Spectrophotometric Determination of Phenolic Compounds in Propolis

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SUMMARY. Three spectrophotometric methods were evaluated in order to determine total phenolic compounds content in propolis from Tucumán, Argentina; applying the Folin-Ciocalteau, Prussian blue and o-phenanthroline methods. The Prussian blue method was the most sensitive one although it was also unstable. The o-phenanthroline method may be more reliable and proved to be quite sensitive and stable. Folin-Ciocalteau method was the most stable and reproducible of all. The statistic analysis for such methods as well as for each of the controls used showed that all the three methods were different at 1% significance levels, except for the Prussian blue and o-phenanthroline methods, which yielded no significant differences for the quercetin control solution.

RESUMEN. “Métodos Espectrofotométricos para la Determinación de Compuestos Fenólicos en Propóleos”. Se evaluaron tres métodos espectrofotométricos para la determinación de compuestos fenólicos totales en propóleos de la provincia de Tucumán, Argentina, mediante los métodos de Folin-Ciocalteau, azul de Prusia y o-fenantrolina. El método de azul de Prusia fue el más sensible aunque inestable; el método de o-fenantrolina fue bastante sensible y más estable que el anterior mientras que el de Folin-Ciocalteau fue el más estable y reproducible. Al comparar estadísticamente los tres métodos usados se encontró que a un nivel de significación del 1% los tres métodos son diferentes, salvo azul de Prusia y o-fenantrolina, en los que no hay diferencias significativas, utilizando el patrón quercetina.

INTRODUCTION

Propolis is a resinous mixture collected from trees 1 by the Apis mellifera bee, which uses it as a building insulating material in the beehive as well as for keeping it in good health. It has important pharmacological properties and it can be used for a wide range of purposes as anti-inflammatory and hypotensive agent, immune system stimulant, and bacteriostatic and bactericidal agent, among many other uses 2,3. All such applications have increased its pharmaceutical demand and have rendered it an interesting subject of study. Its fairly complex chemical composition 4,5 includes phenols, tannins, polysaccharides, terpenes, aromatic acids and aldehydes, among other compounds.

In Argentina, the INAL (The National Food Institute) recognized propolis as a diet supplement in 1995 (file 2110-003755-4 in the Argentine Food Code).

Up to the present, Argentina has no regulations regarding propolis quality. A number of techniques are being compiled so as to comprise several parameters of importance for this natural product. Such regulation 6, IRAM 15935.2000 - Chart A - item 3.1, on the quality of propolis and its by-products, states:

Raw Propolis. A group of resinous, gummy, and balsamic substances of viscous consistency that is collected by bees (Apis mellifera) from some vegetable species (pines, willows, birches, several species of poplars, ash trees, oaks, and the like), in which flavonoids and phenolic compounds are the most important substances.

KEYWORDS: Phenolic compounds, Propolis, Spectrophotometry.

PALABRAS CLAVE: Compuestos fenólicos, Espectrofotometría, Propóleos.
In Annex A in the same Regulation:
Major Propolis compounds. One hundred and sixty compounds have been identified. Over fifty percent are phenolic compounds thought to have pharmacological properties.

The mean phenolic compound content in samples from North-western Argentina has been reported to be 25%.

There is a wide range of methods for determining total phenolic compounds in vegetables such as column chromatography in aluminum oxide and gas-liquid chromatography, as well as some spectrophotometric methods.

Due to the significance of the total phenolic compounds quantification in propolis, this paper assesses the response of three spectrophotometric methods such as Folin-Ciocalteau, Prussian blue, and o-phenanthroline methods so as to select the fastest and most reliable one to be included in future regulations for propolis’ quality in Argentina.

MATERIALS AND METHODS
Reagents
All the reagents used were pro analysis: gallic acid, quercetin dihydrate, 3,4-dihydroxybenzoic acid, caffeic acid, vanillin, potassium ferrocyanide, ferric chloride and 1,10-phenanthroline (Sigma); absolute ethyl alcohol, sodium molybdate dihydrate, sodium tungstate dihydrate, 85% phosphoric acid, 36% hydrochloric acid, sodium carbonate and sodium acetate (Merck).

Equipment
UV-Vis Beckman DU 7500 spectrophotometer; Anton Paar DMA 58 digital Densimeter; Vicking thermostatic bath Dubnoff model.

Calibration curves
Five substances were chosen out of all the compounds frequently found in propolis: 3,4,5-trihydroxybenzoic acid (gallic acid); 3,3',4',5,7-pentahydroxyflavone (quercetin), 3,4-dihydroxybenzoic acid; 3,4-dihydroxyphenylacetic acid (caffeic acid); and 4-hydroxy-3-methoxybenzaldehyde (vanillin).

Control solutions of each phenolic compound at concentrations between 1.10^{-3} and 2.98.10^{-3} M were prepared. The control curves were obtained by applying the following spectrophotometric methods: Folin-Ciocalteau, Prussian blue, and o-phenanthroline.

