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Physicochemical characterization of propolis collected by *Apis mellifera L (Hymenoptera: Apidae)* in Pacho and Bogotá Cundinamarca.

Caracterización fisicoquímica de propóleo colectado por *Apis mellífera L (Hymenoptera: Apidae)* en Pacho y Bogotá Cundinamarca

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Abstract

The objective of this work was to characterize and implement a reliable, viable and effective physicochemical methodology for the quality of *Apis* mellifera propolis collected in two apiaries located in Pacho and Suba-Bogotá, Cundinamarca, Colombia. A qualitative analysis was designed and validated to diagnose and evaluate the quality of propolis for laboratory analysis. Samples were collected by the scraping method, ethanolic extracts of propolis (EEP) were obtained and results were compared under several Latin American standards (Cuban, Brazilian and Salvadoran standards). The presence of phenols and flavonoids was determined by means of solubility in lead acetate, ferric chloride, sodium hydroxide, pW, humidity, oxidizing property, UV-vis spectroscopy profiles, pH, mechanical masses, dry mass, percentage of phenolic compounds, ashes, determination of major and minor minerals and quantity of flavonoids in quercetin. The results showed that the solubility in lead acetate and sodium hydroxide were positive for the 10 samples in duplicate, plus the 3 validation samples; the results showed significant differences between locations and compared with current standards.

Key words: Antioxidants, Phenols, Flavonoids, bee products.

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Resumen

El objetivo de este trabajo fue caracterizar e implementar una metodología fisicoquímica confiable, viable y eficaz para la calidad del propóleo de *Apis* mellifera recolectado en dos apiarios ubicados en Pacho y Suba-Bogotá Cundinamarca Colombia. Se diseñó y validó el análisis cualitativo para diagnosticar y evaluar la calidad de propóleos para analizarlo en el laboratorio. Las muestras fueron colectadas por el método de raspado, se obtuvieron extractos etanólicos de propóleos (EEP) y resultados que fueron comparados bajo varias normas a nivel latinoamericana (Normas cubana, brasilera y salvadoreña). Se determinó la presencia de fenoles y flavonoides por medio de solubilidad en acetato de plomo, cloruro férrico, hidróxido de sodio, pW, humedad, propiedad oxidante, perfiles de espectroscopio UV-vis, pH, masas mecánicas, masa seca, porcentaje de compuestos fenólicos, cenizas, determinación de minerales mayores y menores y cantidad de flavonoides en quercetina. Los resultados evidenciaron que la solubilidad en acetato de plomo e hidróxido de sodio fueron positivos para las 10 muestras por duplicado, más las 3 muestras de validación; los resultados presentaron diferencias significativas entre las localidades y comparados con las normas vigentes.

Palabras Clave: Antioxidantes, Fenoles, Flavonoides, productos apícolas.

Introduction

The name propolis comes from the Greek propolis *Pro* "front or defense" from and *polis* "city" in this case the beehive, this product is highly appreciated for its anti-inflammatory, antitoxic, anesthetic, antioxidant, bactericidal, antiseptic and healing properties, in addition to its wide use in human medicine, veterinary, agriculture, joinery and food concentration (Rodríguez Martín, 2014).

The production of propolis in Colombia and in the world is scarce because the volumes produced are small and the beekeepers are unaware of the benefits of propolis and its characteristics, but because of the profit they can receive from its production, it could be a product of commercial interest. However, there is no standardized technology that allows it to be collected efficiently in order to strengthen the increase in production and consolidate the subsector in the face of future demand for the product. (Patiño, 2001).

The main producers of royal jelly, pollen and propolis in the world are Japan, Poland, France, Netherlands, Germany and the United States; The world's largest importer of propolis is Japan with 7,100 tons and the largest consumer of propolis in the planet is Germany with 4,600 tons and the main exporters are Brazil, Argentina, China, Mexico (Rodríguez & Nuñez, 2014).

