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Bee-Pollen Structure Modification by Physical and Biotechnological Processing: Influence on the Availability of Nutrients and Bioactive Compounds

Carlos M. Zuluaga*[,a,b], Juan C. Serrato[b], Marta C. Quicazan[a]

[a]Instituto de Ciencia y Tecnología de Alimentos. Universidad Nacional de Colombia. Carrera 30 #45-03 Edificio 500C, Bogotá D.C., Colombia
[b]Departamento de Ingeniería Química y Ambiental. Facultad de Ingeniería. Universidad Nacional de Colombia. Carrera 30 #45-03 Edificio 453, Bogotá D.C., Colombia
cmzuluagad@unal.edu.co

Bee-pollen is recognized by its nutritional and bioactive value, related to protein, lipids, vitamins or polyphenols. However, reports indicate it is insufficiently digested once enters to the human digestive system, so compounds are not completely assimilated into the body. This is due to an outer layer highly resistant to degradation, known as exine. Because of this, bee-pollen can be considered as a raw product and it would require of processing in order to enhance bioavailability. In Colombia bee-pollen is a promising product, especially to be used to supply the protein needs in part of the population who cannot even cover their daily nutritional requirements or simply have a limited consumption of animal origin foodstuff. A physical treatment by employing temperature and pressure was first made in order to evaluate the effect on the exine. Subsequently, two independent treatments were carried out, one in which a fermentation by using commercial lactic acid bacteria was induced, and the other, by using protease enzymes to obtain a hydrolyzed product. Performed physical-chemical analyses were *in vitro* digestibility, total phenolics and antioxidant activity (ABTS and FRAP). Results showed that high temperature-pressure processing degraded the exine of bee-pollen, and then inner compounds were more available. Higher percentages for antioxidant activity (43 % for ABTS and 22 % for FRAP), total phenolics (11 %) and digestibility (55 %) were found after this procedure, in relation to raw bee-pollen. In addition, even higher values for fermentation were found in comparison to physical-treated bee-pollen, in particular digestibility (8%) and total phenolics (11 %); nevertheless, better results for hydrolyzated bee-pollen for both parameters (10 % and 33 %) were found. Transformed bee-pollen, specifically after thermal and enzymatic treatments, may be considered as a complete food due to its enhanced availability of nutritional and bioactive compounds with special regard to their already known nutritional-physiological implications.

1. Introduction

Pollen is the male gametophyte of flowers used as a mean for the reproduction of plants. Different insects, including bees, take advantage of pollen as a source of protein, fat, vitamins and minerals (Seeley, 2006). When bees visit flowers, they cover their bodies with pollen dust and form pellets with their saliva; they then attach the pellets to the corbiculae of their hinder legs to carry them to the hive and thus provide nutrients to their offspring (Campos et al., 2008). Although there are over 500 species of bees (Vit et al., 2013), the term “bee-pollen” is usually used for the product collected by species *Apis mellifera* L. Beekeepers have designed traps set in the hives to collect the excess of bee-pollen, and then, to undergo it to basic processes of suitability, especially those of drying and removal of impurities, before being offered on the market (Bogdanov, 2011).
In recent years, the recognition of bioactive and nutritional value of bee-pollen has promoted its incursion as food for humans (Campos et al., 2010). Among the biologically active substances that have been reported in bee-pollen, phenolic compounds, flavonoids and anthocyanins are found (Morais et al., 2011). In terms of

