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Additional Information

Standardizing the analysis of phenolic profile in propolis

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ABSTRACT

The analysis of propolis is controversial, hampering the comparison of its biological properties and estimation of its commercial value. This work evaluates the effectiveness of combining maceration and ultrasonication extraction techniques on the yield, total phenol content (Folin-Ciocalteau) and the specific phenolic compounds (HPLC-UV), on propolis from different origins. The extraction method was not significant in any case; therefore ultrasonication is recommended (time-saving) but only when a double extraction is performed. Propolis yield varies significantly between samples, as it includes impurities, consequently the results should be expressed considering the yield (as balsam) instead of raw propolis. Of the 13 quantified phenolic compounds, CAPE and pinocembrin (803 and 701 mg/g balsam) stand out. The phenolic profile of a propolis must be fixed using both total phenol content (with a consensus reference compound) and the specific phenolic compounds, since the latter provides information about compounds that can play a significant antioxidant role.

Keywords: Propolis, phenolics, Folin-Ciocalteau, extraction methodology

1. Introduction

Bees (*Apis mellifera*) use propolis as a sealant to protect their hives against invaders, heat, humidity and wind. They produce this product by collecting resinous substances from the exudates of certain plants that are modified by mixing with enzymes, pollen and wax; therefore, it is composed of resins (50%), waxes (30%), essential oils (10%), pollen (5%) and other organic substances (5%) (Pietta,

Gardana, & Pietta, 2002; Gómez-Caravaca, Gómez-Romero, Arráez-Román, & Segura-Carretero, 2006; Cheng et al., 2013; Pellati, Prencipe, & Benvenuti, 2013). Propolis has traditionally been used for its antioxidant and medicinal properties (antimicrobial, antiviral, antiinflammatory, antitumoral, hepatoprotective and immunomodulary activies) (Osés, et al., 2016). Among the more than 300 compounds identified in this product, phenolic compounds should be highlighted, as they are mainly responsible for its pharmacological and biological activity (Díaz-Carballo, et al., 2008; Pellati, et al., 2013; Yang et al., 2015; Sforcin et al., 2016; Sampietro, Vattuone, & Vattuone, 2016; Freires, de Alencar, & Rosalen, 2016; Alm-Eldeen, Basyony, Elfiky, & Ghalwash, 2017; Soltani, et al, 2017; de Francisco et al., 2017). The presence in these bioactive compounds in propolis is strongly influenced by the vegetation and climate in the region where the bees are kept (Bankova, 2005; Cheng et al., 2013; Soltani et al., 2017).

Different solvents may be used for extraction of the active components of propolis. Among them, an ethanol/water mixture (70/30) is the most commonly used as it is non-toxic and very efficient at extraction, specially of polyphenols and flavonoids, commonly used as quality criterion in this substance (Cunha, et al., 2004; Popova, et al., 2004; Trusheva, Trunkova, & Bankova, 2007; Popova, et al., 2007; Sampietro, et al., 2016; Alm-Eldeen et al., 2017).

The final extraction of the bioactive compounds depends on the type and quantity of solvent, temperature and time, and even the procedure used to interact with the crude propolis (Sawaya, da Silva Cunha, & Marcucci, 2011). Maceration is the traditional extraction procedure, although in recent years sonication and microwaves have also been recommended due to their efficiency, time saving and selectivity (Trusheva et al., 2007; Sforcin, 2016).

Spectrophotometry, especially the Folin-Ciocalteu method, is the most widely used for the routine determination of total content of phenols and certain groups of flavonoids in propolis (Gonzalez et al., 2003; Kumazawa, Hamasaka, & Nakayama, 2004; Popova et al., 2004; Cottica et al., 2015). However,

other spectrophotometry methodologies have also been used: (DPPH) 1,1-diphenyl-2-picrylhydrazyl (Laskar, Sk, Roy, & Begum, 2010; Cottica et al., 2015); (DNP) 2,4-dinitrophenylhydrazine (Popova et al., 2007) and (ABTS) 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acids) (Gülçin et al., 2010; Sun, Wu, Wang, & Zhang, 2015). There is a significant discrepancy in the results reported in the bibliography about total phenolic content. This is mainly due to the difference in the reference compounds chosen for the construction of the calibration curves necessary to express the quantitative result (Cicco, et al., 2009).

Chromatographic methods, especially HPLC, are used for the separation and quantification of the specific constituent compounds of the phenolic profile, although they are not recommended as routine procedures due to their high cost (Popova et al., 2004; Castro et al., 2014).

The discrepancy found in everything related to the analysis of propolis (method of extraction or quantification, and criteria to express the results) by researchers and laboratories decisively influences the disparity of results (Andrade et al., 2017; de Francisco et al., 2017). Consequently, it is difficult to compare the biological properties of different "types" of propolis. For this reason, it is necessary to standardize an analytical procedure to determine valid common criteria, and therefore accurately classify propolis according to its composition and commercial value.