Flavonoids are the main characteristic of propolis for its antioxidant properties for disease prevention, in the early twentieth century medicine officially recognized propolis with therapeutic properties, English surgeons proved the healing action of propolis and decreasing mortality from gangrene. (Morales, 2012).

In Colombia the use and exploitation of propolis has had little disclosure, this hinders the study to implement a characterization of propolis and avoids establishing quality measures and a classification of raw propolis, to have initiative it is necessary to have quality protocols required to characterize it and thus obtain an added value that allows beekeepers to be encouraged and have an alternative productive activity for an apiculture in crisis. (Gil, Viloria, Durango, & Garcia, 2012)..

The composition of propolis depends on the botanical origin, bees collect raw material from plant resins, buds, branches, young fruits, bark and mechanical impurities that they collect from various plants and certain trees in the collection area. From the resinous substances they collect together with secretions from their glands they obtain propolis, their scent can be from tree species, honey and waxes. (Núñez R. et al., 2018)..

Bees disinfect the inside of the hive with propolis, close cracks and reduce access routes, cover the corpses of enemies that try to enter the hive to prevent their decomposition. This property of propolis was already known to the Egyptians and the priests who used it to mummify the dead. (Carrillo, Castillo, & Mauricio, 2011).

Propolis in agriculture is used to treat diseases such as fungal diseases in fruit trees, vegetables and viticulture; propolis acts as a systemic treatment, i.e. it does not act only in contact areas but penetrates the plant sap and distributes it, it is not preventive but curative when the fungus is present. Propolis can be combined with other preventive treatments such as nettle slurry, Bordeaux mixture for downy mildew and powdered sulfur for powdery mildew. (Mercedes & Modroño, 2019).

Phenolic compounds originate from the plant kingdom, as they are one of the main secondary metabolites in plants and in the animal kingdom by ingestion. Phenols are synthesized by plants and are genetically regulated.

Bactericidal properties were demonstrated with staphylococci, streptococci and salmonellae, for fungal properties it was shown that they cause parasitic conditions or mycosis and shows that *Apis mellifera has a* total of seven types of antibiotics that protects bees. Its food, propolis venom and beeswax, for anesthetic properties is linked by its volatile oil content that is granted effects superior to those of cocaine and novocaine.(Muñoz, Linares and Narváez, 2011)..

In the agroecological-social area, the objective was to analyze the quality of propolis to avoid losses of propolis in the production system and to have it as an efficient cycle. Agroecology has tools available for the service of agricultural production, incorporating bees because this improves the quality of the fruits due to the effect of pollination and small producers benefit from a small amount of income from the use of honey and in some cases pollen. However, propolis can contribute from the economic factor due to the small amount of income as products for sale. From the social component it can be used for self-consumption, from the environmental part it will avoid generating waste, which is why the benefit of this raw material closes the cycle of beekeeping production from the agroecological vision.

Materials and methods

The propolis samples were collected by the scraping method, 5 from Suba-Bogotá Cundinamarca and 5 from Pacho Cundinamarca and one more for the validation of the methodology.

The propolis samples were taken in Suba - Bogotá 4°44'38.4 "N 74°04'22.8 "W with an altitude of 2560 masl, temperatures from 7°C to 19°C, an annual precipitation of 797mm, wind speed of 4.1km/h-7.9km/h, relative humidity 77- 83%, the second location was in Pacho-Cundinamarca in the village of llano la hacienda 5°09'29.8 "N 74°07'44.2 "W at 2134 masl with temperatures from 12°C to 23°C, wind speed 3.5 km/h to 5.7km/h, relative humidity 57%, precipitation 51mm - 205mm monthly and 1440mm annually.

