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ORIGINAL RESEARCH ARTICLE

Influence of the harvesting procedure and extracting process on the antioxidant capacity of ethanolic propolis extracts

Diego Archaina[a,b], Roy Rivero[a,b], Natalia Sosa[a,b] and Bertha Baldi Coronel[a]*

[a]*Facultad de Bromatología, Universidad Nacional de Entre Ríos, Gualeguaychú, Argentina;* [b]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina*

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Under article 1339 of the Argentine Food Code, food containing propolis is considered dietary. Propolis is a complex mixture of over 200 compounds, mainly polyphenols, which are classified as bioactive, and have important biological effects. Its composition depends on the origin of the sample, and therefore the relationship between flavonoids and biological effects is of interest to assess those constituents. Propolis may be suitable for use in the formulation of functional foods or potentiate the biological effects of other food constituents, combining and acting together. Propolis samples were obtained by scraping and trapping. Extractions were also carried out with dissolutions in 70, 80, and 85% ethanol at room temperature, by mixing and stirring; and by the Soxhlet method. The concentration of the resulting extracts was achieved at 30 and 40 °C. In all cases the antioxidant capacity was measured by the Trolox method, the efficiency of the extraction and the concentration of the total flavonoids and phenols were calculated. We found that the temperature which is used to concentrate them has influence on their antioxidant power, the latter being lower at lower temperature. Soxhlet extraction decreases antioxidant capacity as well as content of flavonoids and phenolic compounds. The resulting extracts showed good antioxidant capacity and didn't show direct relationship with ethanol concentration.

Influencia del procedimiento de recolección y del proceso de extracción en la capacidad antioxidante de los extractos etanólicos de propóleos

En virtud del artículo 1339 del Código Alimentario Argentino, los alimentos que contienen propóleo se consideran dietéticos. El propóleos es una mezcla compleja de más de 200 compuestos, principalmente polifenoles, los cuales están clasificados como bioactivos, y tienen importantes efectos biológicos. Su composición depende del origen de la muestra, y por lo tanto la relación entre los flavonoides y los efectos biológicos es de interés para evaluar a sus constituyentes. El propóleos puede ser adecuado para su uso en la formulación de alimentos funcionales o potenciar los efectos biológicos de otros componentes de los alimentos, combinándolos y actuando juntos. Se obtuvieron muestras de propóleos por raspado y recogida. Las extracciones también se llevaron a cabo con disoluciones en 70, 80, etanol 85% a temperatura ambiente, mezclando y agitando; y por el método de Soxhlet. La concentración de los extractos resultantes se logró a 30 °C y 40 °C. En todos los casos la capacidad antioxidante se midió por el método de Trolox, se calculó la eficiencia de la extracción y la concentración de los flavonoides totales y fenoles. Se encontró que la temperatura que se utiliza para concentrarlos tiene influencia en su poder antioxidante, siendo este último inferior a la temperatura más baja. La extracción Soxhlet disminuye la capacidad antioxidante así como el contenido de flavonoides y compuestos fenólicos. Los extractos resultantes mostraron una buena capacidad antioxidante y ninguna relación directa con la concentración de etanol.

Keywords: propolis extract; antioxidant capacity; flavonoids; trolox

Introduction

Raw propolis is a resinous substance prepared by bees as a result of mixing the resin obtained from plants with their salivary secretions, and its composition varies depending on geographic location, botanical origin, climatic factors, and seasonal effects in their areas of origin (Agüero et al., 2014; Falcão et al., 2013; Lima et al., 2009; Mendes da Silva, de Souza, Matta, Ribeiro De Andrade, & Nova Vidal, 2006). It is composed of around 50% resins (flavonoids and phenolic acids), 30% waxes, 10% essential oils, 5% pollen, and 5% of various organic compounds (Baldi Coronel, 2010; Falcão et al., 2010).

Propolis have been ascribed many medicinal properties such as: binder, immunomodulatory, antibiotic, antimicrobial, antifungal, anti-inflammatory, hepatoprotective, antioxidant, anti-hemorrhagic, dewormer, stimulating regeneration of epithelium, reducing cholesterol, energizing, detoxifying, tonic, anticancer, and antitumor, therefore has been studied worldwide (Agüero et al., 2011; Herrera, Alvear, Barrientos, Montenegro, & Salazar, 2010; Pistellii & Giorgi, 2012; Saavedra et al., 2011). Also, it exerts inhibitory effect against various viruses. This activity is attributed to the content of phenolic compounds, especially caffeic acid, caffeic esters, ferulic

*Corresponding author. Email: bbaldi@fb.uner.edu.ar

acid (3-methylbut-2-enyl caffeate, 3-methylbutyl ferulate), and aglycones flavónicas (luteolin and quercetin), very active against the virus herpes (El-khawaga, Salem, & Elshal, 2003; Hegazi & Abd El Hady, 2002).