For the aforementioned reasons, the objective of this work was to evaluate the effectiveness of the most used bioactive compound extraction techniques (maceration and ultrasonication) applying different extraction combinations (double maceration, double ultrasonication and maceration followed by ultrasonication) on the yield (with respect to crude propolis), on the total phenol content and on the quantification of specific compounds of the phenolic profile of propolis.

2. Materials and Methods

2.1. Raw samples

Crude propolis from different countries were used in this study to consider a possible range of variability in the phenolic profile. Specifically, 3 samples from Rumania (Suceava County), 2 from Spain (Gestalgar and Montroy municipalities, in Valencian region) and 1 from Honduras (municipality of Siguatepeque, department of Comayagua) were analysed. The samples were collected at the end of summer and beginning of autumn. Each sample from Romania and Spain was harvested from a specific professional apiary, composed of no less than 150 hives. In the case of Honduras the sample came from wild hives collected by the Lencas communities. The samples were stored at -18°C until analysis.

2.2. Reagents and solutions

The standards used: caffeic acid, rutin, p-coumaric acid, ferulic acid, m-coumaric acid, quercetin, trans-cinnamic acid, apigenin, genistein, kaempferol, chrysin, pinocembrin, caffeic acid phenylethyl ester (CAPE), and acetonitrile were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gallic acid as well as Folin-Ciocalteau reagent were acquired in Scharlab (Barcelona, Spain). All reagents and standards used were HPLC grade, and purified water from a Milli Q system was used throughout the experiments.

2.3. Extraction procedure

Each crude sample (10-15 g) while still frozen, was ground to homogenize it before extraction. Three different extraction methods were carried out: double maceration (MM), double ultrasonication (UU) and maceration followed by ultrasonication (MU). Each extraction was executed in triplicate.

Maceration-Maceration (MM): One gram of pulverized sample was weighed and dissolved in 30 mL of 70% ethanolic solution (70:30 ethanol:water). Then, this solution was stirred constantly for 24 hours in a dark room. After that, a 5 min centrifugation (5000 rpm at 5 °C) was carried out and the supernatant was separated from the residue by filtration (Whatman 3) (1st extraction). This process

was repeated on the residue, to obtain a second supernatant (2nd extraction). Both supernatants (1st + 2nd extraction) where collected in a volumetric flask and topped up to 100 mL using the same 70% ethanol solvent.

Ultrasonication-Ultrasonication (UU): Again one gram of pulverized sample was weighed and dissolved in 30 mL of 70% ethanolic solution (70:30 ethanol:water). Then, rather than the solution being stirred constantly for 24 hours in a dark room, the extraction process was carried out in an ultrasonic bath at 25ºC for 30 minutes. Then, centrifugation and filtration was carried out as for MM.

Maceration–Ultrasonication (MU): The first extraction was carried out in the same manner as described for maceration, and then submitted to a second extraction as described for ultrasonication.

In the UU and MU extraction methods the same number of extracts were considered as described in the MM method: 1st extraction, 2nd extraction and 1st + 2nd extraction.

The extracts were preserved at -18 °C until the quantification of the "propolis yield" (balsam content), and the determination of the total polyphenols (by spectrophotometry) and specific compounds (by HPLC).

2.4. Establishing a standardized quantitative criterion

In order to standardize the expression of phenol compounds present in a propolis extract, the yield of the propolis samples (eliminating the impurities) was calculated. The yield was expressed as balsam content (soluble ethanolic fraction) and determined according to Popova el al., 2007. To this end, an aliquot (2.0 mL) of each ethanolic extract was evaporated in a vacuum oven to constant weight. The percentage of yield was calculated following the equation (1):

$$Yield = \left(\frac{\text{weight of the dry ethanolic extract}}{\text{weight of crude propolis}}\right) * 100 \qquad \text{equation (1)}$$

2.5. Spectrophotometric determination of total phenolic content

116 The method used to determine the total phenolic content of the propolis extract was based on a slight
117 modification of the procedure described by Cicco et al. (2009). 100 μL of each extract of propolis (1^{th},
118 2^{nd} and 1^{st} + 2^{nd}) plus 1900 μL distilled water were placed in a glass tube and then the solution was
119 oxidized by adding 100 μL of Folin-Ciocalteau reagent. After exactly 2 minutes, 800 μL of 5% sodium
120 carbonate (w/v) was added. This solution was maintained in a water bath at 40°C for 20 min, and then
121 the tube was rapidly cooled with crushed ice to stop the reaction. The generated blue colour was
122 measured using a spectrophotometer at 760 nm. As the result of the total content of phenolic
123 compounds is clearly dependent on the reference substance used in the calibration curve, three
124 different standards were essayed in this study: rutin, gallic acid and a mixture of pinocembrin/galangin
125 (2:1) (Popova et al., 2004; Woisky, & Salatino, 1998). In order to prepare the stock standard solutions,
126 25 mg of rutin, gallic acid or a pinocembrin/galangin mix (2:1) were dissolved to a final volume of 25
127 mL methanol in each case and stored at -20 ºC. The calibration curves were carried out at the beginning
128 of each working day from six working standard solutions, which were prepared by appropriate dilution
129 of each stock standard solution with 70 % ethanol. Concentration ranges were: 50-600 μg/mL for
130 rutin, and 50-300 μg/mL for both gallic acid and pinocembrin/galangin (2:1). The ethanolic solution
131 was used as a blank.

