Assessing the performance of analytical methods for propolis. A collaborative trial by the International Honey Commission

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Abstract

Propolis is a resinous beehive product with extraordinary bioactivity and chemical richness, linked with the botanical sources of the resin. The potential of this product keeps captivating the scientific community, conducting to continuous and growing research on plant sources, composition or applications in agriculture, cosmetics, pharmacy, odontology, etc. In all cases, the quality assessment is a requirement and relies on methods to extract the bioactive substances from the raw propolis and quantify different components. Unfortunately, besides the absence of international quality requirements, there is also a lack of standardized analytical procedures, despite the presence of several methodologies with unknown reliability, often not comparable. To overcome the current status, the International Honey Commission established an inter-laboratory study, with propolis samples from around the globe, to harmonize analytical methods and evaluate their accuracy. A common set of protocols was matched between twelve laboratories from nine countries, for quantification of ash, wax and balsamic content in raw propolis, and for spectrophotometric evaluation of total phenolics, flavone/flavonol and flavanone/dihydroflavonol in the extract. A total of 3428 results (97% valid data), were used to assess the methods accuracy following ISO-5474 guidelines. The within-laboratory precision, revealed good agreement levels for the majority of the methods, with relative variance below 5%. As expected, the between-laboratory variance increased, but, with exception of the flavanone method that revealed a clear lack of consistency, all the others maintained acceptable variability levels, below 30%. Because the performance of ultrasounds procedures was low, they cannot be recommended until further improvements are made.

Keywords: Propolis; Collaborative study; Methods harmonization; Standard methods; International Honey Commission

Introduction

Propolis is well known for its extraordinary bioactivity but also for its chemical complexity, making it an outstanding source of continuous research. Every day we are able to find new publications on this bee product describing a new singularity, a novel substance with high bioactivity never yet described or a new application. At date, more than 7700 documents can be found on Scopus database with a clear increase year rate (above 670 publications/year), focusing in different fields of science such as agriculture, biology, chemistry and biochemistry, where production and quality issues are discussed, but also in pharmacology, medicine, immunology, dentistry, veterinary, engineering or environmental sciences, exploring the potential applications. Just in 2021, more than 100 review papers have been published, dealing with propolis plants sources and production (Dezmirean, Paşca, Moise, & Bobiş, 2021; Mountford-McAuley, Prior, & Clavijo McCormick, 2021; Popova, Trusheva, & Bankova, 2021; Salatino, Salatino, & Negri, 2021) quality and composition (Alvarenga et al., 2021; V. Bankova, Trusheva, & Popova, 2021; Farag et al., 2021; Shahinozzaman, Obanda, & Tawata, 2021; Shanahan & Spivak, 2021; Šuran et al., 2021), food technology (Irigoiti et al., 2021; Yong & Liu, 2021), pharmacologic interactions (Arentz et al., 2021; Asfaram, Fakhar, Keighobadi, & Akhtari, 2021; Ekeuku & Chin, 2021; Forma & Bryś, 2021; Masadah, Ikram, & Rauf, 2021; Rivera-Yañez et al., 2021; Zulhendri, Felitti, Fearnley, & Ravalia, 2021) or even about propolis impact on SARS-CoV-2 (Ali & Kunugi, 2021; Elmahallawy et al., 2021; Merarchi, Dudha, Das, & Garg, 2021).

This continues increment in scientific information, undoubtedly relevant, involve frequently systematic comparison between studies, standing on the qualitative and quantitative assessment of the composition of raw propolis or its extracts. A true comparison requires the use of the same methods and procedure, which is not always fulfilled. Changes on parameters that induce compositional variation, such as solvent and its polarity, temperature

or time of extraction, are frequently observed between studies but overdue when it comes to compare final results. Minor individual modifications on the analytical methodologies or the use of different chemical standards to quantify the phenolic composition and bioactivity of propolis are frequently ignored, resulting merely in a numerical comparison of meaningless values. This lack of scientific exactitude and the unknown impact of the methodology modification on the methods performance, allied to the compositional complexity of the matrix, compromises the true value of propolis and creates obstacles for its recognition by the international authorities (Efsa Panel on Dietetic Products & Allergies, 2010).