Due to these characteristics, propolis is considered a functional ingredient, and in recent years has been considered as an important substance which can be used as component of biocosméticos and health food for multiple purposes (Bankova, de Castro, & Marcucci, 2000; Burdock, 1998; Wollenweber & Buchmann, 1997).

Moreover, it is noteworthy that propolis is a very viscous material and has many impurities. For use it must be purified by solvent extraction, removing the wax and organic waste preserving polyphenolic fraction containing most of the bioactive components (Kalogeropoulos, Konteles, Troullidou, Mourtzinos, & Karathanos, 2009). The most widely used solvent is ethanol, and by successive extractions dewaxed propolis extracts rich in polyphenolic components are obtained which give it its importance as a natural product of high biological value (Araújo da Silva et al., 2014).

Considering that in recent years the world has been invaded by new forms of production and consumption; a marked concern was generated in consumers by the unstoppable deterioration of natural resources. This leads people to think in consuming less artificial foods and return to processed foods with natural components that establish in the organism some specific function and beneficial. In this sense, a growing demand was observed in the industry of propolis, because it could be used as an ingredient in the formulation of nutritious food and with high added value; their inclusion is highly justified due to the important properties possessed since it is a concentrate of flavonoids (Araújo da Silva et al., 2014). The phenolic compounds, including the flavonoids represent clearly the quality of the final product. If the percentage of these fractions is higher, the propolis will be purer and of better quality (Ahn, Kumazawa, Hamasaka, Bang, & Nakayama, 2004). Regulations in Argentina and other countries like Brazil set the expected values of these compounds in order to establish minimum quality requisites to comply with.

The aim of this work was determine the influence of harvesting method and extraction process in the antioxidant capacity of ethanol extracts propolis optimizing a methodology to conserve natural active ingredients (flavonoid content and antioxidant power).

Materials and methods

Harvest and conditioning of crude propolis

Samples of propolis were collected from apiaries located in Gualeguaychú, Entre Rios, Argentina, in summer during the months of January–February 2013 using different harvest methods: sample 1 (M_1) obtained by the scraping method, which employs a stainless steel spatula to remove product stuck in the sides, cover, and between covers of the hive. Sample 2 (M_2) obtained by matrixed plastic mesh that once covered the material, is removed from the hive and stored in freezer at −20 °C to facilitate removal of the product. The third sample (M_3) was obtained by mixing the above two to obtain a representative sample as industrial scale is made. The sampling was done at random.

Ethanolic extract of propolis

The Ethanolic Extracts of Propolis (EEP) were obtained using two methodologies successive extractions employing stirring at room temperature (a) and Soxhlet extraction (b). The EEP were identified as shown in Table 1.

Successive extractions employing stirring at room temperature

The extraction was performed using different concentrations of ethanol (70, 80, and 85%). The EEP were obtained using the modified methodology of Sawaya et al. (2004), where 5 g of crude propolis was mixed with 50 ml of ethanol solution and stirred using a magnet stirrer for 30 min. The liquid was separated and the residue was re-extracted with the same portion of alcohol three times, obtaining a total volume of 200 ml, which is then kept in a refrigerator at 4 °C for 48 h and then filtered to separate the waxes of the extracts. The filtered extract was divided into two portions of 100 ml, which were subjected to different thermal treatments (30 and 40 °C) for 1 h to evaluate the influence of temperature.

Soxhlet extraction

This was performed according to the modified method of Cunha et al. (2004). First, an extraction was performed employing hexane in order to remove waxes and then ethanol 96° was then added to obtain propolis resins. Two grams of sample was mixed with 150 ml of hexane for the first extraction and 150 ml of ethanol for the second extraction. The liquids coming from the extractions with hexane and ethanol were stored for the subsequent determination of the percentage of waxes and resins, respectively.

Table 1. Code used to identify the different extraction methods.