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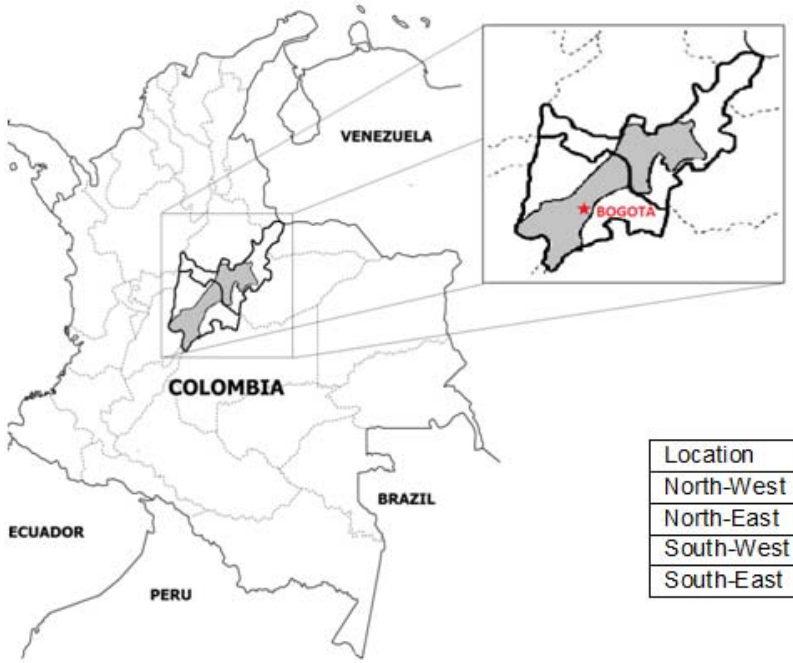
nutrition, bee-pollen has a protein content ranging from 20 and 25 %, lipids between 1 % and 10 %, and carbohydrates ranging from 20 % to 35 % (Del-Risco, 2002). However, regardless of the characteristics of this food, there are reports that show a reduced availability of nutrients and bioactive compounds once the bee-pollen is ingested. This is mainly due to the strong chemical structure that covers the outer layer of the grain (Bogdanov, 2011). The pollen cell walls consist of a series of stratified concentric layers. The outer wall is known as exine and is very flexible, elastic, strong and firm; it is made of sporopollenin, a compound that provides chemical resistance to pollen and preserves the compounds which are within it (Atkin et al., 2011). This suggests that the bee-pollen for human consumption must undergo transformation processes; one, as a strategy to increase its shelf life, and two, to improve the nutritional and functional quality indicators.
On the other hand, in Colombia beekeepers have recognized the geographical advantages of the region known as the Cundiboyacense Highland, where about 90 % of the bee-pollen domestic production is concentrated (Martinez, 2006). This area presents yields per hive of near 40 kg of product/y, 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). With respect to Colombian bee-pollen from the Cundiboyacense Highland, several reports have described its physicochemical composition (Fuenmayor et al., 2014). As for the botanical species, there is a tendency to a greater presence of dandelion (Hypochaeris radicata) and red clover (Trifolium pratense) pollen (Montoya, 2011).



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)

Even though there are plenty of studies that focus on the research of the characterization of physical-chemical properties of bee-pollen, there are actually few reports on potential treatments to suit it as a food with enhanced nutritional and bioactive value for humans. Consequently, the need to strengthen the beekeeping productive chain, particularly in aspects related to innovation and new product development, is clear (Martinez, 2006). The objective of this work was to develop alternatives of valorization of bee-pollen by employing transformation processes. Initially, a physical treatment by employing temperature and pressure was first made in order to evaluate the effect on the exine. Subsequently, two independent treatments were carried out, one in which a fermentation by using commercial lactic acid bacteria was induced, and the other, by using protease enzymes to obtain a hydrolyzed product.

2. Material and Methods

2.1 Bee-pollen

Bee-pollen samples were collected in the Colombian Cundiboyacense Highland region. Bee-pollen was stored in bags and kept in refrigeration until analyses.

2.2 Thermal treatments

Performed thermal treatments on bee-pollen consisted in several assays under conditions of temperature and pressure, carried out in an autoclave. In this experiment, time of treatment was evaluated at the times of 5 min, 10 min and 15 min, always at a temperature of 121 °C. All conditions were evaluated by triplicate.

