SCIENCE AND FOOD TECHNOLOGY

# Digestibility and availability of nutrients in bee pollen applying different pretreatments

CIENCIA Y TECNOLOGÍA DE ALIMENTOS

# Evaluación de la digestibilidad y disponibilidad de nutrientes del polen apícola al aplicar diferentes pretratamientos

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**Abstract**

Bee pollen is characterized by its high nutritional value that could be used in human diet, specifically for its value in protein and antioxidant capacity. Different studies emphasize that pollen shows a restriction in nutrient absorption caused by its complex external cell wall, being not easily digestible by monogastric species as bees and humans. The objective of this study was to apply different pretreatments: enzymatic, alkaline, dry thermal and wet thermal. In order to evaluate the effect of each pretreatment, protein by Bradford method, *in vitro* digestibility, antioxidant capacity and total phenols were quantified. Protein estimated by Bradford method decreased in pretreated pollen, due to the breakage of peptide bonds, and the digestibility raise from 62% in untreated pollen to 85-98% in pretreated pollen. In relation to antioxidant capacity, it showed a non-representative decrease regarding other vegetable matrices, with the exception of a raise in phenols for some pretreatments. Such results coincide with microstructural changes observed in pretreated pollen micrographs. Finally, the variables assessed by principal component analysis showed differences for every pretreatment.

***Keywords:*** *Cell wall, chemical, enzymatic, hydrolysis, thermal.*

**Resumen**

El polen apícola se caracteriza por su alto contenido nutricional que podría ser empleado en la alimentación de los seres humanos, específicamente por su valor en proteína y capacidad antioxidante. Diferentes estudios resaltan que el polen presenta restricción en su absorción de nutrientes por su compleja pared celular externa, siendo poco digerible para especies monogástricas como abejas y seres humanos. El objetivo de este estudio fue aplicar diferentes pretratamientos: enzimático, alcalino, térmico seco y térmico húmedo. Para evaluar el efecto de los pretratamientos, se cuantificó proteína por el método de Bradford, digestibilidad *in vitro*, capacidad antioxidante y fenoles totales. Se encontró una disminución de proteína para el polen pretratado por el método de Bradford, debido al rompimiento de los enlaces peptídicos, así como el incremento en la digestibilidad del polen sin tratar del 62% al 85-98% del polen pretratado. En cuanto a la capacidad antioxidante presentó una disminución no representativa respecto a otras matrices vegetales, excepto para los fenoles al revelar un incremento para algunos pretratamientos, resultados que concuerdan con los cambios microestructurales de las micrografías del polen pretratado. Finalmente todos los pretratamientos presentaron diferencias al correlacionar las variables evaluadas mediante un análisis de componentes principales.

***Palabras Clave:*** *Enzimático, hidrólisis, pared celular, químico, térmicos.*

## 1. Introduction

Bees perform an important role in vegetable biodiversity maintenance ensuring plant reproduction and genetic diversity by pollination (Saavedra et al., 2013). Floral pollen is the secretory product in male organs of plants, it is the male gamete of seeds (gymnosperm and angiosperm). The body of bees is especially provided to collecting floral pollen, material that is their main source of nutrients (protein, lipids, vitamins, minerals, antioxidants, among others). Bee forms small spheres or pellets of floral pollen by means of agglomeration by nectar and salivary secretion addition, then bee transports them to the hive, carrying one pollen pellet or sphere in the corbicula of each hind leg. Therefore the product prepared by bees is called bee pollen or corbicular pollen (Rodriguez, 2012).

The consumption of bee pollen has increased as being considered as a healthy and therapeutic product, regarding its nutritional properties, source of proteins, lipids, vitamins, minerals (Campos et al., 2008) amino acids, carotenoids, flavonoids, phenolic compounds, antioxidants, carotenes and xanthophylls (Jean-Prost, 2007), being significant for new life styles which look for healthy eating habits. Accordingly, pollen is the second bee product with high potential of commercialization in Colombia, in terms of its advantages in weather and botanical conditions, geographical position, and biodiversity, which brings extensive economic and social benefits for the country (LABUN, 2006; Martinez, 2006).