The demand for exploring propolis similarities and somehow search for common pathways towards propolis standardization is becoming evident within the entire propolis value-chain and can only be achieved through the harmonization of extraction processes, the use of common reference standards and by expressing the results following the same procedure (Vassya Bankova et al., 2019; Lopes, 2017; Osés et al., 2020; Zaccaria et al., 2019).

The International Honey Commission, a worldwide voluntary network targeting to the development and implementation of new analytical methods for quality control of bee products, within the Propolis Working Group, defined as a priority to strength the scientific studies on propolis and establish a background for future definition of quality standards for industry, producers and laboratories. In a first stage, an inter-laboratory study was set to harmonize basic analytical methods and evaluate their accuracy (repeatability and reproducibility). The study protocol and data handling was implemented accordingly to the international standards guidelines established within the ISO 5725-2. (ISO, 1994) Real and diverse propolis types were used independently of the botanical source of the resin or the

procedure used to gather the propolis, generating a wide range at the parameters levels and so enabling an indirect evaluation of the methods robustness.

It is important to highlight that the International Standard Organization recently created a subcommittee, ISO/TC 34/SC 19, dedicated exclusively to the standardization of bee products and within this, a specific working group (WG2) of international experts, aiming to standardize the terms, definitions, classification, traceability, analytical methods and the minimum compositional requirements for authenticity and quality of propolis. The relevance of this collaborative trial here presented is therefore of major importance for the propolis trade and effectively served as a scientific contribution to the standards currently being prepared within ISO.

Materials and methods

Participants

The international collaborative study was accomplished by 12 laboratories from 9 countries (Bulgaria, Germany, Greece, France, Italy, Portugal, Slovenia, Spain and Turkey), and include analytical, industrial and research laboratories with different levels of experience in propolis analysis. Additionally, the consortium incorporated one company, experienced in propolis processing and trade.

Propolis samples

Each of the participants in the trial were asked to supply approximately 1 kg of propolis from its region, no matter the collection mode. All the samples were shipped to the partner Allwex Food Trading GmbH, which was responsible to apply a common preparation procedure to each sample: after reception, the sample was codified, homogenized within a

proper mill, and divided into 12 sub-samples, which were then distributed to each participant laboratory, so all the labs analyzed the same samples. Overall, each laboratory received 15 sub-samples gather from different origins around the globe, specifically: Baltic region, Brazil (green and poplar propolis), Bulgaria, France, Italy, Poland, Portugal, Slovenia, Spain, Turkey and Ukraine, which represent a wide set of propolis types. Once received, the propolis samples were kept refrigerated at -20 ºC until further analysis.

Protocols establishment

The parameters to be tested were previously agreed within the meeting of the IHC-Propolis WG held in September 2014 in Opatija, Croatia. After discussion with all the participants, it was established that the first analytical methods to harmonized/evaluate should focused on the common parameters used by the international community to characterize propolis samples. In that context, the decision felt, for raw propolis, on ashes, wax and balsam content, and total phenols and flavonoids for the propolis extract.

To establish a specific protocol for each parameter, all laboratories were first asked to describe the methods they currently applied. The different specifications of the analytical procedures where then discussed based on three principles: (i) the method should be scientifically consistent; (ii) the technical requirements and chemicals should be easily accessible in any analytical laboratory around the globe; (iii) when possible, the quickest and simplest procedure should be chosen. Concluded the discussion period, one protocol was established for ashes, total phenols, flavone/flavonol and flavanone/dihydroflavonol, while for wax and balsam content the consortium agreed to explore two alternative analytical options. In both cases, the central point was to reduce the time consumption. The harmonized