Folin-Ciocalteau Method. The reagent consists of 100 ml of sodium tungstate dihydrate, 25 of sodium molybdate dihydrate, 50 ml of 85% phosphoric acid solution and 100 ml of 36% hydrochloric acid solution. The volumes of control solution used ranged from 5 µl up to 400 µl; 2 ml of 15% Na_{2}CO_{3} solution and 0.5 ml of the reagent were added to each one. Finally, distilled water was added until a final volume of 10 ml was reached. The preparation was heated for five minutes in a 50 °C thermostatic water bath. After cooling at room temperature, absorbance was measured at 765 nm.

Prussian blue Method: the volumes of control solution used ranged from 5 µl up to 400 µl. The following mixture was added to each dilution of the control solutions: 400 µl of 0.0008M K_{2}Fe(CN)_{6} and 400 µl 0.1M FeCl_{3} in 0.1 M HCl solution. The final volume was 10 ml. Seven minutes later, absorbance was measured at 700 nm.

o-Phenanthroline Method: the volumes of control solution used ranged from 5 µl up to 400 µl. Then, 300 µl of 0.2M CH_{3}COONa were added to each one in order to keep the solution within a range from 3 to 4 pH values. 200 µl of 0.1M FeCl_{3} and 200 µl of 0.5% o-phenanthroline solution were added until a 10 ml final volume was obtained. Absorbance was measured after 24 h in the dark at 500 nm. Precautions were taken so that both the solution to be measured and the corresponding control showed the same pH values.

Absorbance versus g x10^{-5} of standard phenol used was plotted for every method.

The calibration curves presented different phenol concentration ranges for the application of the Lambert-Beer’s Law: the Folin-Ciocalteau method yielded the highest range (4.25 10^{-6} up to 1.5 10^{-4} g), the Prussian blue method showed an intermediate range (1.64 10^{-6} up to 1.14 10^{-4} g) and was the most sensitive one; finally, the o-phenanthroline method presented the lowest range (2.53 10^{-6} up to 3.74 10^{-5} g). All the calibration curves showed statistically good R^2 values ranging from 0.990 up to 0.999, as shown in figures 1-3.

Samples and ethanolic extracts
Fourteen propolis samples from northwestern Argentina were used. They came from different Tucumán districts: Trancas, El Manantial and Amaicha del Valle (2000 m over sea level) situated 164 km from San Miguel de Tucumán. All the samples were collected from different sites inside the beehive (Table 1).

Propolis samples were kept in the dark in a freezer. They were mechanically cleaned and pulverized when cold until very fine particles were obtained.
Consequently, 0.1 g of each sample was weighed and 30 ml of 80% ethanol were added. After 12 h maceration, it was shaken for 30 min at 30°C and 50 rpm. Supernatant was filtered 5 h later. The procedure was repeated five times and phenol extraction completion was confirmed when FeCl₃ test strips turned green in the presence of such compounds. In all cases a 250 ml gauge glass was used for filtration. The volume was later on increased with 80% ethanol obtaining propolis ethanolic extracts (PEE) of measured density of 0.84571 to 0.85480 g/ml.

PEE aliquots were taken and analyzed by the Folin-Ciocalteau, Prussian blue and o-phenanthroline methods. The complete procedure described elsewhere for the calibration curves was performed three times for each one of the studied samples.

The presence of mean phenolic compound values were obtained from the absorbance readings by using the corresponding calibration curves and expressed according to each phenolic standard. When absorbance readings gave very low values for any of the methods, the measurements were repeated with a higher quantity of PEE, reaching always final volumes of 10 ml.

RESULTS AND DISCUSSION

Total phenolic compounds concentrations in propolis samples analyzed and obtained by the spectrophotometric methods applied in this work, were expressed in weight percent as gallic acid, quercetin, 3,4-dihydroxybenzoic acid, caffeic acid and vanillin. The values are shown in Table 2.

Figure 1. Folin-Ciocalteau Method. Calibration Curves for: Gallic acid (R = 0.999); Quercetin (R = 0.997); 3,4-dihydroxybenzoic acid (R = 0.998); Caffeic acid (R = 0.997); Vanillin (R = 0.999).