As a consequence, the Bee and Apiculture Productive Chain – CPAA established the action strategic plan 2011-2025 (CPAA, 2011), that aims for the acknowledgment of the potential and issues of the apiculture sector, by means of identification of threatens, weaknesses, strengths and opportunities for producers and consumers. This organization has also projected a growth of 5% in pollen production for 2015 with an index of 38 kg/hive/year, and by the year 2025 a growth of 8% with an average of 40 kg/hive/year. The framework of the National Policy of Competitiveness and Productivity considers environmental sustainability as a competitiveness factor, useful in compliance with the laws and environmental regulations, to promote productive process and to motivate development of business opportunities (Martinez, 2006; CONPES, 2008). In this sense, the strategic plan established alliances with different actors of the CPAA, to promote the competitive development through several strategies that enhance production, commercialization and research.

There are reports, however, about the complex pollen structure that interferes negatively in the digestibility of nutritional compounds, presenting a low absorption in the intestinal tract of humans and bees (Hesse et al., 2009; Zuluaga et al., 2014). Pollen is surrounded by a complex protective outer membrane called exine, that is characterized for being resistant and firm, and composed of Sporopollenin, which are biopolymers as cutin (composed of fatty acids to protect the cell surface), suberin (polymer of long-chain fatty acids) and lignins (phenolic compounds) (Zuluaga et al., 2014). Those biopolymers protect pollen from desiccation during long periods of time and avoid the death of the cell. In contrast, the pollen inner wall called intine, that covers the protoplasm, is delicate with low resistance, composed of cellulose and pectin. Additionally, the pollen grain is reported containing other compounds as tocopherols, provitamin A, vitamin D and phytosterols, which protect it against oxidation; as well as selenium, a mineral that preserves pollen from damage caused by ultraviolet radiation (Gavarayeba, 1996).

This project aims to be an alternative of valorization for pollen as food, through the development of transformation processes applying different pretreatments in order to increase availability of nutritional compounds, and improve pollen digestibility, for potential use in human diet.

## 2. Materials and methods

### 2.1 Feedstock

*Bee pollen*

This study used dried commercial pollen distributed by the company "Apiario Los Cerezos", located in the municipality of Viracachá, Department of Boyacá (Colombia). The samples were stored in polystyrene bags and protected from light for their commercialization.

### 2.2 Analytic methodology used in monitoring the experiments

The methods adopted to evaluate the effect of pretreatments on bee pollen are explained in the following sections.

#### 2.2.1 Dry heat thermal pretreatment

The pollen sample was mixed with water (ratio 1:2 pollen/water) and then was neutralized with NaOH 5N to make use of the methodology described by Fuenmayor (2009) y Salazar (2014), applying their best treatment, sterilization at 121°C during 15 min, to obtain a microbiological stable pollen.

#### 2.2.2 Weat heat thermal pretreatment

The pollen sample was adjusted in a ratio of 1:2 to water, then it was placed in a closed container approximately at 130°C and 2 atm, during 2 min after reaching such pressure, to be neutralized with NaOH 5N. Finally, it was sterilized at 121°C during 15 min.

#### 2.2.3 Enzimatic pretreatment

The methodology described by Castro et al. (2014) was considered with some modifications as initiating with a pollen in natural state, using the commercial enzyme Protamex TM supplied by Coldaenzimas Ltda. The pollen sample was adjusted with NaOH 0,1N altering its pH from 5,5 to 7,5. The ratio 0,01 g enzima /g polen was applyed, and then it was taken to thermostatic bath STUART SBS40 (USA) to 37 ± 2 ºC, under constant agitation 200 RPM x 4 h, doing pH control each 2 hours. Finally, the treated pollen was sterilized at 121°C during 15 min.

*Protamex™ Enzime*

This enzyme is characterized by being a protease complex of *Bacillus* used in different studies for hydrolysis of food proteins. Its optimal work conditions are pH 5.5 to 7.5 at temperature from 35 to 60°C, and it has an activity of 1,5 Anson Units (AU-NH)/g enzyme (Liaset et al., 2003).

#### 2.2.4 Alkaline pretreatment

It was used NaOH 3% (w/v) with a ratio 1:1 g of pollen /mL de solution, then it was taken to a water bath at 70 °C during 10 min. After that, water was added in a ratio 1:1 to hydrolyzed pollen, and finally it was sterilized.