	Stirring			
	Ethanol (%)			
Extraction process	70	80	85	Soxhlet
A	●			
B		●		
C			●	
D				●

Efficacy of the extraction

The theoretical content of resins extracted by the stirring method ($R_{Stirring}$) was determined (in quadruplicate samples) gravimetrically in an oven Ionomex MCH (Buenos Aires, Argentina) at 100 °C to constant weight. The results were expressed as percentage in dry basis (% d.b.). The performance of the extracts was calculated by comparing these values with the resins' actual content extracted by Soxhlet ($R_{Soxhlet}$) refer to the amount of actual resin in the crude sample. Comparing the theoretical amount with the actual the efficacy of the extraction using Equation (1) was determined . This relationship was used to determine the effectiveness because the Soxhlet method, used as reference method, extracts 100% of resins (INTA IRAM-15935-2/2008).

$$\text{Extraction efficacy} = \frac{R_{Stirring}}{R_{Soxhlet}} x100 \qquad 1$$

where $R_{Stirring}$ is the grams of resins obtained for agitation present in 100 g of sample and $R_{Soxhlet}$ is the grams of resins obtained for Soxhlet present in 100 g of sample.

Determination of the antioxidant power of EEP

The antioxidant activity was determined by the TEAC (Trolox Equivalent Antioxidant Capacity) assay according to the procedure proposed by Re et al. (1999) using the 2,2′-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid] to produce the cationic free radical $ABTS^{\bullet+}$. ABTS was dissolved in distilled water to yield a 7 mM solution. Radical cation solution was prepared by incubating .0194 g of ABTS and .0033 g of potassium persulfate for 16 h in darkness at room temperature and subsequently diluted with phosphate buffer pH 7.4 to a final absorbance of 1.00 ± .01 at 734 nm.

For antioxidant power determination, .1 ml of dilution 1/1000 of EEP was added to a cuvette containing 1.9 ml of the ABTS solution incubated for 30 min (t_{30}) at 25° C and absorbance was monitored using a spectrophotometer Jenway 6505 ultraviolet-visible at 734 nm. The Trolox stock (Sigma-Aldrich) solution 4 mM (1 mg/ml) was used to construct a calibration curve in a range of concentrations between .02 and .12 mg/ml of Trolox (r^2 = .9980). The results were expressed as milliequivalents of Trolox per gram of EEP in dry basis (meq Trolox/g EEP). All measurements were performed in triplicate and the results were reported as the mean ± standard deviation.

Total flavonoid contents

Total flavonoid contents in EEP were determined by the method of Kumazawa, Hamasaka, and Nakayama (2004), with minor modifications. To .1 ml of EEP solution, .5 ml of 2% $AlCl_3$ ethanol solution was added. After 30 min at room temperature, the absorbance was measured using a spectrophotometer Jenway 6505 ultraviolet–visible at 425 nm. Solutions of quercetin (Sigma-Aldrich) between 2 and 10 mg/ml were used to construct the calibration curve (r^2 = .9997). Total flavonoid contents were calculated as mg of quercetin equivalent per gram of EEP in dry basis (mg QE/g EEP). This procedure was performed with each of the studied propolis in triplicate and the results were reported as the mean ± standard deviation.

Total polyphenol content

Total polyphenol contents in EEP were determined by the Folin-Ciocalteau colorimetric method (Prior, Wu, & Schaich, 2005). EEP solution (.1 ml) was mixed with water (10 ml) and 1.0 ml of the Folin–Ciocalteau reagent (Merck), was stirred and allowed to stand for 2 min at room temperature. Subsequently 4 ml of 20% Na_2CO_3 was added, made up to volume with distilled water up to 25 ml placed in water bath at 50 °C for 5 min and the absorbance was measured using a spectrophotometer Jenway 6505 ultraviolet–visible at 765 nm after 1 h cooled to room temperature. Solutions of gallic acid (Sigma-Aldrich) between .1 and .5 mg/ml were used to construct the calibration curve (r^2 = .9984). The results were expressed as mg of gallic acid equivalents per gram of EEP in dry basis (mg GAE/g EEP). This procedure was performed in triplicate samples and all results were reported as the mean ± standard deviation.

Statistical analysis

The results were statistically analyzed by the analysis of variance (ANOVA) to determine significant differences between the samples. The analysis of the means was performed through the LSD Fisher procedure at $p < .05$ using the software Infostat v.2008 (Di Rienzo et al., 2008). The simple linear regression parameters were analyzed using the software XLSTAT, version 2012.6.08. The correlation between parameters was determined by measuring the linear correlation coefficient (r) ratio of the linear relation between two variables, random and quantitative.