147 protocols are described below, step by step, to disseminate and potentiate its use on future
148 researches.
149
150 ***Ash Content***
151 • Heat a silica or platinum crucible to redness for 30 min, and allow to cool in a desiccator
152 and weigh (W1).
153 • Weight 1 g of raw propolis (W2) in the dried crucible previously weighted.
154 • Incinerate the sample in a muffle furnace at 600°C during 3H, or until white or light
155 cream colored ashes are obtained.
156 • Cool in a desiccator and weight (W3).
157 • Repeat the incineration process (additional 30 min), cooling and weighing until constant
158 weigh (W3).
159 • All the procedure must be performed in triplicate.
160 Ash content was calculated as % Ash=[(W3-W1)/(W2-W1)]x100
161
162 ***Wax Content***

163 The wax protocol was performed with two options. Option 1 required Soxhlet
164 extraction, while option 2 required ultrasounds.
165 *Option 1 (Soxhlet)*
166 • Extract 2 g of propolis (W1) with petroleum ether in a Soxhlet apparatus for 6H.
167 • Evaporate the extract to dryness under reduced pressure.
168 • Leave the residue to cool in a desiccator until constant weigh (W2).
169 • All the procedure must be performed in triplicate.
170 Wax content was calculated as % wax1= (W2/W1)*100

Option 2 (Ultrasounds)

- Extract 2 g of propolis (W1) with 100 mL of petroleum ether with ultrasounds for 30 min.
- Cool at room temperature, filter and wash the filter residue with petroleum ether.
- Evaporate the filtrate solution to dryness under reduced pressure.
- Leave the residue to cool in a desiccator until constant weigh (W2).
- All the procedure must be performed in triplicate.

Wax content was calculated as % wax2= (W2/W1)*100

Balsam content

The extraction procedure was available in two options. Option 1 required stirring at room temperature for 24H, while option 2 required ultrasounds.

Option 1 (stirring);

- Weight 1 g of propolis sample (m_p) in 30 mL of 70 % ethanol/water;
- Keep the mixture under mechanical agitation at room temperature;
- After 24H, filter the mixture through a filter paper;
- To confirm the absent of phenolics in the remaining solid, add a few drops of $FeCl_3$ (5% in methanol). If positive result is observed (colour development) the extraction procedure must be repeated under the previous conditions;
- After the second/third extraction, all the extracts must be combined in a 100 mL volumetric flask and the volume adjusted with 70 % ethanol/water.
- The previous five steps of the extraction procedure must be done in triplicate.
- For evaluation of the balsam content, combine 2 mL of each extraction solution (3x2 mL) and evaporate to dryness.
- Leave the residue to cool in a desiccator until constant weigh (m_e).

The balsamic content will be expressed as $\%BC_1 = \frac{m_e}{m_p} \times \frac{50}{3} \times 100$ (m_e= mass of dry extract; m_p= average mass of propolis in the triplicate; 50/3 is the dilution factor).

Option 2 (ultrasounds);

- Weight 1 g of propolis (m_p) sample in 30 mL of 70 % ethanol/water
- Keep the mixture in an ultrasonic bath.
- After 20 minutes, filter the mixture through a filter paper;
- To confirm the absence of phenolics in the remaining solid, add a few drops of $FeCl_3$ (5% in methanol). If positive result is observed (colour development) the extraction procedure must be repeated under the previous conditions;
- After the second/third extraction, all the extracts must be combined in a 100 mL volumetric flask and the volume adjusted with 70 % ethanol/water.
- The previous five steps of the extraction procedure must be done in triplicate.
- For evaluation of the balsam content, combine 2 mL of each extraction solution (3x2 mL) and evaporate to dryness.
- Leave the residue to cool in a desiccator until constant weigh (m_e).

The balsamic content will be expressed as $\%BC_2 = \frac{m_e}{m_p} \times \frac{50}{3} \times 100$ (m_e= mass of dry extract; m_p= average mass of propolis; 50/3 is the dilution factor)

Total phenolic content

- *Working solution*: Pipette 1.5 mL of propolis extract solution (combine 0.5 mL of each extract solution) to a 10 mL volumetric flask and dilute with 70 % ethanol/water. This procedure should be performed, independently, for each extraction option (stirring or ultrasounds).

