressed as µmol trolox / g bee-bread (dry basis).
Ferric reducing ability of plasma FRAP. The determination of the antioxidant activity towards the ferric radical complex-2,4,6-tripyridyl-s-triazine (TPTZ) reaction was performed (Benzie and Strain, 1996). 20µL of extract, 450 µL of FRAP solution (a mixture of a buffer acetate-acetic acid, TPTZ and $FeCl_3$ solutions) and 735 µL de distilled water were mixed and kept in darkness during 30 min at 40 °C. The results were expressed as µmol trolox/g bee-bread (dry basis).

2.4 *In vitro* digestibility

1.5 g of dried-defatted sample is mixed with 150 mL of a 0,002% pepsine in HCl 0.075 N solution and kept in agitation by 16 h at 45°C. Then, the content is filtered and the protein content of both the non-digestible and digestible portions are determined by Kjehldahl method. Digestibility will be the ratio among the protein content of the digestible part and the original protein content of the sample. Result is expressed as the g of protein digested/100 g bee-bread total protein (ICONTEC, 1994).

3. Results and Discussion

The bee-bread samples for this study were taken from the most important region for this apicultural activity in Colombia. This region is the Cundiboyacense high plateau which is located at an altitude higher than 2,500 m.a.s.l., on the central part of the Colombian eastern Andes. It consists of flat and high lands between the Cundinamarca and Boyacá departments, having a 15 °C mean temperature which can range from 0°C to 24

C. The Cundiboyacense high plateau has an Andes orobiome, characterized by the presence of mixed agricultural areas, pasture for dairy production, shrubland and damp moorshaving endemic vegetation in the highest parts (Chamorro-Garcia et al., 2013).
In this region a large number of plant species can be found simultaneously providing pollen for *Apis mellifera* bees. Previous studies revealed that *Melastomatacea* and *Fabaceae* (including its three sub-families), *Asteracea, Myrtaceae, Euphorbiacea* were the most important plant families providing pollen from Cundiboyacense Highland (Chamorro-Garcia et al., 2013). For the physicochemical results of Colombian bee-bread, the mean and standard deviation were calculated for all data. Table 1 presents average bee-pollen composition. On the other hand, Tables 2 and 3 shows results for average bioactive compounds and antioxidant activity and a comparison of values for digestibility among bee-pollen and bee-bread, respectively.

Table 1: Nutritional content of bee-bread

Moisture (%)		Ash (%)*		Lipids (%)*		Protein (%)*	
Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max
15.6 ± 3.6	7.8 - 19.1	2,45 ± 0,18	2.19 - 2.60	3,40 ± 1,08	1.65 - 5.50	23,1 ± 2,9	19.1 - 27.3

**Dry basis*

Table 2: Bioactive composition and antioxidant activity of bee-bread. Dry basis.

Total flavonoids (mg eq-quercetine/ g bee-bread)		Total phenolics (mg eq-gallic acid / g bee-bread)		FRAP (µmol trolox / g bee-bread)		TEAC (µmol trolox / g bee-bread)	
Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max
3.2 ± 1.0	1.9 - 4.5	8.9 ± 3.1	2.5 - 13.7	46.1 ± 13.0	35.0 - 70.1	61.5 ± 10.2	46.1 - 76.3

Table 3: Digestibility values for bee-pollen and bee-bread.

Bee-pollen digestibility (g protein digested / 100 g total protein)		Bee-bread digestibility (g protein digested / 100 g total protein)		t-student test p value
Mean ± SD	Min - Max	Mean ± SD	Min - Max	< 0.01
63.9 ± 16.2	38.7 - 85.3	79.1 ± 16.0	45.7 - 94.7	

Moisture in Colombian bee-bread varied from 7.8 to 19.1 g/100 g in the present study and had 15.6 (± 3.6) g/100 g mean value, this is a notorious lower value in comparison to bee-pollen, which usually is in the order of 32 % of moisture (Zuluaga, 2010). On the other hand, Bee-bread protein content is one of its most regarded nutritional features; this ranged from 19.1 to 27.3 g/100 g (dry basis) evaluated by using factor N x 6.25, having a 23.1 (± 2.9) g/100 g mean. It is expected that the content of protein in bee-bread be similar to bee-pollen (about 23.8 g/100 g) (Fuenmayor et al., 2014), since the biochemical process induced by bees is aimed at degrading the outer layer of the pollen grain, without any damage of inner content.
Total lipid content was highly variable and ranged from 1.65 to 5.50 g/100 g (mean 3.40 (± 1.08) g/100 g). The content of lipids is highly variable and dependent on the amount of fatty acids, carotenes and vitamins present in pollen. Mean ash content found for Colombian bee-bread was 2.45 (±0.18) g/100 g (dry basis) ranging from 2.19 to 2.60 g/100 g. These results agreed with previous findings for bee-pollen from the Cundiboyacense Highland (Fuenmayor et al., 2014), giving the ash content remain invariable along the process.