Results

Efficacy of the extraction process

In order to investigate the efficacy of the process to obtain EEP, different ethanol concentrations were used so as to calculate the amount of resin (%) present in the propolis samples which were extracted by the methods of stirring and Soxhlet. Table 2 shows how the effectiveness of the extraction process increases when the concentration of ethanol increases. Besides, a significant difference is observed between the percentages of extraction efficiency for the same concentration

Table 2. Efficacy of the process of stirring according to the different ethanol concentrations used. Resin contents extracted by the stirring and Soxhlet methods.

Samples	Extraction process	$R_{Stirring}$ (%)	$R_{Soxhlet}$ (%)	Extraction efficiency (%)
M_1	A	41.36 ± 1.77^{f}	55.76 ± 3.04^{c}	74.17
	B	49.24 ± 3.23^{de}		88.30
	C	51.60 ± 3.05^{cd}		92.53
	D			100.00
M_2	A	47.65 ± 2.99^{e}	$74.02 \pm .50^{a}$	64.37
	B	53.35 ± 3.92^{bc}		72.08
	C	56.15 ± 3.44^{ab}		75.86
	D			100.0
M_3	A	48.83 ± 1.59^{de}	60.44 ± 1.53^{b}	80.79
	B	48.77 ± 3.13^{de}		80.69
	C	57.47 ± 2.94^{a}		95.08
	D			100.00

Note: The averages (n = 3) with different letters (a,b,c,d,e,f) are significantly different. ($p < .05$).

of ethanol due to harvesting method. The percentage is slightly higher compared M_1 to M_2, because the sample 1 was obtained by scraping, method that extracts propolis accompanied by external impurities incorporated by the producer when collected. The presence of impurities in the EEP throws an error by default in the gravimetric method. For M3, which is a mixture of the two, the percentage of effectiveness is observed about the average.

Antioxidant capacity

Figure 1 shows the results of the antioxidant capacity obtained for the EEP according to the procedures of extraction and subsequent heat treatment at the studied temperatures (30 and 40 °C). As for the antioxidant capacity, it can be seen that the ABTS values vary significantly from one extract to other ($p < .05$). There was no direct relation between the ethanol concentration and the antioxidant power. Figure 2 presents the resins' content and capacity antioxidant of EEP obtained by the stirring method, heated at 30 °C corresponding to samples M_1, M_2, and M_3.

Flavonoid content and total phenolic compounds

Figure 3 shows the flavonoid contents (Figure 3(a)) and total phenolic compounds (Figure 3(b)) present in the EEP, which were obtained in different ethanol concentrations and treated at different temperatures. The

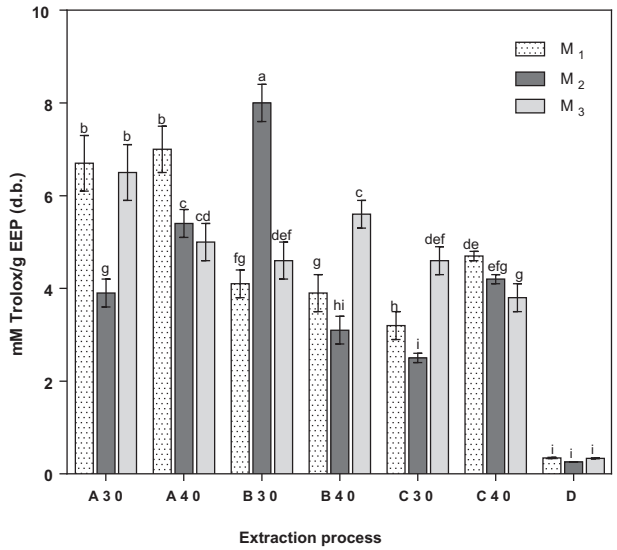

Figure 1. Antioxidant capacity of the EEP corresponding to samples M_1, M_2, and M_3 extracted by the stirring method, heated at 30 °C (A30, B30, C30) and 40 °C (A40, B40, C40). Soxhlet method (D).
Note: The averages ($n = 3$) with different letters are significantly different. ($p < .05$).