- Mix an aliquot of the working solution (0.2 mL) with 1.5 mL of water and 0.4 mL of the Folin-Ciocalteu's reagent.
- Then, add 0.6 mL of a sodium carbonate solution (20%) to the mixture, and adjust the final volume (5 mL) adding 2.3 mL of distilled water.
- Keep the mixture in the dark for 2H at room temperature and measure the absorbance at 760 nm.
- Prepare the blank in the same conditions as the samples, using instead of the sample, 0.2 mL of 70 % ethanol/water.
- For the quantification, a calibration curve of gallic acid should be prepared using the same procedure as for the samples (5 points at the following concentrations: 0.025; 0.050; 0.100; 0.200; 0.300 mg/mL).
- If the sample absorbance does not follow within the calibration curve, the concentration of the working solution should be adapted.

Total phenolic content should be calculated as: $P_f = \frac{c \times V \times 100}{S_V \times M} \times 100$

where, P_f- Percentage of phenolic compounds in raw propolis (calculated as gallic acid equivalents); c- Concentration obtained from the calibration curve, mg/mL; V – Final volume of the working solution (10 mL or other); S_V – Volume of the sample extract used to prepare the working solution (1.5 mL or other); M – Mean value of the propolis weight used in the three parallel extractions, mg.

Flavone/Flavonol

- *Working solution*: Pipette 1.5 mL of propolis extract solution (combine 0.5 mL of each extract solution) to a 10 mL volumetric flask and dilute with 70 % ethanol/water. This

procedure should be performed, independently, for each extraction option (stirring or ultrasounds).

- In a 25 mL volumetric flask mix 1 mL of the working solution with 10 mL of methanol and 0.5 mL of 5% $AlCl_3$ solution (5g in 100 mL of methanol). Adjust the final volume with methanol.
- The mixture is left in the dark for 30 min at room temperature. After the reaction, measure the absorbance at 425 nm.
- Prepare the blank in the same conditions as the sample, using, instead of the sample, 1 mL of 70 % ethanol/water
- For the quantification, a calibration curve of quercetin should be prepared using the same procedure as for the samples (5 points at the following concentrations: 0.005; 0.020; 0.050; 0.100; 0.250 mg/mL).
- If the sample absorbance does not follow within the calibration curve, the concentration of the working solution should be adapted.

Total flavone/flavonol content should be calculated as: $P_{fl} = \frac{c \times V \times 100}{S_V \times M} \times 100$

where, P_{fl} is the percentage of flavone/flavonol compounds in raw propolis (calculated as quercetin equivalents); c- concentration obtained from the calibration curve, mg/mL; V – Final volume of the working solution (10 mL or other); S_V – volume of the sample extract used to prepare the working solution (1.5 mL or other); M – mean value of the propolis weight used in the three parallel extractions, mg.

Flavanone/dihydroflavonol

- *Working solution*: Pipette 9 mL of propolis extract solution (combine 3 mL of each extract solution) to a 10 mL volumetric flask and dilute with 70 % ethanol/water. This

procedure should be performed, independently, for each extraction option (stirring or ultrasounds).

- Mix an aliquot of the 1 mL of the working solution with 2 mL of DNP solution (1 g DNP in 2 mL 96% sulfuric acid, diluted in a 100 mL volumetric flask with methanol);
- Heat the solution at 50 ^{o}C for 50 min in a water bath with shacking;
- After cooling to room temperature, dilute the mixture in a 10 mL volumetric flask with 10 % KOH in methanol (w/v).;
- Add an aliquot (0.5 mL) of the resulting solution to 10 mL of methanol and dilute in a 25 mL volumetric flask with methanol.
- Measure the absorbance at 486 nm.
- Prepare the blank in the same conditions as the samples, using 1 mL of methanol instead of the propolis solution.
- For the quantification, a calibration curve of naringenin should be prepared using the same procedure as for the samples (5 points at the following concentrations: 0.10; 0.20; 0.50; 1.00; 2.50 mg/mL).
- If the sample absorbance does not follow within the calibration curve, the concentration of the working solution should be adapted.