With regarding to bioactive compounds, total flavonoid content ranged from 1.9 to 4.5 mg eq-quercetine/ g bee-bread. Flavonoids are the secondary components of most importance in bee-bread. These compounds influence the visual appearance of the grain (pigmentation) and flavour (astringency and bitterness) [9], [10]. In pollen grains, most of flavonoids exist as glycosides, known as aglycones, being quercetin the major compound [11]. Although there is not a recommended daily ingest for flavonoids, it is suggested an intake of about 200 - 100 mg per day [12]. Average total flavonoid content in bee-pollen of this region has been established in 5.16 mg eq-quercetine/g bee-pollen (Zuluaga et al., 2014), a higher value than in bee-bread, due to possible differences in botanical origin of pollen and also the fact that a degradation of the outer layer of the grain makes more available bioactive compounds to degraded by environmental conditions.
Not only flavonoids, but phenolic compounds are considered among the largest contributors to the antioxidant potential of natural food products (Larson, 1988). Total phenolics content in bee-bread ranged from 2.5 to 13.7 mq eq-gallic acid / g bee-bread. The antioxidant activity of polyphenols is due to their redox properties, which play an important role in scavenging free radicals and oxygen species or decomposing peroxides (Nijveldt et

al., 2001); such properties are closely related to its chemical structure, especially with the number of hydroxyl groups on the aromatic ring and conjugated double bonds. In addition to their individual effects, antioxidant molecules interact synergistically so that one can protect other against oxidative destruction (Foti et al., 1996; Damintoti et al., 2005).

In addition, the main in vitro approach for assessing the antioxidant activity of a sample is through the determination of its ability to neutralize or scavenge free radicals, since anti-radical activities are very important in preventing spoilage of foods and biological systems (Gulcin et al., 2007). In this study, two techniques were used for estimating antioxidant activity under this approach: TEAC assay, which is based on the inhibition by antioxidants of the absorbance of radical cation ABTS•, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), and FRAP assay based on the reaction of ferric radical complex-2,4,6-tripyridyl-s-triazine, a relatively stable free radical capable of accepting an electron or hydrogen radical to become a stable molecule and therefore gets reduced in the presence of an antioxidant. In our study, FRAP and TEAC reported values ranged from 35.0 to 70.1 and from 46.1 to 76.3 µmol Trolox/ g bee-bread. In comparison to reported values for bee-pollen, 67.0 76.2 µmol Trolox/ g bee-pollen for FRAP and TEAC, respectively, it can be noticed the reduction in values, corroborating the loss of activity as it was found in total flavonoid analysis.

Finally, digestibility parameter have shown significant differences between the found value for bee-bread (79.1 g protein digested/100 g total protein) and bee-pollen (63.9 g protein digested/100 g total protein). Despite being an in vitro test, it reflects the variation in the structure of pollen and the greater availability of nutritional and bioactive compounds in bee-bread.

4. Conclusions

Physicochemical and functional characteristics of bee-bread from the Cundiboyacense Highland could play an important role for quality control of this product. Particularly, high levels of protein and lipids were found in analysis which have remained unaltered compared to bee-pollen. There is actually a loss in values of bioactive compounds for bee-bread, as compared to bee-pollen, possibly due to a breaking of the outer layer of the grain which makes more available bioactive compounds to be degraded by environmental conditions. Even so, it is important to establish, in further studies, if despite the breaking of the cell wall of the grain and the reduction on the content of bioactive compounds, a greater absorption of bioactive compounds in the human gastrointestinal tract can be achieved. High protein and lipids levels, combined with an appreciable content of bioactive compounds indicate the possibility of using bee-bread as a dietary supplement. Further analysis focused on in vivo tests for evaluating bioactive components and digestibility properties for fully characterizing Colombian bee-bread and differentiating them locally and from other varieties from around the world. It is expected that this work would be an important tool for cataloguing and recognizing Colombian bee-bread as being a beneficial source of natural nutrients.

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