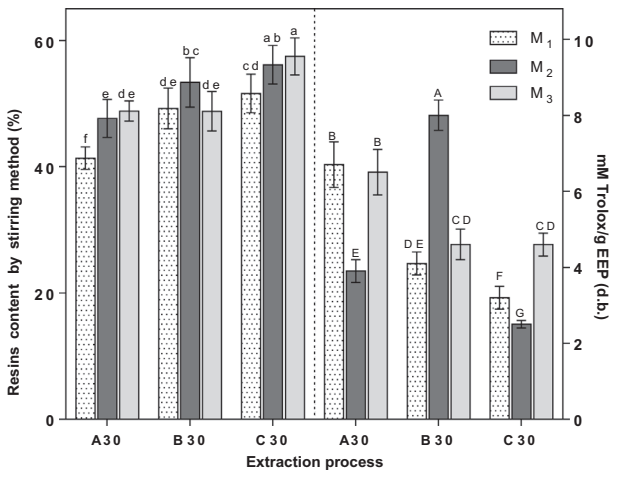

Figure 2. Resins content (left *Y* axis) and capacity antioxidant (right *Y* axis) of EEP obtained by the stirring method, heated at 30 °C (A30, B30, C30) corresponding to samples M_1, M_2, and M_3.
Notes: The mean ($n = 3$) corresponding to bars of resins contents containing a different lowercase letter are significantly different ($p < .05$). The mean ($n = 3$) corresponding to bars of capacity antioxidant containing a different uppercase letter are significantly different ($p < .05$).

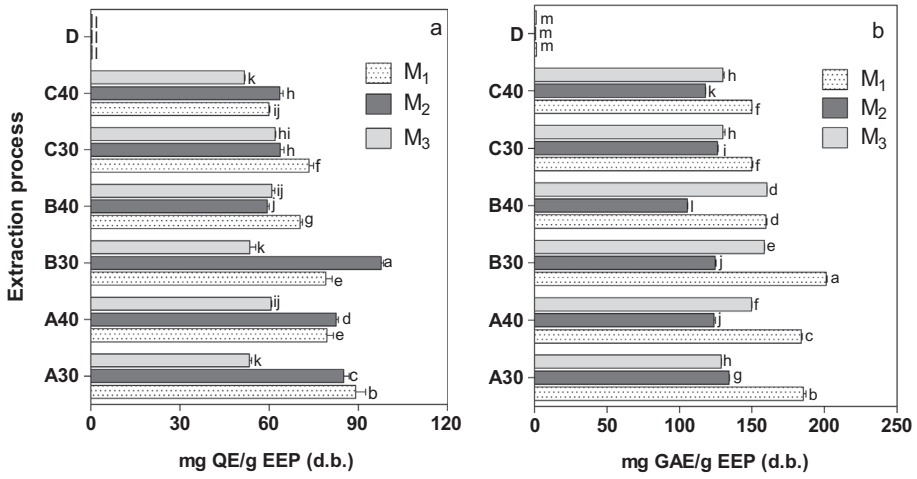

Figure 3. Contents of flavonoids (a) and total phenolic compounds (b), according to extraction process: Stirring at 30 °C (A30, B30, C30) and 40 °C (A 40, B40, C40), Soxhlet method (D).
Note: The averages (n = 3) with different letters are significantly different. (p < .05).

results indicate that M_1 at 30 °C and at the different ethanol concentrations maintains an average content of flavonoids of 80.5 mg QE/g EEP, which decreases 13.2% at 40 °C. With respect to M_2, we observed that the lowest temperature showed an average content of 82.2 mg QE/g that decreases 16.7% at 40 °C. As for M_3, minimum significant differences were observed in the total flavonoids at 30 and 40 °C.

Correlations and relations

Figure 4 shows the parameters of simple linear regression for the relations between the flavonoid contents and the total phenolic compounds. The analyzed EEP presented a linear relation of .8714, confirming good correlation, due to the fact that when this value is near 1, the grade of association between variables is higher. Moreover, being positive, this indicates a direct relation, i.e., they tend to vary in the same direction. The coefficient of determination (r^2) was measured. This enables us to quantify the goodness of fit of the linear relation (r). Our results, r = .8714 and r^2 = .764, show a good level of linear association between the variables because 76.4% of the variation can be explained through the adjusted regression line.