132 *2.6. Chromatographic determination of the phenolic profile*

133 Individual stock solutions of each standard were prepared in methanol at 10 mg/mL, and stored at -
134 20ºC. The working standard mixture solutions were made by diluting the appropriate amount of each
135 stock standard solution to obtain 5 calibration levels (final concentrations of 5, 10, 20, 200 and 1000
136 μg/mL).

137 An HPLC Agilent 1120 Compact LC, consisting of a binary pump with integrated degasser, an
138 autosampler injector, column oven and UV/VIS detection was used to determine the phenolic profile
139 and EZChrom Elite software for data analysis. Chromatographic separation was carried out on a C18

column (250 mm x 4.6 mm, 5 μm, Tecnokroma). Chromatographic method was based on information provided by Pellati, F., Orlandini, G., & Benvenuti, S. (2011) with slight modifications. The run time was 30 min, with 1 min post run time. Details about the method are as follows: column oven (30 °C); mobile phase A (5% formic acid); mobile phase B (acetonitrile); flow rate (0.8 mL/min); needle wash (100% acetonitrile); injection volume (1 μL); detection (295 nm). The gradient applied was: 0 min (10% B); 3 min (15% B); 18 min (40% B); 24 min (40% B); 27 min (66% B); 33 min (70% B); 40 min (80% B).

A blank injection was performed in all the trials to check chromatographic interference in the resolution. The retention times of all the standards were confirmed by individual standard injections. A standard mixture to check the retention times was injected each working day. The samples were filtered through a 0.2 μm pore size membrane filter prior to chromatographic analysis. The analysis were performed in triplicate.

2.7. Statistical Analysis

A multifactor ANOVA (with LSD test and α=0.05), using the Statgraphics Centurion program, was applied to evaluate the effect of the method of extraction and the type of propolis sample. The interaction between both factors was also considered. A Principal Component Analysis (PCA), with the software Unscrambler X.10 was also applied to describe the relationship between the methods of extraction and the variables analysed: total phenols and the phenolic profile.

3. Results

3.1. *Yield of the propolis samples*

Table 1 shows the average and standard deviation of the yield in propolis (expressed as g balsam/100 g crude propolis) obtained by applying three different extraction methods (double maceration “MM”, double ultrasonication “UU” and maceration followed by ultrasonication “MU”). Data were obtained

after a first extraction (1^{st}), after a second extraction (2^{nd}) and after the combination of both extracts (1^{st}+2^{nd}). In addition, this table shows the result of the multifactor ANOVA (P-value, F-ratio and minimum and maximum LSD values) carried out considering the factors: "method of extraction" and "sample". The respective double interaction of both factors (data not shown) was not significant. The "number of extraction" was not considered as a factor because of the obvious difference between them: 1^{st} extract, 2^{nd} extract and 1^{st}+2^{nd} extracts.

Significant differences between samples were observed in relation to the propolis yield. Sample 1 (Romania 1) and sample 5 (Spain 2) showed the highest values for the three methods of extraction applied; whereas sample 2 (Romania 2) and sample 3 (Romania 3) had the lowest. Differences in propolis yield are mainly due to impurities such as: wax, insect remains, splinters of wood from the beehives and other debris in the crude propolis samples. This demonstrates that beekeeping practices decisively influence the final yield of this product, because of the presence of foreign material in the propolis (Stan, Mărghitaş, & Dezmirean, 2011). This is obviously very important when the quality and price of the propolis samples on the market is to be fixed. Therefore, it seems more coherent to express the results of the content of active ingredients in propolis with respect to the yield in terms of the "balsam", instead of the weight of crude propolis. The expression of the yield as balsam has been used by other authors as a way to state the percentage of extracted matter from crude propolis Liu et al., 2006; Trusheva, et al., 2007).