2.3 Bee-pollen fermentation

This process was done once it was selected the best thermal treatment of those mentioned in numeral 2.2, based on exine degradation and minimum damage of bioactive compounds. For fermentation, a commercial culture CHOOZIT MY800 (Danisco, Denmark) was employed, which is a mixture of microorganisms *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Fermentation was done by employing a mixture bee-pollen:water in ratio 1:1. Prior to inoculation, the culture was activated in a 9 mL tube with MRS broth and left incubating for 24 h at 37 °C. Then, a massive cultivation was done in a petri dish with MRS agar and kept for 24 h at the same conditions. Finally, formed colonies in the agar were collected and introduced in a 90 mL tube with saline solution until reaching a turbid level comparable to a McFarland #5 standard, equivalent to a microorganism concentration of 10^8. An aliquot of 1 mL was taken and mixed with 9 mL of MRS broth and 1 g bee-pollen, and incubated by 48 h. Once concluded, the content of this mixture was used as inoculums. Fermentation process lasted 72 h at 37°C under anaerobic conditions.
At the end of fermentation, a viable lactic acid bacteria count was done. 11 g of fermented bee-pollen were taken and mixed with 100 mL of peptone water. From this mixture, dilutions were done by taking aliquots of 1 mL and passing them to 9 mL tubes of peptone water. Dilutions equivalent to CFU of 10^6, 10^7 and 10^8 were cultivated by immersion in MRS agar.

2.4 Bee-pollen enzimatic hydrolisis

Bee-pollen were added, suspended in one volume of distilled water, and homogenized (Ultra-Turrax, IKA-Werke, Germany), and pH of the suspension was adjusted at 7.0 using NaOH 0.01 N. The digestion was performed by addition of Protamex (Novozymes, Denmark) 1.5 AU/g at 37 °C with constant stirring at 200 RPM. Protamex is an endoprotease classified as serine peptidase, which was selected since it was very effective in preliminary assays done on bee-pollen. After 4 hours, hydrolysis was stopped by boiling for 2 minutes. The obtained hydrolysates were filtered and then dried at 40°C by 6 h.

2.5 Physicochemical analysis

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).
Preparation of ethanol extracts for total phenolics and antioxidant analysis. 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 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 735µL 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).

2.6 Statistical analysis

Data obtained from all treatments were compared for each variable by employing a one-way analysis of variance (ANOVA). In case of significant differences, a Tukey test was done at a 95% confidence level.

3. Results and Discussion

3.1 Thermal treatments

Samples of bee-pollen were subjected to thermal treatments in autoclave. In Table 1, the results of bioactive compounds and antioxidant activity are shown, for both treated and fresh bee-pollen.

Table 1: Results for bioactive compounds and antioxidant activity of bee-pollen. Dry basis.

	Fresh	5 min	10 min	15 min
Digestibility (g digested protein / 100 g total protein)	79.07 ± 6.39^{a}	79.40 ± 7.14^{ab}	83.45 ± 6.71^{ab}	86.80 ± 1.71^{b}
Total phenolics (meq gallic acid / g bee-pollen)	13.34 ± 3.61^{a}	16.34 ± 6.22^{ab}	20.35 ± 4.18^{b}	18.00 ± 5.40^{ab}
FRAP (µmol TROLOX / g bee-pollen)	75.71 ± 12.74^{b}	72.95 ± 10.80^{ab}	68.06 ± 12.53^{ab}	55.05 ± 20.26^{a}
TEAC (µmol TROLOX / g bee-pollen)	75.13 ± 16.82^{ab}	68.83 ± 17.17^{ab}	88.85 ± 18.04^{b}	66.26 ± 12.19^{a}