In this research the parameters of pH, pW, dry extract, oxidant property, determination of flavonoids and phenols, mechanical masses were evaluated according to the methodology described by Tagliacollo (2010) and from the book "Origin, nature, physicochemical properties and therapeutic value of propolis" of (Salamanca, 2017) ash and moisture were determined, an adaptation of the methodology was made for reading in uv-vis spectrophotometry and the results of all parameters were compared under the Cuban agricultural branch standard NRAG 1994, Russian (Joint Resolution SPRI No 94/2008 and SAGPA No. 357/2008**)**, Brazilian, Mexican, Salvadorian, Salvadorian (Joint Resolution SPRI No 94/2008 and SAGPA No. 357/2008**),** Brazilian, Mexican, Salvadorian, Salvadorian and Cuban standards.Salvadoran (Lemus, Rodriguez, & Orellana, 1995)Argentina (Kozlowski, 2002)For the quantification of flavonoids, it was carried out under the methodology of (Palomino G, García P, Gil G, Rojano, & Durango R, 2009) Finally, for the determination of minerals, the following elements were determined: manganese, zinc, copper, iron, magnesium, calcium, potassium, sodium by means of flame atomic absorption spectrophotometry, and 5 g of raw propolis ash, since this represents the mineral content of the propolis sample.

Propolis was stored in amber jars, marked and deposited in cool, dark and dry places, kept at 4°C for 24 hours, the following analyses were performed applying a validation: reproducibility, accuracy or trueness, average. (Tagliacollo, 2014)

STATISTICAL DESIGN: A completely randomized design was used in which the following statistical measures were employed: mean, reproducibility, coefficient of variation, accuracy or trueness.

The data obtained were analyzed using SPSS ® Statistics 25 software, where their normal distribution was checked and the Student's t-test was applied to accept or reject the null hypothesis for each of the parameters evaluated, and to confirm the assumption of normal distribution, the Shapiro Wilk test was applied due to the fact that there is a group of less than 30 samples.

According to the statistical analysis performed with SPSS ® Statistics 25R software, the hypothesis of equality of variances for phenols, oxidative property, dry extract, moisture, mechanical masses and pW is rejected, so that it is concluded that with a significance level of 95% there are significant differences between the samples taken in Pacho and the samples taken in Suba-Bogota, but for pH and ash for the results obtained in the laboratory there is a significance level between each of the variables for each zone.

Result

Results and comparison under the regulations A parameterization was elaborated for the qualitative tests depending on the aspect of the result of the final solution, which was applied for the determination of phenolic compounds with sodium hydroxide, ferric chloride; and for flavonoids the reaction of the propolis extract with lead acetate according to the regulations in force.

The first comparative result is the mineral content in each of the duplicate samples from the two study zones (see Table 1).

Table 1 *Mineral content in propolis*

Minerals	Pacho (mg/g)	Bogotá (mg/g)
Potassium	738,424136	421,0838995
Sodium	16,7900821	28,64315058
Calcium	607,207669	350,668352

Magnesium	125,185616	79,55285195
Iron	53,7146915	26,27389793
Copper	1,43265741	0,54945489
Zinc	24,3871311	9,629088324
Manganese	7,85367682	7,111313004

A table was prepared to represent the qualitative tests of major and minor minerals in crude propolis ash (see Table 4).

Determination Of Phenolic Compounds:

Reaction With Sodium Hydroxide:

Table 2 *Parameters for the determination of phenolic compounds in samples of ethanoic extract of propolis*

Solution appearance	Qualitative test result
Slightly granular	+
Granular	++
Highly granular	+++

The reaction of the propolis extract with sodium hydroxide must be positive in order for the propolis to determine if phenolic compounds are present and thus be accepted according to the regulations. The reaction and the basis is positive when the sodium hydroxide breaks the C ring of a flavonoid which can be a flavonone or a flavone and this rupture causes the formation of a chalcone, evidenced by the yellow coloration that varies its intensity in the reaction (See Table 3).

Table 3 *Determination of phenols location 1*

NaOH reaction analysis	Sample Suba - Bogota	Qualitative test result	Coloring
1	10%	++	Yellow
	5%	++	
	10%	++	Yellow
	5%	++	
	10%	+	Yellow
	5%	+	
	10%	+++	Yellow
	5%	+++	
5	10%	++	Yellow
	5%	++	

The following table shows the results of the sodium hydroxide analysis in Pacho (see Table 4).