In the present study, propolis yield did not vary significantly between the extraction methods (1^{st} + 2^{nd} extraction) since the range of variability is similar in the three cases (MM: 51-80.5 g), MU: 51-79.8 g and UU:48.3 to 76.9 g). However, analysing the 1^{st} and 2^{nd} extraction separately, it is observed that results of the first extraction (1^{st}) for MM and MU are very similar (MM: 46.5-69.6 g balsam/100 and MU: 47.0- 9.0 g balsam/100 g crude propolis) and slightly higher than those obtained from the first extraction of UU (42.0-67.3 g). Moreover, in the second extraction, the opposite behavior was

187 shown: MM (4 to 6.2 g), MU (from 3.4 to 6.6 g) and UU (from 5.5 to 11.2 g). It is evident that a
188 second extraction in the case of UU compensates for the lower yield observed after the first extraction,
189 with respect to the other methods.

190 In view of the yield results, the UU method applying 2 extractions seems more recommendable,
191 even though the final result is very similar to the other two methods, there is considerable time-saving:
192 UU (1 hour), MU (24.5 hours) and MM (48 hours). Liu et al. (2006) and Trusheva et al. (2007) also
193 observed that ultrasonication was the most efficient method in comparison to microwaves or
194 maceration for propolis and medicinal plants respectively. However, in the present study it was shown
195 that ultrasonication is only the best procedure when two consecutive extractions are carried out on the
196 same propolis sample.

197 *3.2. Total phenolic content*

198 The average values and the standard deviation of total phenolic content quantified in the propolis
199 samples and the ANOVA multifactor ("method of extraction" and "sample") are shown in Table 2.
200 The three different extraction methods (double maceration "MM", double ultrasonication "UU" and
201 maceration followed by ultrasonication "MU") were also considered to evaluate the effectiveness of
202 the extraction method in the recovery of the total phenolic content from crude propolis.

203 Total phenolic content was expressed in three ways considering different compounds as calibration
204 references: rutin, gallic acid and pinnocembrin/galangin, as they are indistinctly used in the reported
205 bibliography to measure the total phenolic content in propolis (Popova et al., 2004; Cottica et al.,
206 2015). All this is necessary to compare the results of other reported propolis and to avoid confusion
207 in terms of their active ingredients.

208 Again, the advantage of the UU extraction procedure is obvious, as the results are very similar to
209 MU and MM, although considerable time is saved. As mentioned in the previous section for yield,

210 although some authors reported the advantages of ultrasonic extraction versus traditional maceration
211 when determining the antioxidant capacity (Trusheva et al., 2007; Azmir et al., 2013), these are only
212 evident when a double extraction of the same sample is performed.

213 Instead of per gram of raw propolis, as is usually reported by other authors, the results were shown
214 as per gram of "balsam" (Kumazawa et al., 2004; Laskar et al., 2010; Cottica et al., 2015). This was
215 considered more accurate because, as mentioned before, propolis yield varies significantly between
216 samples as it includes impurities that should be excluded from the weight.

217 Table 2 shows that figures for gallic acid and pinocembrin/galangin are of the same order, whereas
218 the figures for rutin are always approximately double (mg/g balsam) for each sample, even though
219 they represent the same level of total phenol content. For instance, total phenolic content expressed as
220 rutin ranged between 169 mg/g balsam in the Honduran sample (sample 6) to 981 mg/g balsam in
221 Sample 5 (Spain 2); whereas if it is expressed as gallic acid or pinocembrin/galangin the values where:
222 84 and 86 mg/g balsam in sample 6, and 442 and 471 mg/g balsam in Sample 5. It is important to
223 consider this when comparing the results reported by different authors and the data generated by the
224 analytical laboratories. The lack of consensus in the reported works when expressing the total phenol
225 content of propolis, complicates comparison between different types of propolis.

226 In the present work, no significant differences were observed for the total phenolic content with
227 respect to the extraction method, but differences were found due to the type of sample. As mentioned
228 before, the sample (S5) from Spain showed the highest average value of total phenolic content and
229 the sample from Honduras (S6) the lowest. The samples from Romania presented intermediate
230 average values but relatively close to those of Spain without significant differences between them.

231 It is difficult to compare the results of the total phenolic content found in the present work with
232 those reported in the bibliography, because even after using the same analytical method (Folin–
233 Ciocalteau procedure), the results could be expressed differently since other reference compounds

(calibration standards) were used. For instance, Popova et al (2004) used a mixture of pinocembrin and galangin as a reference (expressing the results as mg/mL extract). In poplar-type propolis these authors reported values from 19.0 to 28.3 mg pinocembrin and galangin /mL of extract. Gonzalez et al., 2003, used gallic acid, quercetin, 3,4-dihydroxybenzoic acid, caffeic acid and vanillin as reference compounds but showed the results as a percentage of raw propolis, while Kumazawa et al., 2004 and Cottica et al., 2015 used gallic acid as a calibration standard, assessing the total polyphenol content as mg of gallic acid equivalent per gram of propolis (mg GAE/ g propolis). The last authors reported values of 199.35 and 65.92 mg GAE/g propolis using ethanol-water extractions for Canadian propolis.