Figure 5 shows the correlation between the antioxidant capacity of the EEP and the total flavonoid contents. It was also observed a direct and positive relation (r = .817 and r^2 = .668) but not completely linear. Figure 6 presents the correlation between the antioxidant capacity and the total phenolic compounds. Values of r = .751 and r^2 = .563 were obtained. It is observed

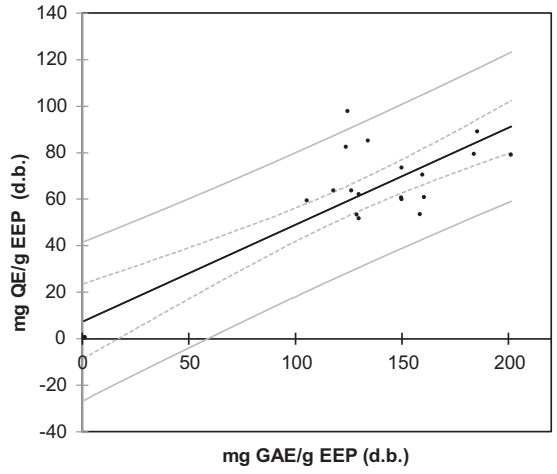

Figure 4. Regression of mg QE/g EEP vs. mg GAE/g EEP (dry basis).
Notes: Active (●), model (black continuous line), confidence interval (Mean 95%) (grey dotted line), confidence interval (Obs. 95%) (grey continuous line).

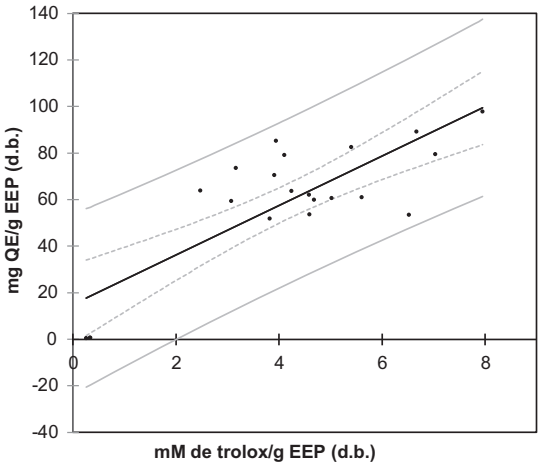

Figure 5. Regression of mg QE/g EEP vs. mM Trolox/g EEP (dry basis), active (●), model (black continuous line), confidence interval (Mean 95%) (grey dotted line), confidence interval (Obs. 95%) (grey continuous line).

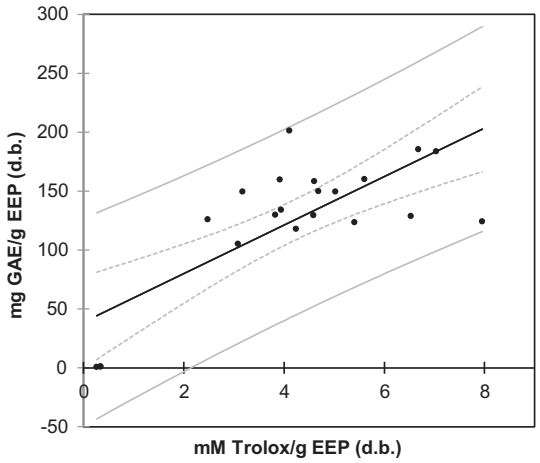

Figure 6. Regression of mg GAE/g EEP vs. mM Trolox/g EEP (dry basis), active (●), model (black continuous line), confidence interval (Mean 95%) (grey dotted line), confidence interval (Obs. 95%) (grey continuous line).

that although the correlation is direct, the percentage was lower (75%) and only 56% of the variation in the values can be explained with the adjusted regression line.

Discussion

Considering that the beekeeping industry manufactures mixtures to obtain composite and homogeneous samples, added to the fact that the general composition of propolis can vary according to the harvest method which is used (Agüero et al., 2014; Mendes da Silva et al., 2006). According to the results shown in Table 2, is fundamental optimizing the process of obtaining the EEP because the percentage of the ethanol fraction is a parameter that is directly related to the extraction yield. These results agree with Martínez Rojas, Fajardo Cárdenas, and Pérez Morales (2005) those found that to higher concentration of ethanol in the mixture hydroalcoholic the highest yield was obtained.

Agreeing with Palomino García (2009) the highest yield of ethanol extract was obtained in propolis collected by the method of mesh, which has a percentage by weight relative to dry matter exceeds 30%.