Table 4 Determination of phenols location 2

NaOH reaction analysis	Pacho Sample	Qualitative test result	Coloring
1	10%	+++	Brownish green
	5%	++	
	10%	+	Brownish green
	5%	+	
	10%	++	Green
	5%	++	
	10%	+	Green
	5%	+	
5	10%	+++	Brownish green
	5%	+++	

Determination Of Flavonoids - Reaction With Lead Acetate

The reaction is positive when it presents a yellow precipitate or a cloudy solution to be accepted by the standards. It is based on the formation of a colored complex between lead and dihydroxyphenols and polyhydroxyphenols such as flavones, flavanones and anthocyanins contained in propolis and it is necessary to have these compounds in order to obtain its antimicrobial, antimicrobial and antiviral characteristics (see Table 5).

Table 5 *Determination of flavonoids location 1*

Sample Suba - Bogota	Coloring	Qualitative result
1	Yellow	-
	Yellow	-
	Yellow	-
	Yellow	-
5	Yellow	-

The following table shows the results for the determination of flavonoids in lead acetate in Pacho (see Table 6).

Table 6 *Determination of flavonoids location 2*

Pacho Sample	Collation	Qualitative Result
1	Green	+
	Brownish green	+++
	Green	+
	Green	+
5	Green	+++

The flavonoid content was determined by the colimetric method with lead acetate and for the quantification of the total flavonoid content it was determined by the method of Kumazawa *et al.* (Kumazawa, Hamasaka, & Nakayama, 2004). with minor modifications, to 0.5 ml of ethanolic propolis extract solution diluted 1:1000 at 5% ALCL3 and after 1 hour at room temperature and avoiding light, the absorbance was measured at 430nm.

CALIBRATION CURVE: For the calibration curve standards, 2.5mg of quercetin was used and dissolved in 25ml of 96o methanol, the total flavonoid content was calculated as quercetin (mg/g), subsequently this stock solution was diluted to 5, 10, 15, 15, 20 and 25µg/mL as follows.

To read the samples a dilution of the ethanolic extract of propolis was made, 0.1ml of EEP was taken and was taken to a volume of 25ml of ethanol to each sample, already having the dilutions 2ml and 2ml of aluminum chloride 5% were taken from each one, they were shaken with a bortex, the samples were left in a place with room temperature and dark for 1 hour and we proceeded to read each one of the samples in triplicate on the calibration curve. Finally, an average of the weight of 0.1ml of EEP was taken from each region.

Flavonoid Quantification

The quantification of flavonoids were few compared to other international propolis, but it was higher than the norm, the highest amount of flavonoids was obtained in Pacho with 6.52 mg/g and the highest amount in Bogota was 4.3 mg/g, being very significant the difference.

Ferric Chloride Reaction

This reaction of propolis extract with ferric chloride is used to identify the presence of phenols in the propolis extract sample, colored compounds are formed as the hydroxyl groups act to activate the benzene ring by donating density, the aromaticity of the ring is weakened and it acquires more the characteristics of a conjugated polyene, the iron (III) ion is a moderate oxidant that induces the conversion of a pyrogallol or a hydroxylated orthoquinone, with higher concentration of pyrogallol it promotes the formation of a polymer of the quinone formed and a very long pi system is produced so that absorption is obtained in the whole spectrum. (Penadillos & Antonio, 2013)

Dry Extract

For the propolis samples from the municipality of Pacho, it was found that most of the samples were below the range established by the Latin American legislative norm, giving a range of 3.026% - 12.496%, therefore 40% (4 samples) of the 10 samples meet the Latin American norms, these being from Suba-Bogotá. It is defined that the range of the results is high and may be related to the botanical origin and the area where the bees collected the resin.

Oxidizing Property

The samples analyzed were below the standards, which suggest a maximum reaction time of 22 seconds to have an optimal oxidation to identify a good quality of propolis due to its high oxidation rate, the time range for the 10 samples was 9.96 - 21.01 seconds. These results can be affected by the relative humidity that favors the natural oxidation of the product during its production, a high percentage of humidity is higher than the 22 seconds established by the regulations is due to a bad storage of the product. (Tagliacollo, 2014).