3.3. Phenolic profile

The average values and standard deviation of the quantified phenolic compounds in propolis samples (expressed as mg of compounds/g balsam) obtained applying three different extraction methods: double maceration (MM), double ultrasonication (UU) and maceration followed by ultrasonication (MU) are shown in Table 3. Data were obtained after the combination of two extracts carried out on the same propolis sample (1^{st}+2^{nd}) to achieve the maximum possible extraction as demonstrated in the previous sections. This table also shows the ANOVA results (F-ratio and significant differences) obtained for two factors: method of extraction and type of propolis.

Most of the compounds are found in the six kinds of propolis, but in different quantitative proportions. Of the 13 quantified compounds, all of them showed significant differences between samples and only one presented significant differences considering the method of extraction. This result demonstrates the influence of the kind of propolis on the quantification of this type of compound. In the present work, the most abundant compounds found were CAPE (771-803 mg/g balsam) and pinocembrin (606-701 mg/g balsam), both in Sample 5 (Spain 2). Furthermore, this sample showed the highest significant values for: kaemferol (323-357 mg/g balsam), chrysin (332-343 mg/g balsam), m-coumaric acid (134-152 mg/g balsam), caffeic acid (79-88 mg/g balsam),

quercetin (38-43 mg/g balsam) and apigenin (36-40 mg/g balsam). The rest of the compounds analyzed were more abundant in sample 1 “Romania 1” (p-coumaric 278-284 mg/g balsam, ferulic 243-260 mg/g balsam, rutin 56-79 mg/g balsam and genistein 7-14 mg/g balsam). Sample 6 (Honduras) stands out both for the absence of 5 out of the 13 compounds analyzed and for the low quantities of the identified compounds. However, this sample is the only one that contains trans-cinnamic acid (48-59 mg/g balsam) together with sample 3 “Romania 3” (55-66 mg/g balsam). The sample from Honduras differs greatly from the other samples, as the chromatograms revealed the presence of abundant unknown peaks. This suggests that this propolis contains other phenolic compounds not considered in this study, which could be the subject of future research.

European propolis from Ukraine and Bulgaria mainly contained pinobanksin (14.7 mg/g of ethanolic extract) and chrysin (120.4 of mg/g of ethanolic extract) (Kumazawa et al. 2004), while propolis from China (Beijing) had caffeic acid (3.74 mg/g propolis) as the major phenolic acid and pinobanksin-3-O-acetate (69.36 mg/g propolis) and as the main flavonoid (Sun et al., 2015).

In order to evaluate the global effect of the method of extraction and the type of propolis on the total phenolic content and the phenolic profile, a principal component analysis (PCA) was performed. Figure 1 shows the PCA bi-plot of scores and loading, where the codes for each point correspond to “method of extraction-number of sample”. Two principal components explained 84% of the variations in the data set: PC1 (58%) and PC2 (26%). The first thing to note when looking at the PCA is that all the samples of propolis are clearly differentiated. It is also noteworthy that there is a certain proximity between samples from the same country: samples 1, 2 and 3 from Rumania (upper quadrants) and samples 4 and 5 from Spain (bottom right quadrant). The approximations between samples indicate some similarity in terms of the variables analyzed, which is logical since the environmental conditions and vegetation decisively influence the characteristics of propolis (Bankova, 2005; Popova et al., 2007).

When analysing the PCA plot in more detail, it can be observed that for the same sample there is practically no difference with respect to the extraction method applied. This indicates that the method of extraction has no effect whatsoever on the analysed parameters as the score points are mainly grouped according to the type of propolis.

The loading plot clearly shows that certain compounds are responsible for differentiation between samples: CAPE, kaemferol, chrysin, apigenin, pinocembrin, caffeic acid, m-coumaric acid and quercetin, associated with PC1 are characteristic of samples 4 and 5, as trans-cinamic is for sample 6 and ferulic acid, p-coumaric, genistein and rutin (corresponding to PC2) are for samples 1 and 2. However, sample 3 has an intermediate position among all of them.

4. Conclusions

An accurate standardization of the phenolic profile of propolis is required in order to define quality criteria and therefore support the estimation of the commercial value of this expensive natural product. This must focus not only on the active chemical composition, but also on the use of adequate analytical protocols defining solvents, extraction procedures, and what is equally important, the criteria to express the results. Working only with standardized methodology (accepted by all the agents involved: scientists, traders, public administration and analytical laboratories) will make it possible to have reliable and comparable data.