According to the Argentine Food Code, it is established in the article 1384: IRAM norms 15935-1 and 15935-2 that EEP must contain at least 30% resin extracted from raw propolis to be marketed safely. This condition has been verified in this study. Considering the data obtained in Figure 1 the temperature values studied in this work have influence on the antioxidant power of the extracts obtained by stirring, being lower at lower temperature and drastically decreasing the antioxidant capacity of the extracts when exposed to very high temperatures, like the ones used in the Soxhlet method (D).

Regarding the content of phenolic compounds, we agree with Ahn et al. (2004), variation in antioxidant capacity would be given by the composition of phenolic components present in the original sample and not in the percentage of EEP. Therefore, it is possible to relate the presence of polyphenols with the antioxidant capacity of the extracts. So if the samples had major contents of phenols and total flavonoids, it would be expected to obtain higher antioxidant capacity.

It is noteworthy that no relationship was observed between the content of total resins extracted in the EEP and antioxidant power value (Figure 2). The values observed in Figure 3 are comparable to the ones reported by Isla, Nieva Moreno, Sampietro, and Vattuone (2001) about propolis in different regions of Argentina, which presented values that ranged from 13.3 to 62.0 mg QE/g extract. Also comply with IRAM Standard 15935-1/2004 which establishes for the phenolics compounds the minimum value of 50 mg GAE/g EEP and flavonoids 5 mg QE/g EEP.

If we relate these values to the content of total phenolic compounds, in the described conditions, it is observed that M_2 presents a higher content of flavonoids and the M_1 has a higher content of total phenolic compounds. This would be connected, on one hand to, with the harvest method, because M_2 was obtained by trapping, what ensures a higher purity. In contrast, the scraping method used in M_1 may affect the quality of the extract containing impurities of external nature, obtaining a lower phenolic fraction in the total resin content. On the other hand, other existent secondary metabolites, different from those of phenolic nature (terpenoids, prenylated organic acid derivatives, lignans) cause a variation in the final values of both parameters (Cuesta-Rubio et al., 2007; Duran et al., 2008; Salamão et al., 2008).

The correlation between content of flavonoids and total phenolic compounds shown in the Figure 4 was positive indicating that a large amount of phenolic content can be derived from the flavonoids contents coinciding with the values obtained by Cottica et al. (2011) who obtained a correlation positive of .8679.

According to that seen in Figure 5, the correlation between the antioxidant capacity of EEP and total flavonoid content is not completely linear, because antioxidant activity assay measure the expression of all the reducing compounds (flavonoids and other compounds with active sites of electron transfers), whereas the method measures only the flavonoid contents. Considering this particular case in which the spots are disperse, the expression (antioxidant capacity) does not depend entirely on the analytical measure (flavonoid contents) (Ahn et al., 2004).

However, the relationship between total antioxidant capacity and total phenolic compounds shown in Figure 6 is different from that occuring with total flavonoids, because the correlation coefficient is only .563. It is assumed that this difference is due to other compounds, apart from flavonoids, in the group of total phenolic compounds, which produce a dispersion of the values of the antioxidant power, as they do not have the same

capacity. Therefore, the antioxidant capacity in the extracts should be mainly due to the composition of phenolic components in the original sample instead of the percentage of ethanol extract.

Furthermore, the Soxhlet extraction proved to decrease considerably the content of flavonoids and phenolic compounds, due to exposure to high temperature and prolonged extraction time, obtaining minimum values of antioxidant power. According to Ahn et al. (2004) it can be inferred that the antioxidant capacity of the extracts will depend on the quality of the propolis, as well as the concentration and type of compound that the extracted resin contains.

On the basis of the results obtained, it has been proved that the evaluated propolis presents a good percentage of resins, (main component of propolis) mainly constituted by phenolic compounds and flavonides, what makes it a good quality product.

In conclusion, the method used in the harvest of propolis influences the quantity of obtained EEP. In general the extracts presented good antioxidant capacity, and did not show direct relation with the ethanol concentration. The temperatures used to concentrate the extracts influence on their antioxidant power. The extraction by Soxhlet decreases significantly the contents of flavonoids and phenolic compounds. The value of the antioxidant capacity of the extracts will depend on the quality of the propolis, i.e., besides the concentration and the type of compounds which the extracted resin contain, and not depend on the EEP percentage.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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