#### 2.2.5 Determination of protein (Bradford method)

A sample of 1g was weighted and added to 25 mL of phosphate buffer with pH: 7,6, under agitation in vortex during 5 min. Then it was taken to an ultrasound 15 min, and centrifuged to 9000 rpm at 4°C during 20 min. After that, 2,5 mL were diluted in 50 mL of distillated water. 1 mL of the extract was added to 1 mL of Bradford reagent, and after agitating the readings were made to 595 nm, using 1 mL of Bradford reagent and 1 mL of distilled water as blank. The result was expressed in percentage of protein (Castro et al., 2014).

#### 2.2.6 Determination of digestibility by in vitro pepsine

The quantitative method described in the Col-ombian Technical Standard NTC 719 was con-sidered for this test (ICONTEC, 1994), contemplating some modifications made in the study of Castro et al. (2014). A sample of 1,5 g of dried and degreased pollen was transferred to an erlenmeyer flask containing 150 mL of a solution 0,002 % of pepsine in hydrochloric acid with a concentration of 0,075 N. Then, it was taken to a thermostatic bath STUART SBS40 (USA) to maintain it under soft agitation and to incubate during 16 h at 45 ºC. The results were expressed in % of digested protein.

**2.2.7 Preparation of the extract for antioxidants**

A sample of 1 g was added to 15 mL of ethanol 96% in a 50 mL Falcon tube. This solution was subjected to untrasound ELMA during 10 min, after that it was taken for centrifugation during 10 min to 7000 rpm at 10°C, then the supernatant of the extract was transferred to a 50 mL volumetric flask. The procedure from the ethanol action was repeated two times more, and finally the volume was completed to 50 mL with ethanol 96%. An aliquot of 5 mL of the extract was transferred to a 10 mL volumetric flask with ethanol 96%, and the extract was stored in an amber flask according to the methodology described by Bernal (2012) and some modifications of the Institute of Food Science and Technology (ICTA) from the National University of Colombia.

**2.2.8 Determination of antioxidant capacity by the TEAC method (discoloration of the radical cation ABTS)**

10 µL of the extract were added to 1 mL of $ABTS^{\bullet+}$solution, and the reaction in the darkness was expected during 6 min. Then it was measured to a wavelength of 734 nm in a UV- visible spec-trophotometer THERMO SCIENTIFIC GENESYS 10S (USA), calculating the change in the absorbance compared to the solution without reaction. A calibration curve with Trolox as antioxidant was obtained to make the calculations, and the results were expressed in mmol Trolox/g pollen (Bernal, 2012).

**2.2.9 Determination of antioxidant capacity for the FRAP method (ferric reducing antioxidant power)**

For this analysis was considered the methodology assessed by ICTA and Bernal (2012). The mixture of 20µL of the extract, 450 µL of FRAP reagent and 735µL of distillated water reacted in the darkness during 30 min counted from the moment the extract was added. The measurements were made in a UV-visible spectrophotometer THERMO SCIENTIFIC GENESYS 10S (USA), in a wavelenght of 593 nm. Finally, the results were obtained by the construction of a calibration curve made with Trolox as antioxidant, consequently they are expressed in mmol Trolox/g pollen.

**2.2.10 Determination of total phenols by folin–ciocalteu method**

The measurement was determined by reading in spectrophotometry in a wavelength of 765 nm. 500 µL of the extract, 25 mL of distillated water, and 2 mL of a sodium carbonate solution 10% were mixed, then, after 10 min 500 µL of Folin were added, and the volume was completed to 50 mL. The solution was stored in darkness during 2 h, afterwards the reading was made in a UV-visible spectrophotometer THERMO SCIENTIFIC GENESYS 10S (USA), using distillated water as blank. The results were obtained by the construction of a calibration curve with gallic acid, and they were expressed in mg gallic acid/g pollen (Bernal, 2012).

**2.2.11 Statistical methodology**

The results were studied by a principal component analysis to apply an univariate or multivariate analysis of variance (ANOVA or MANOVA) accor-dign to the principal component obtained, using the software SPSS Statistics version 22, Statgraphics Centurion version 22, MATLAB.