In the present work, it was found that propolis type had a greater influence on the yield, on total phenol content and on individual phenolic compounds than did the method of extraction. This paper has demonstrated that ultrasonication is more suitable than maceration as a method for extraction of phenolic compounds in propolis, as this procedure saves a considerable amount of analysis time. However, this is the case only if a double extraction is performed on the same sample; since it requires only 1 hour even after performing the extraction twice. The results should be expressed considering the yield and not the raw propolis, because of the impurities included in the propolis samples. The

value of total phenol content is comparable between propolis only when the same reference compounds are used. It seems daring to define the antioxidant capacity of a propolis by means of analyzing only the total phenol content, since this total value does not necessarily reflect the presence of certain compounds that can play a significant antioxidant role.

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Figure Caption

Figure 1. Scores (A) and loading (B) plots of the two principal components of the PCA model for phenolic content (total expressed as pinocembrin/galangin and individual phenolic compounds quantified in propolis samples obtained applying three different extraction methods: double maceration (MM), double ultrasonication (UU) and maceration followed by ultrasonication (MU). Data were obtained after the combination of two extractions carried out on the same propolis sample.

Table 1. Yield (average percentage and standard deviation with respect to crude propolis: g balsam /100 g crude propolis) in propolis samples obtained applying three different extraction methods: double maceration (MM), double ultrasonication (UU) and maceration followed by ultrasonication (MU). Data were obtained after a 1^{st} extract, after a 2^{nd} extract and after the combination of both extracts (1^{st}+2^{nd}). ANOVA results (P-value, F-ratio, and minimum and maximum LSD values) obtained for two factors: method of extraction and type of propolis.

	Method of extraction								
	MM			**UU**			**MU**		
	Number of extraction			**Number of extraction**			**Number of extraction**		
	1^{st}	2^{nd}	1^{st}+2^{nd}	1^{st}	2^{nd}	1^{st}+2^{nd}	1^{st}	2^{nd}	1^{st}+2^{nd}
Sample 1 (*Romania 1*)	69.6 (0.9)	6.2 (0.3)	80.5 (0.5)	67.3 (1.1	7.0 (0.3)	76.9 (1.0)	69.0 (0.9)	6.6 (0.4)	79.8 (2.3)
Sample 2 (*Romania 2*)	46.5 (1.4)	4.6 (0.4)	51.0 (0.8)	42.4 (1.7)	11.2 (1)	54.0 (1.9)	47.0 (0.8)	3.9 (0.4)	52.9 (2.4)
Sample 3 (*Romania 3*)	48.4 (1.6)	4.0 (0.4)	54.5 (1.5)	42.0 (3.2)	6.8 (0.3)	48.3 (2.0)	47.3 (0.5)	3.4 (0.3)	51.0 (2.3)
Sample 4 (*Spain 1*)	56.8 (3.9)	5.0 (0.2)	64.0 (2.9)	50.9 (1.0)	5.5 (0.6)	59.2 (3.6)	51.6 (2.9)	3.8 (0.2)	56.7 (2.5)
Sample 5 (*Spain 2*)	69.4 (2.3)	5.0 (0.4)	74.9 (3)	66.8 (3.4)	11.1 (1.3)	75.4 (1.1)	68.1 (0.5)	4.0 (0.3)	70.7 (3.9)
Sample 6 (*Honduras*)	46.5 (1.5)	5.7 (0.5)	55.8 (2.8)	43.1 (2.9)	10.6 (0.8)	56.9 (1.1)	47.4 (3.3)	5.5 (0.5)	55.3 (3.1)

ANOVA RESULTS

ANOVA Sample factor	**ANOVA Method of extraction factor**
P-value (0.0000)	**P-value (**0.9246)

F-ratio (5.95)	**F-ratio (**0.08)
LSD (average); (min/max)	**LSD** (average); (min/max)
S1 [b,c] (50.00); (42.53 / 57.46)	MM (45.51); (40.62 /50.40)
S2 [a,b] (42.02); (835.10 / 48.94)	UU(46.32); (41.23 / 51.41)
S3 [a,b] (40.88); (33.97 / 47.80)	MU (44.89); (39.90 / 49.88)
S4 [b] (47.52); (40.61 / 54.44)	
S5 [c] (59.16); (52.25 / 66.08)	
S6 [a] (33.87); (26.67 / 41.06)	

Samples followed by the same letter are not significantly different.

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423 **Table 2**. Total phenolic content in propolis samples obtained applying three different extraction methods: a double maceration (MM), double
424 ultrasonication (UU) and maceration followed by an ultrasonication (MU). Data are obtained after a 1st extraction, after a 2nd extraction and
425 after the combination of both extracts (1st+2nd). The results are expressed as rutin (mg/g balsam), gallic acid (mg/g balsam) and a mixture of
426 pinocembrin/galangin (2:1) (mg/g balsam). ANOVA results (F-ratio and significant differences) obtained for two factors: method of extraction
427 and type of propolis.