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The presence of mean phenolic compound

<table>
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<tr>
<th>Sample</th>
<th>Area</th>
<th>Sampling Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aguadita (Amaicha del Valle)</td>
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<tr>
<td>2</td>
<td>Aguilares</td>
<td>Inside the beehive</td>
</tr>
<tr>
<td>3</td>
<td>El Manantial</td>
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<tr>
<td>4</td>
<td>D.A.(Trancas)</td>
<td>Inside the beehive</td>
</tr>
<tr>
<td>5</td>
<td>J.L. (Trancas)</td>
<td>Opening of beehive</td>
</tr>
<tr>
<td>6</td>
<td>El Pozo (Trancas)</td>
<td>Opening of beehive</td>
</tr>
<tr>
<td>7</td>
<td>Locadio Paz G.B. (Trancas)</td>
<td>Opening of beehive</td>
</tr>
<tr>
<td>8</td>
<td>El Molino (Amaicha del Valle)</td>
<td>Inside the beehive</td>
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<tr>
<td>9</td>
<td>La Banda (Amaicha del Valle)</td>
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<tr>
<td>10</td>
<td>E.R.P. (Amaicha del Valle)</td>
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<tr>
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<td>Fronterita. Los Zazos (Amaicha del Valle)</td>
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<td>12</td>
<td>J.L.II (Trancas)</td>
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<td>13</td>
<td>El Pozo (Trancas)</td>
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<td>14</td>
<td>El Mirador (Amaicha del Valle)</td>
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Table 1. Origin and source of sample collection in the beehive.
Methods

<table>
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<th>B</th>
<th>C</th>
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<th>G</th>
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**Table 2.** Percentage concentrations of phenolic compounds expressed as, **G:** gallic acid. **Q:** quercetin. **B:** 3,4-dihydroxybenzoic acid. **C:** caffeic acid. **V:** vanillin.

method was more sensitive than the Folin-Ciocalteau method for the same samples. On the other hand, although showing low sensitivity, the Folin-Ciocalteau method is the most reproducible when immediate results are expected. Since an undesirable precipitate is produced and enhanced with time, a highly skilled operator is of the utmost significance when using the Prussian blue method. When o-phenanthroline was employed, the reactive mixture required a 24 h period before it was able to stabilize the color as well as strict pH checking so that the blank reagent and the samples had the same acidity. This involves a time-consuming procedure for the obtention of results.

The difference in phenolic compounds values obtained from the three methods applied and for the same phenolic control is accounted for by the reagent sensitivity, the different redox potential of the systems used, and in particular by the higher reducing capacity of the phenol group in a basic medium. It should be noted that the reaction medium was different for the methods studied.

In the Folin-Ciocalteau method, the phosphotungstic reagent acting in a basic medium ionized phenols, which rendered them more reducing agents. When the Prussian blue and o-phenanthroline reagents were used in an acid medium, phenols were not ionized. This fact may explain their modest reducing capacity.
Samples Quality

With regard to propolis sample quality, according to regulation RST 317-77 from the former USSR which require values greater than 30% for phenolic compounds, the highest qualities were observed in the samples 1, 8, and 11 (Table 2).

Only sample 8 from El Molino (Amaicha del Valle) collected from inside the beehive reached the required value for phenolic compounds content.

The propolis collected from the beehive opening (samples 5, 6, 7, 9, 12, and 14) contains low values of phenolic compounds, which is probably due to solar and light exposure. Such an affirmation is based on the literature stating that propolis must be protected from external factors in order to preserve its properties, thus preventing oxidation of the bioactive compounds.

Statistic Analysis of Results

The phenol percent variable resulting from the Folin-Ciocalteau, Prussian blue, and o-phenanthroline methods was used with 5 control solutions (gallic acid, quercetin, 3,4 dihydroxybenzoic acid, caffeic acid, and vanillin).

First, the variance analysis performed for a factor that involves each combination of method-control solution at a level of significance of 1%, suggests that comparisons among different factors are statistically different (Table 3).

Then, in order to eliminate the effect of the various controls employed, comparisons among methods for each control used were carried out. The t test analysis for matched samples was performed (Table 4).

CONCLUSIONS

Operatively, due to its reproducibility, the Folin-Ciocalteau method is recommended for propolis with high phenolic compound concentrations.

The formation of the Prussian blue complex is a sensitive and rapid method for the spectrophotometric determination of total phenols. Therefore, it would be recommendable for very diluted PEE although the handling of the reactive mixture can be rather laborious.

The method using o-phenanthroline as a reagent required a longer procedure for obtaining good results.

Statistically, all the comparisons performed with the three methods for each control solution at 1% significance level, proved to be different.

However, when the Prussian blue and the o-
phenanthroline methods using quercetin as control were used no significant differences were found. Both methods are strongly recommended (Table 4).

As a result of the statistical analysis performed, it would be highly convenient to express the results in terms of quercetin owing to its high solubility in water, excellent reproducibility in the measurements, small SD as well as its low student t parameter.

However, when such a control is unavailable, vanillin can be used because it shows similar behaviour although it presents an acceptable value for the statistical t parameter.

Thus, a contribution to typifying such a diet supplement is presented here in order to complement the IRAM 15935.2000-Chart A-Norms in an attempt to propose selected methods which are fast and reliable to be included in Argentina’s regulations for propolis’ quality.

Acknowledgements. The financial support from the Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT) and to Susana Chauvet for the statistical analysis.

REFERENCES