**2.2. 12 Analysis of pollen structure**

*Morphological staining of pollen*

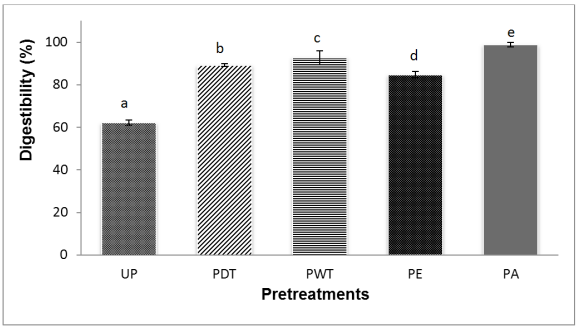
The morphology of raw pollen and pollen under different pretreatments were evatuated by placing the sample on a microscope slide to verify its micro-structure, specifically the outer layer of polen.

The metodology described by Ortiz, Cogua (1989) was followed with some modifications. Glicerine jelly mixed with fuscine over a mi-croscope slide were used and then the direct observation was made in an optical microscope.

## 3. Results and discussion

### 3.1 Comparison of bee pollen protein, without treatment and with different pretreatments, determined by Bradford method

Figure 1, shows a comparison of average protein content determined by Bradford method in pollen without treatment and pretreated. It is important to emphasize that such method is applied in different vegetables matrices for being a sensible and practical method.



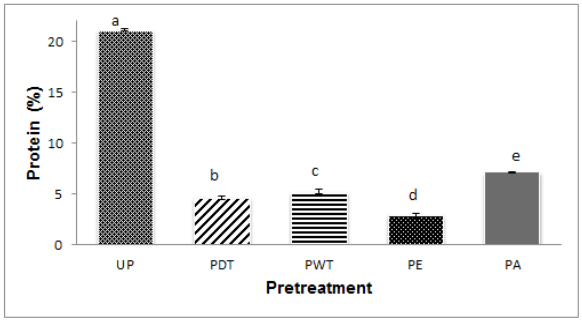

*Protein by Bradford method. Different letters for each bar indicates significant differences among pretreatments, with a confidence level of 95%.

***Figure 1.*** *Comparison of protein for untreated pollen: UP, pollen with dry thermal pretreatment: PDT, pollen with wet thermal pretreatment: PWT, pollen with enzymatic pretreatment: PE, pollen with alkaline treatment: PA.*

According to different protein reports for bee pollen, was obtained a value of 21,11%, which coincides with a report made in another research about the proximity of this value to the Chilean variety *Cryptocarya alba* 22% (Montenegro et al., 2013). In fact, figure 1 shows a significant lower value of protein in treated pollen with different pretreatments, in comparison to untreated pollen, which is caused by the breakage of peptide bonds. Enzymatic hydrolyzed pollen, however, presented the lowest protein content among the pretreatments. In accordance to some authors this result is due to a possible combined action made by enzymes, producing the breakage of certain peptide bonds that are not quantified and releasing particular amino acids (Castro et al., 2014; Sánchez, 2010), although other studies report that the reagent could only react with *arginine* and *lysine* residues specifically (Grintzalis et al., 2015).

### 3.2 Determination of digestibility in untreated and pretreated pollen

Few reports present the weakening of primary pollen wall with technological purposes (Zuluaga et al., 2014), since they are focused in morphology and specie definition, without considering the search of a pollen with a high amount of available nutrients to be incorporated in the human diet. In the figure 2 is compared the pollen digestibility of untreated pollen or in its original state, to the pollen obtained after applying the different pretreatments of this research. Certain authors have studied the dry thermal pretreatment with this vegetal matrix, however they have focused on obtaining a microbiological stable pollen rather than studying it as a bioprocess (Fuenmayor, 2009; Salazar, 2014), which emphasizes the lack of research about a high digestible pollen.




*Different letters for each bar indicates significant differences among pretreatments, with a confidence level of 95%.