	Method of extraction		
	MM	**UU**	**MU**

		Number of extraction			Extractor number			Extractor number		
		1st	2nd	1st+2nd	1st	2nd	1st+2nd	1st	2nd	1st+2nd
Sample1										
(Romania 1)										
	Rutin	554 (56)	58 (10)	584 (84)	547 (43)	62 (6)	648 (26)	582 (36)	65 (12)	650 (89)
	Gallic acid	249 (24)	26 (4)	264 (31)	246 (19)	28 (3)	298 (12)	261 (16)	29 (5)	293 (34)
	Pinocembrin/Galangin	265 (26)	28 (5)	281 (36)	262 (20)	30 (3)	315 (13)	279 (17)	31 (5)	312 (39)
Sample 2										
(Romania 2)										
	Rutin	679 (17)	37 (1)	712 (19)	521 (25)	127 (15)	711 (20)	623 (14)	50 (5)	750 (14)
	Gallic acid	306 (8)	17 (1)	320 (8)	228 (11)	57 (7)	319 (9)	281 (6)	23 (2)	337 (6)
	Pin/galang	326 (8)	18 (1)	342 (9)	242 (12)	61 (7)	341(9)	299 (7)	24 (2)	359 (6)
Sample 3										
(Romania 3)										
	Rutin	604 (18)	54 (3)	719(11)	507 (42)	79 (9)	726 (6)	598 (22)	50 (5)	707 (11)
	Gallic acid	273 (8)	25 (1)	322 (5)	232 (19)	36 (4)	327 (2)	271 (10)	23 (2)	318 (5)
	Pin/galang	290 (8)	26 (1)	344 (5)	246 (20)	38 (4)	349 (3)	288 (10)	24 (3)	339 (5)
Sample 4										
(Spain 1)										

	Rutin	638 (23)	60 (3)	792 (23)	620 (51)	44 (6)	701 (54)	692 (88)	44 (3)	774 (23)
	Gallic acid	286 (10)	27 (1)	353 (10)	279 (22)	20 (2)	305 (24)	311(38)	20 (1)	347 (10)
	Pin/galang	306 (11)	29 (1)	378 (11)	298 (24)	21 (3)	330 (25)	332 (41)	21 (1)	370 (11)
Sample 5										
(Spain 2)										
	Rutin	801 (22)	59 (1)	981 (46)	840 (86)	74 (1)	981 (52)	927 (65)	49 (1)	1052(47)
	Gallic acid	363 (10)	26 (0)	442 (0)	286 (38)	33 (0)	442 (23)	419 (29)	22 (0)	474 (21)
	Pin/galang	386(11)	28 (0)	471 (22)	306 (41)	35 (0)	471 (25)	446 (31)	24 (0)	505 (22)
Sample 6										
(Honduras)										
	Rutin2	126 (16)	33 (4)	169 (7)	144 (15)	29 (2)	192 (27)	136 (10)	22 (5)	163 (6)
	Gallic acid	60 (7)	15 (3)	84 (3)	69 (7)	13 (1)	97 (13)	65 (4)	10 (2)	83 (3)
	Pin/galang	62 (7)	16 (4)	86 (3)	72 (7)	14 (1)	99 (14)	67 (5)	11 (2)	85 (3)

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ANOVA RESULTS

ANOVA Sample factor			**ANOVA *Method of extraction factor***		
Rutin	Gallic acid	Pinocenbrin+galangin	Rutin	Gallic acid	Pinocenbrin+galangin
P-value (0.0000)	**P-value (0.0000)**	**P-value (0.0000)**	**P-value (0.9263)**	**P-value (0.9314)**	**P-value (0.9312)**

F-ratio (13.88)	**F-ratio (13.39)**	**F-ratio (13.58)**	**F-ratio (0.08)**	**F-ratio (0.07)**	**F-ratio (0.07)**
LSD	**LSD**	**LSD**	**LSD**	**LSD**	**LSD**
S1[b] (382) (297/466)	S1[b] (172) (134/210)	S1[b] (183) (143/224)	MM (417) (344/490)	MM (188) (155/221)	MM (200) (165/235)
S2[b,c] (465) (355/575)	S2[bc] (209) (160/259)	S2[b,c] (223) (170/276)	UU (413) (340/485)	UU (187) (162/228)	UU (199) (164/233)
S3[b](449) (338/559)	S3[b] (202) (153/252)	S3[b] (216)(163/269)	MU (432) (360/504)	MU (195) (154/219)	MU (207) (173/242)
S4[b,c] (508) (398/619)	S4[bc] (229) (179/278)	S4[b,c] (244) (191/297)			
S5[c] (618) (508/728)	S5[c] (278) (228/328)	S5[c] (296) (243/349)			
S6[a] (101) (7/186)	S6[a] (49) (11/87)	S6[a] (51) (10/91)			