Solubility Of Eep Particles In Lead Acetate And Sodium Hydroxide

For both analyses, the objective is to verify the homogenization of the extract particles in a saline solution to identify phenols and flavonoids. All the samples from the studied areas were positive.

pH

The range of the sample results is between 3.69 - 4.8 which indicates that the samples tend to be acidic. The standards do not establish a value, but the literature on pH in alcoholic media reports that the pH value is close to 5.0.

Mechanical Masses

The mechanical masses are wood shavings, wax, plant remains, the regulations show that they constitute 40 - 50% of the total mass, the rest is mass that is biologically active. In the samples analyzed a range of 59.79 - 26.68% was obtained, the quality of propolis is inversely proportional to the content of mechanical masses since if the propolis is harvested in traps it is purer in terms of propolis content since if it is harvested with the scraping system more mechanical masses are obtained.

Ash In Dry Mass

In the locality of Suba-Bogotá, high ash indices were obtained with 1.5% and in Pacho with 0.8%. The propolis from the city of Bogotá complies with Brazilian standards, with 100% of the 5 samples collected in Suba-Bogotá complying with the standards and 10% complying with the standards for the 5 samples collected in Pacho.

Humidity

The results obtained do not exceed the percentage of Brazilian standards. A range of 3.6% to 4.9% was obtained for Pacho and Bogotá with respect to the regulations, which indicate that the maximum result can be 8%, and the dry base (pW) did not exceed 8% because the two locations had a range of 3.7% to 5.2% for moisture on a dry base.

Uv-vis spectrophotometry

A sweep of each of the samples diluted 1:1000 was made to read them in the spectrophotometer, a base line with deionized water was needed for the spectrum to read it based on that line and compare it with the propolis samples and proceed to read the 10 samples by washing and purging the cuvette to avoid contaminating the sample with the other samples. Next, one of the sweeps that the spectrophotometer marked is observed, being 230 the maximum absorbance peak in Pacho.

According to the methodology reported by (Palomino G et al., 2009) adjustments were made to the calibration curve adapted from the methodology of (Lemus et al., 1995).

Determination of minerals by atomic absorption spectrophotometry

For the determination of minerals there are regulations and methodology that establishes some percentage of certain minerals in propolis, the results obtained between 0.5 - 728.4 mg/g of propolis were made in duplicate and an average was made for each of the samples, Pacho has the highest amount of minerals than in Bogota, the highest amount that has the crude propolis is in potassium and the lowest amount of minerals was obtained in copper as follows: Potassium<Calcium<Magnesium<Iron<Zinc<Sodium<Manganese<Copper.

In the extraction process of propolis by centrifugation 60% of the samples presented a milky and opaque turbidity because the water contained in the crude propolis causes insolubility of waxes and less hydrophilic components (Grosso, 2017).

Regarding the mechanical masses the result may vary due to the collection method, for the determination of ashes the results of Suba-Bogotá were very low with respect to the legislation established for it to be a quality propolis, but in comparison at national level the articles reviewed (Bastos, Guzman, Figueroa, Tello, & Scoaris, 2011) in Caldas it was found that the percentage of ashes with the scraping methodology is 0.22 - 0.78 on the other hand for the studied zones (Pacho and Suba-Bogotá) with the same methodology it is 0.8% for Suba-Bogotá and 1.5% for Pacho. (Julián Martínez, Carlos Garcia, Diego Durango, & Jesús Gil, 2012). According to the (Salamanca Grosso, 2017) the amount of ash in the samples is the amount of total fixed solids that represents the mineral content of the propolis, since the muffle incinerates the organic matter of the propolis, i.e. the more ash content the sample has the better because it fixes a nutritional rate in the product.