Different letters in a same row indicate significant differences

Although no significant differences in digestibility between fresh bee-pollen and treated at 5 and 10 min were found, a progressive increase in this variable respect to time in an autoclave at a temperature of 121 °C is observed, demonstrating that moist heat affects the rigid structure of pollen. After 15 min of treatment, values even higher than 85 % are reached, a digestibility significantly higher than found in fresh bee-pollen.
In addition, the analysis of the bioactive compounds and antioxidant activity showed a progressive growth at 5 and 10 min compared to fresh pollen, however, these values decline in the treatment of 15 minutes. For total phenols and TEAC is observed a significant increase in the treatment at 10 min. The reduction in values of bioactive compounds and antioxidant capacity at 15 min of exposure could have happened because the heat treatment achieve a breaking of the outer cell wall of pollen, and the time of exposure to heat lasts enough that the bioactive compounds are sufficiently available and to be degraded by effect of temperature.
The report of antioxidant activity in the case of FRAP were inconclusive, however TEAC results were consistent with those found in the analysis of total phenols. In the FRAP assay, reported values are usually lower than in TEAC (when trolox is used as standard for both techniques), because the first practically measures non-protein total antioxidant capacity. In the TEAC method, the antioxidant effects of proteins are pronounced (Erel, 2004). This is an important issue, and maybe the reason why TEAC was more useful in this research, considering that Colombian bee-pollen has a protein average content of about 28% (Fuenmayor et al., 2014).
For the mentioned reasons, it can be concluded that autoclaving 10 minutes achieves improved digestibility and the availability of bioactive compounds, so these conditions were used for subsequent biotechnology experiments.

3.2 Fermentation and Enzymatic Hydrolysis

The results of fermentation and enzymatic hydrolysis are shown in Table 2. These information were compared to the thermal treatment of 10 minutes, in order to establish if any significant difference can be attributed to the biotechnological process. A microbiological test of commercial sterility, showed that no microorganisms were viable after exposing bee-pollen to 10 min at 121C in autoclave.

Table 2: Results for bioactive compounds and antioxidant activity of hydrolyzed and fermented bee-pollen. Dry basis.

	10 min Thermal treatment	Hydrolyzed	Fermented
Digestibility (g digested protein / 100 g total protein)	83.45 ± 2.71^{a}	89.7 ± 2.92^{b}	84.86 ± 3.05^{a}
Total phenolics (meq gallic acid / g bee-pollen)	20.35 ± 2.18^{a}	27.75 ± 2.46^{b}	18.89 ± 2.24^{a}
FRAP (µmol TROLOX / g bee-pollen)	68.06 ± 12.53^{ab}	72.27 ± 0.61^{b}	58.63 ± 4.79^{a}
TEAC (µmol TROLOX / g bee-pollen)	88.85 ± 4.04^{a}	94.66 ± 4.39^{b}	55.11 ± 1.90^{c}

Different letters in a same row indicate significant differences

Statistical analysis showed that for digestibility, the content of phenols and TEAC antioxidant capacity, significantly greater values were obtained in the enzymatic treatment. A comparison with the thermal treatment showed that this result is different from the effect achieved only by temperature. Although the FRAP activity did not differ significantly, it can be observed a trend toward increasing its value in the hydrolyzed pollen. In the case of fermentation, found values did not show favorable effects with respect to thermal treatment, and on the contrary, a reduction in the antioxidant activity assessed by TEAC was obtained.
In other studies, Fuenmayor used strains of the microorganism Lactobacillus plantarum, which achieved an increasing in bioactive compounds content and antioxidant capacity. It is clear, then, that this lactic acid bacteria had a better performance than the commercial culture employed in this study (Fuenmayor, 2009). Moreover, Marinova and Tchorbanov produced a hydrolysate from bee-pollen, with no previous thermal treatment, by using proteinase and aminopeptidase enzymes. Results concluded that an improvement in the terms of antioxidant capacity and total phenolic content was achieved, which is a similar finding to our study (Marinova and Tchorbanov, 2010).
Into consideration, although the outer layer of bee-pollen has no protein compounds, it is estimated that the thermal treatment achieved the opening of the grain leaving exposed peptide structures, over which the protease could act, releasing amino acids and phenolic compounds linked to these, and increasing the values in variables studied in this work.

4. Conclusions

In this work, a number of successive steps for bee-pollen processing was achieved, in order to increase the digestibility, bioactive compounds and antioxidant activity. A thermal treatment at 121 °C for 10 min, combined with an enzymatic hydrolysis using a protease, yielded an overall increase of 10 % in digestibility, 14 % in total phenolics, and, 13 % in TEAC antioxidant capacity. Bee-pollen is a food containing valuable nutrients and bioactive compounds which may be included in the diet as a nutritional supplement due to its functional characteristics.

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