***Figure 2.*** *Comparison of digestibility for untreated pollen: UP, pollen with dry thermal pretreatment: PDT, pollen with wet thermal pretreatment: PWT, pollen with enzymatic pretreatment: PE, pollen with alkaline treatment: PA.*

A general and significant increase in *in vitro* protein digestibility was found in pretreated pollen in relation to the untreated pollen, fact that can be explained by the protein denaturalization due to high temperatures and chemical reactions that take place in certain pretreatments, which could benefit the exine deformation. Enzymatic pretreatment has a rise of 84% in digestibility, that could be related

to protein decrease (see figure 1), coinciding with results obtained by Castro et al. (2014), that reported amino acid release after applying an enzymatic treatment to pollen in fresh state.

Certain studies highlight different protein hy-drolysis, since they can improve pancreatic digestion and increase nutrient avalilability, as a result of breaking the molecular structure, considering that a protein disgestion in food is easier when it is denaturalized, increasing nutrient absorption in the human intestine (Gonzalvo, 2001; Martinez, 2006). Additionally, some authors point out bees do not consume pollen in its fresh state, transforming it into bee bread that has a digestibility of 66% (Castro et al., 2014). The protein digestibility of pretreated pollen is close to the value reported for animal proteins, 95% to lactic proteins and 85% to egg, emphasizing that fermented milks can present a higher digestibility and protein absorption when compared to milk in natural state (Castillo & Menestres, 2004).

Regarding the antioxidant capacity FRAP and ABTS (Table 1), they showed a decrease in most of the pretreatments, except for alkaline pretreatment in ABTS that did not present a significant difference compared to the untreated dried pollen. The reduction of antioxidant capacity of pollen could be consequence of effects produced by different treatments applied (Araya et al., 2006; Salazar, 2014), coinciding with other authors that point out technological processes could decrease the antioxidant activity of certain vegetable matrices (Perea-Villamil et al., 2009). On the other hand, some studies reported a raise in antioxidant capacity of some food products as a favorable effect of a thermal pretreatment or a dry process, fact that is probably associated to the Maillard reaction (Fuenmayor, 2009; Salazar, 2014).

***Table 1.*** *Comparison of antioxidant capacity and phenols for pretreated and untreated pollen.*

| Sample | FRAP (mmol Trolox/g pollen) | ABTS (mmol Trolox/g pollen) | Phenols (mg gallic acid/ g pollen) |
|---|---|---|---|
| Untreated pollen | $0{,}029\pm0{,}001_{a}$ | $0{,}079\pm0{,}014_{a}$ | $15{,}19\pm0{,}58_{a}$ |
| Dry thermal pretreatment | $0{,}006\pm0{,}002_{b}$ | $0{,}024\pm0{,}001_{b}$ | $9{,}91\pm0{,}01_{b}$ |
| Wet thermal pretreatment | $0{,}011\pm0{,}001_{c}$ | $0{,}023\pm0{,}001_{b}$ | $12{,}19\pm0{,}13_{c}$ |
| Enzymatic pretreatment | $0{,}006\pm0{,}000_{b}$ | $0{,}015\pm0{,}004_{b}$ | $7{,}00\pm0{,}06_{d}$ |
| Alkaline pretreatment | $0{,}022\pm0{,}52_{d}$ | $0{,}074\pm0{,}006_{a}$ | $20{,}27\pm0{,}63_{e}$ |

The values of phenols showed a slight reduction for every pretreatment in comparison to pollen in its natural state, except for alkaline pretreatment that presented a raise, these results are similar to those reported by Castro et al. (2014). This fact could be explained in terms of the complex structure of pollen, as the outer layer called exine which is a polymer composed for fatty acids and lignins of phenolic compounds, releases certain phenols when it is subjected to some pretreatments producing a degradation in the cell wall of this matrix and a fracture in the polymer lignin (Zuluaga, 2014).

In spite of the decrease found in antioxidant pro-perties of pretreated pollen, the values are still significant when compared to other matrices as blackberry and barberry (Bernal, 2012). Finally, it was demonstrated that every pretreatment benefits pollen digestibility in relation to pollen in natural state, considering that pretreated pollen could be consumed.