Regarding moisture and pW, it was determined in the samples and considers the water loss of the sample after having removed moisture in the field and pW is the percentage of moisture that the propolis sample can retain at room temperature without alteration from an external factor. In this case, the samples from Suba-Bogotá contain more moisture than those from Pacho due to the environmental conditions in Pacho, which also affects other propolis characteristics such as the oxidizing property. The levels of oxidizing activity for the alcoholic extracts of propolis are 22 seconds, Pacho has a less oxidizing activity than Suba-Bogota since Pacho had 12.37 seconds as opposed to Suba-Bogota that obtained 18.93 seconds, these results favor the product during its production, if it has any alteration to the maximum limits established by the regulations it is due to a bad storage of the product, this would also be an indication of possible fermentations generating unwanted products or contaminants being toxic and harmful to health.(Delgado Aceves, Andrade Ortega, & Ramírez Barragán, 2018).

The results obtained in Pacho contain a higher level of acidity than Suba-Bogotá.

The samples analyzed with respect to the dry extract varied from 5.8% to 9.8%, 11% being the value established by Brazilian regulations, therefore 40% of the samples of 10 samples analyzed comply with the current Brazilian legislation, these samples from the town of Pacho being more in accordance with the regulations.

The content of phenols present in the extract varied in 2.6 for Bogota and 6.14 for Pacho being 0.5% the ideal content of phenols according to Brazilian regulations therefore all samples exceed and are consistent with the regulations in terms of quality, the high content of phenols that has the propolis increases the value of apitherapy in the international market for its antimicrobial effect, as well as other substances present in the propolis (Tagliacollo, 2014)The phenolic compounds are recognized for their high biological, anticarcinogenic, anti-inflammatory, analgesic and oxidant activity. (Bastos et al., 2011).

The components of propolis are different depending on the flora and climatic conditions, according to the results obtained, the less disturbed natural environment has a higher concentration of phenols as in Pacho Cundinamarca improves the quality of propolis, from the statistical analysis it is concluded that apart from the fact that there are significant differences there is the possibility of having a higher quality propolis, The hypothesis under the established parameters the propolis of the two zones are of the same quality and statistically if there are differences between the two and the social benefit for small producers at an economic level and own consumption for their health, bronchodilator benefits of propolis by high contents of phenols, aromatic acids, esters, aldehydes, camarinas and triglycerides.

Conclusions

The difference in color in the reactions for the determination of phenols and flavonoids is due to the difference in flora and climatic conditions in which each of the samples was collected. The most important characteristic that gives the difference in quality of propolis is the amount of phenolic compounds, because the one from Suba-Bogota is of better quality due to its content of phenolic compounds.

Propolis does not have a chemical formula, it is a resin made up of several elements from different plants collected by Apis mellifera bees. Propolis contains 2.6% - 6.14% phenols in the areas of Pacho and Suba - Bogota respectively, favoring human health due to its pharmaceutical properties and its biological potential, mainly its antioxidant activity.

Environmental benefit, the use of tree resins by bees and continuous visits to the flora, promote forest conservation and protection of natural resources, use for their hives for their antimicrobial properties.

Most of the ethanolic extracts analyzed meet the regulations of the Brazilian legislation, the ash content obtained in the propolis of Suba-Bogotá make them promising as nutritional additives for the human diet, the ash content also indicates the mineral content since when calcining a sample evaporates much of the amount of organic matter and remains only the minerals, highlighting that minerals are very important for biological activity and cellular metabolism, for the human being can prevent arteriosclerosis and the increase of the immune capacity, the mineral content is used to characterize geographical areas where propolis is produced since it can be an indicator of environmental pollution of the environment where Apis mellifera collects its raw material to produce its propolis, the presence of iron, chromium, nickel, copper and lead are considered to be stable complexes that form the same flavonoids. (Betances, 2018).

The propolis environments were different, the environment of Pacho was rural, a eucalyptus forest and the environment of Bogota was more urban, full of bushes, flowers and various trees, according to the literature, eucalyptus trees contain more phenols and flavonoids, therefore it may affect the content of flavonoids and phenols is higher in Pacho, also depends on the climate affects the oxidative property which is an index of the amount of phenols and flavonoids in propolis.

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