Total flavanone/dihydroflavonol content should be calculated as: $P_{fln} = \frac{c \times V \times 100}{S_V \times M} \times 100$

where, P_{fln} is the percentage of flavanone/dihydroflavonol in raw propolis (calculated as naringenin equivalents); c- concentration obtained from the calibration curve, mg/mL; V – Final volume of the working solution (10 mL or other); S_V – volume of the sample extract used to prepare the working solution (9 mL or other); M – mean value of the propolis weight used in the three parallel extractions, mg.

Chemicals and instruments

The following list describe, in general, the chemicals and equipment used, nevertheless, suppliers and models may vary accordingly to the laboratory. A full description used by each laboratory can be consulted in the supplementary material, Table S1. Folin–Ciocalteu`s reagent was from Panreac (Barcelona, Spain). Aluminium chloride, potassium hydroxide, ferric chloride, sulphuric acid was from Sigma Chemical Co (St Louis, MO, USA) and 2,4-dinitrophenylhydrazine (DNP) from Fluka (Buchs, Switzerland). Gallic acid, quercetin and naringenin were obtained from Sigma Chemical Co (St Louis, MO, USA). HPLC-grade methanol, ethanol and petroleum ether were purchased from Fisher Scientific (Leics, UK). The laboratory equipment used was: a muffle furnace SNOL, Optic Ivymen System (Utena, Lithuania), a Soxhlet apparatus Behr Labor Technik, Model R 106 T, (Düsseldorf, Germany), an ultrasounds J.P. Selecta (Barcelona, Spain), a rotary evaporator from Heidolph, model Heizbad Hei-VAP (Schwabach, Germany), and a spectrophotometer from Analytikijena, model Specord 200 (Jena, Germany). The water was treated in a Milli-Q water purification system (TGI pure system, Houston, TX, USA).

Statistical analysis

All participant laboratories received a standard form codified for the lab and for the samples, into which the results were entered in triplicate. A previous pre-evaluation on the data was performed by the study coordinator to detect any discrepancy and the need to repeat experiments. Once conclude the experimental design, the individual data were evaluated following the international guidelines ISO 5725(ISO, 1994), which are supported on the analysis of variance. If the individual data significantly differ from the normal distribution above 95% of confidence, the result is considered as a straggle. If the confidence level of 99% was reached, the value is defined as an outlier. Extreme values, or outliers, were

315 removed based on Mendel's *k* statistics and Cochran's test, to guarantee the homogeneity of
316 variances at certain levels, and using Mendel's *h* statistics and Grubb's test, to guarantee the
317 consistency of the laboratories average (Dispas et al., 2018). Once gathered the statistically
318 relevant values for each parameter and sample, the method was checked for its consistence
319 based on mean, repeatability and reproducibility values (ISO, 1994). The ratio between the
320 reproducibility and repeatability standard deviation (S_R/S_r) was used to evaluate whether
321 means between laboratories results are in agreement, setting values below 3, between 3 and
322 6 and above 6 as good, fair of unsuitable performance. (Henderson et al., 2014) The Relative
323 standard deviation values (RSD) were interpreted as tolerance levels for sample-to-sample
324 and lab-to-lab variability. The performance of each laboratory was also assessed based on
325 Z-scores (Vander Heyden & Smeyers-Verbeke, 2007). Statistical analysis was performed
326 using R Software Version 3.2.4 and RStudio, applying *metRology* and *outliers* open access
327 libraries.
328
329 **Results**

330 A total of 3531 final values were gather, however, not all the laboratories were able to
331 perform the entire set of experiments, Table S2, either because they did not comply with
332 technical requirements to perform the analytical procedures, or due to the lack of valid
333 results. The higher participation was attained for ash, wax content (with Soxhlet) and
334 extraction (using mechanical agitation at room temperature), with 9 laboratories presenting
335 full valid results. The application of ultrasounds was clearly the procedure where a lower
336 number of laboratories fulfilled the technical requirements, and so, it can be regarded as a
337 handicap if the goal is the widespread use of the method.