### 3.3 Statistical estimation of variables evaluated in the pretreatments in a combined way

The definitive data analysis was made by a Principal Component Analysis (PCA), to evaluate in a clear way the effect of each evaluated variable as protein by Bradford method, digestibility, antioxidant capacity and total phenols. Figure 3 shows the *score plot* and the *loading plot* of the principal component extracted that explained a 96% of variance in the data. Considerable differences are noticeable between dried pollen (UP) and pretreated pollen, in terms of higher digestibility and lower protein content. Regarding antioxidants, the general tendency is a reduction in the different pretreatments except for phenols.

For the principal component extracted was applied ANOVA (Table 2) to evaluate the effect of pretreatments and to verify by Tukey that all the pretreatments showed different mean vectors, indicating a significant difference on each mentioned variable in relation to pretreatments. The exceptions were alkaline and dry thermal pretreatments.

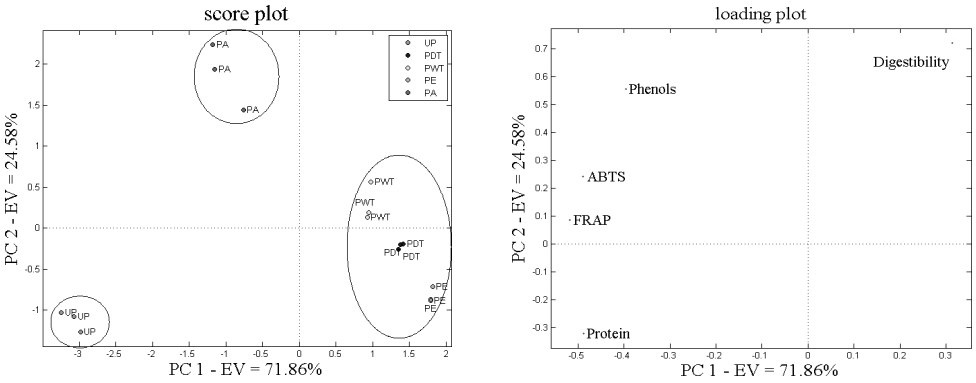



***Figure 3.*** *Score plot and Loading plot of the Principal Component Analysis (PCA) of the variables evaluated in different pretreatments.*

***Table 2.*** *Summary of the univariant Analysis of Variance (ANOVA) from the principal component extracted.*

| Source | Sum of Squares | Gl | Mean Square | Ratio-F | P-value |
|---|---|---|---|---|---|
| Among groups | 14.4623 | 5 | 2.89246 | 193.19 | 0.0000 |
| Intragroups | 0.179667 | 12 | 0.0149722 | | |
| Total (Corr.) | 14.642 | 17 | | | |

**Multiple range test for the principal component extracted**

Method: 95.0 percentage Tukey HSD

| *Sample* | *Cases* | *Mean* | *Homogeneous group* |
|---|---|---|---|
| PWT | 3 | 0.766667 | X |
| PDT | 3 | 1.21333 | X |
| PA | 3 | 1.31667 | X |
| PE | 3 | 1.66 | X |
| UP | 3 | 3.53667 | X |

| Contrast | Sig. | Difference | +/- Limits |
|---|---|---|---|
| PA - PE | * | -0.343333 | 0.335626 |
| PA - UP | * | -2.22 | 0.335626 |
| PA - PWT | * | 0.55 | 0.335626 |
| PA - PDT | | 0.103333 | 0.335626 |
| PE - UP | * | -1.87667 | 0.335626 |
| PE - PWT | * | 0.893333 | 0.335626 |
| PE - PDT | * | 0.446667 | 0.335626 |
| PS - PWT | * | 2.77 | 0.335626 |
| PS - PDT | * | 2.32333 | 0.335626 |
| PWT - PDT | * | -0.446667 | 0.335626 |

*(a) UP: Dried untreated pollen, (b) PDT: Pollen with dry thermal pretreatment, (c) PWT: Pollen with wet thermal pretreatment, (d) PE: Pollen with enzymatic pretreatment, (e) PA Pollen with alkaline pretreatment.*

### 3.4 Staining of pollen morphology

Considering that evaluating the incidence of pretreatments on pollen structure was a desired outcome of this study, the figure 4 presents measurements of optical microscopy made for dried untreated pollen and treated pollen in thermal, enzymatic and alkaline form, in order to observe the structural modification of pollen after being exposed to different process.