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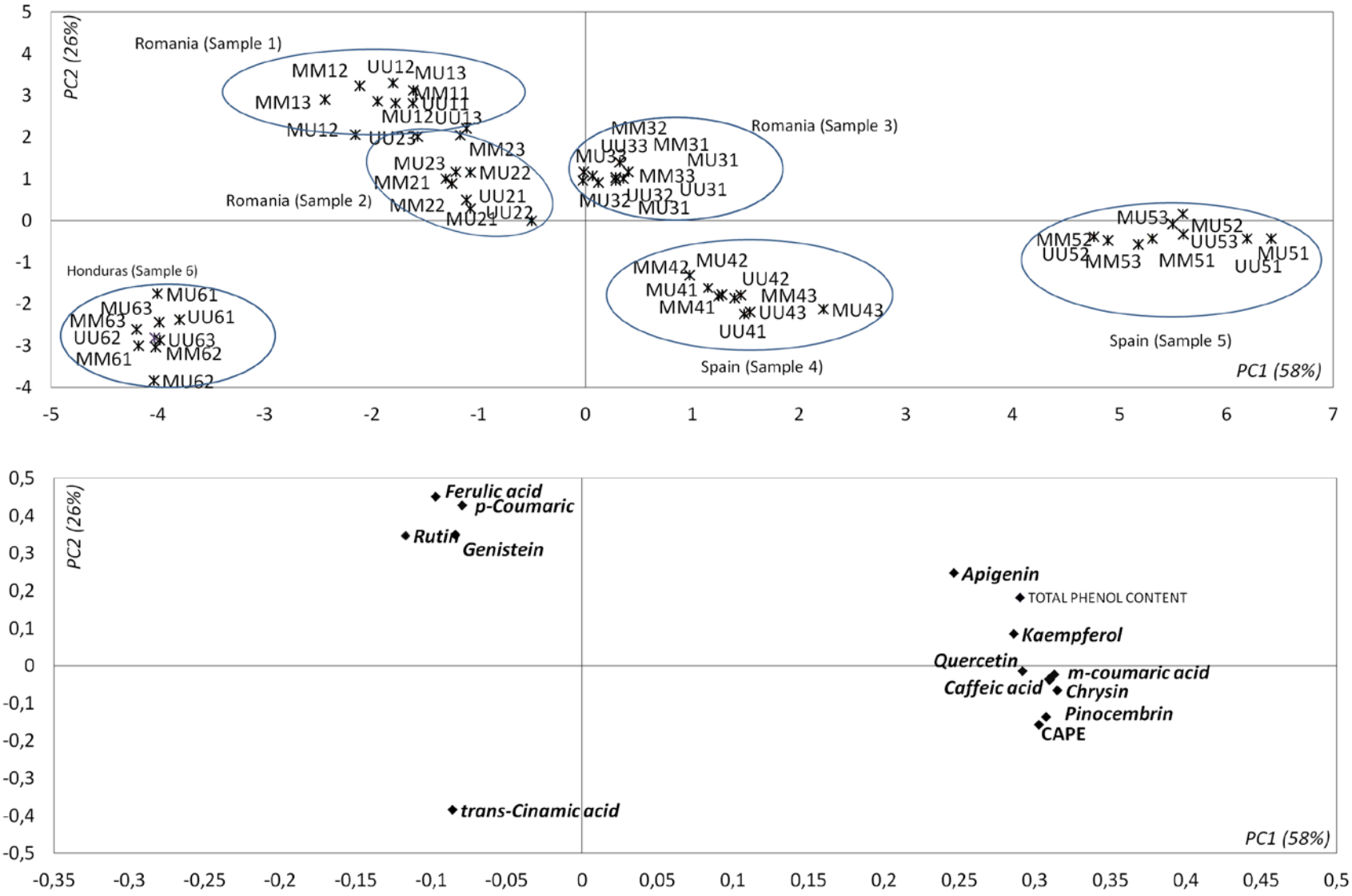
PC2 (26%)
PC1 (58%)
Romania (Sample 1)
Romania (Sample 2)
Romania (Sample 3)
Honduras (Sample 6)
Spain (Sample 4)
Spain (Sample 5)
MM12 UU12 MU13 MM11 MM13 UU11 MU12 UU13 MU12 UU23 MM23 MU23 MU22 MM21 UU21 MM22 UU22 MU21
MM32 UU33 MM31 MU31 MU33 UU32 UU31 MU32 MU31
MU53 MU52 MM52 UU53 MU51 UU52 MM53 MM51 UU51
MM42 MU42 UU42 MU41 MM43 MM41 UU43 MU43 UU41
MU61 MU63 UU61 MM63 UU62 UU63 MM62 MM61 MU62
Ferulic acid
p-Coumaric
Rutin
Genistein
Apigenin
TOTAL PHENOL CONTENT
Kaempferol
Quercetin
m-coumaric acid
Caffeic acid
Chrysin
Pinocembrin
CAPE
trans-Cinamic acid

Figure 1