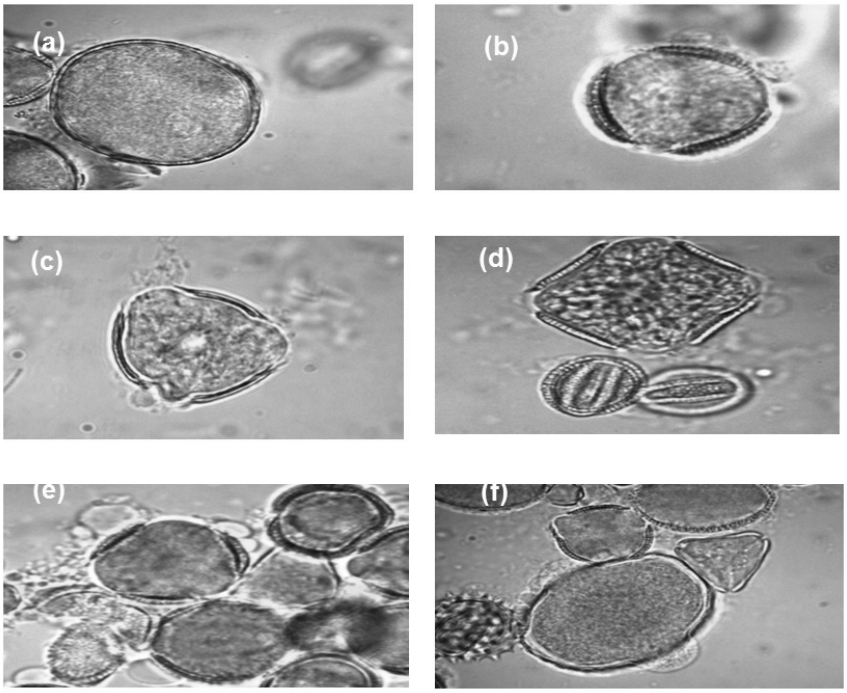



***Figure 4.*** *Micrographs at 100X magnification of bee pollen untreated and with pretreatments.*

*(a) Morphology 1 of dried untreated pollen, (b) Morphology 2 of dried untreated pollen, (c) Pollen with dry thermal pretreatment, (d) Pollen with wet thermal pretreatment, (e) Pollen with enzymatic pretreatment, (f) Pollen with alkaline pretreatment.*

Regarding to figure 4, (a) shows an oval homogeneous morphology without fissures, while (b) differs in being circular and having three wade edges in the primary wall, although in these morphologies is evident a well-defined structure where exine is highlighted. In (c) is presented the thermal treated pollen with a deformation in the cell wall of various morphologies. Morphology (d) displays a deformation in the structure caused by wet thermal pretreatment, furthermore (e) has a degradation in the outer structure of pollen by the action of enzymatic pretreatment. Finally, (f) which belongs to alkaline treated pollen, shows a fissure and exine deformation.

On the other hand, this type of technique is referred in most of studies as being focused in botanical origin of pollen, however it was demonstrated that it could be applied for other kind of analysis.

## 4. Conclusions

The different thermal, chemical and enzymatic pretreatments applied to bee pollen generated significant changes in protein by Bradford method. Enzymatic pretreatment, in particular, affected the bee pollen extensively, presenting an evident decrease in protein and more available nutrients.

When 62% of digestibility of pollen in natural state is compared to 85% of digestibility for pollen with enzymatic pretreatment, 89% for dry thermal pretreatment, 92% for wet thermal pretreatment and 98% for alkaline pretreatment, it is found a

significant increase that implies obtaining a matrix with better characteristics than the initial pollen, in terms of availability of nutrients that could benefit its consumption.

The antioxidant properties experienced a reduction for pretreated pollen in respect to pollen without treatment, however those changes were not pronounced compared to other vegetable matrices. According to the results of this study, pretreatments are a good alternative to increase the digestibility and improve the availability of other nutrients in bee pollen.

Enzymatic, thermal and chemical pretreatments perform a considerable effect in pollen microstructure, in accordance to the deformation of exine observed by optical microscopy.

## 5. Acknowledgements

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