The average results and standard deviation, for each sample, before statistical treatment, are shown in Table 1. The ashes level on the samples varied between 0.4 and 3%, fitting within the range commonly described for propolis worldwide. (Bogdanov, 2017; Cunha et al., 2004; Falcão, Freire, & Vilas-Boas, 2013) In respect to the wax content, the variation is expressive between samples, with values ranging from almost 10 to 50%, in total content. Although some of the samples present high amounts of wax, what may reflect its origin and the collection procedure, the amplitude on the values is a great opportunity to check the robustness of the methods at different concentration levels. A first approach between the two methods under evaluation clearly indicates that there is no statistically significant difference in the obtained results, no matter the level of wax in the samples. The same result was observed when comparing the extraction procedures explored, with ultrasounds and mechanical agitation at room temperature. The balsam content for the samples oscillated from 48 to 73%, again reflecting samples with distinctive compositional characteristics.

The phenolic composition of the propolis, evaluated through the total phenolic content, flavone/flavonol and flavanones/dihydroflavonol did not differ in respect to the extraction method, however, clear differences were found between samples, Table 1. Samples S13 and S14, revealed high values for all the three parameters, while sample S07, with a low content in the total phenolics, is relatively poor in terms of the flavone/flavonol content but leveled in respect to the other group of flavonoids. The phenolic composition obtained from spectrophotometric methods, is always expressed in equivalent terms, and so, is directly dependent on the chemical compound used as a reference.

Discussion

Results consistency and outliers

The statistical evaluation of the results was executed following the guidelines of ISO 5275-2 (Dispas et al., 2018; ISO, 1994). As a first approach, the consistency within the laboratories was inspected using the Mandel's *k* plot. For a specific level (sample), if the within-laboratory standard deviation and the mean standard deviation differed above the 5% level of significance it was considered as a straggle and, if above the 1% significance, it was marked as outlier. All straggles and outliers were then reevaluated using Cochran's test. If the test confirmed the value as an outlier, it was removed, and a new Mandel's *k* plot was generated in an iterative procedure, until no more outliers were found. At least two replicates must be valid for each sample, otherwise the full data on that sample, for that specific laboratory, was discharged.

Figure 1 is an example of this approach: the results for the full data set, Figure 1a, clearly indicates the presence of several potential outlier results, in laboratories 8, 9, 10 and particularly 12. The latter, with several samples above the threshold of 1%. After removal of abnormal replicates, when possible, or the entire sample for the laboratory, a significant diminishing in the number of results above the 1% level was achieved. For this particular parameter, and to achieve a full set of valid results, Figure 1b, it was required two iterations and the removal of samples from laboratories 8 and 12. At this stage, it is important to mention that values above the 1% level on Mandel's plot do not require automatically its removal from the study, only if the outlier situation is also confirmed using the Cochran's test.

After the data was validated for within-lab consistency, a similar approach was applied to check the between-laboratory variability, in this case using the Mandel's *h* for inspection, and the Grubb's test for outlier removal decision. Figure S1 correspond to the graphical representation used for the inspection of the results from the balsamic content obtained under

mechanical agitation at room temperature. For this parameter, the full data show the presence of at least two possible outliers in Laboratories 1 and 12, Figure S1a, which required the removal of some data samples from these laboratories. Besides the outlier's inspection, Mandel's *h* plot allows also the comparison of the pattern between laboratories to assess the presence of markedly deviatory behaviors, systematic deviations. The presence of positive and negative values, Figure S1, confirms a common valid profile, (ISO, 1994) with laboratories showing samples with both positive or negative *h* values but also an even number of laboratories with either positive or negative patterns.

The application of the procedure in the entire set of data lead to the exclusion of 103 outliers, which corresponds to an average of 3% removal, Table S3, with maximum for ashes, wax and flavone content, that reached 5, 6 and 7%, respectively. For other side, the data for total phenolics at room temperature were considered all valid. The final mean value for each sample and for all the parameters, after statistical clearness, is displayed in Table S4, together with the observed variation range between laboratories. Overall, there are no relevant differences in the mean values between the raw data and after outlier's discharge, but, it is clearly noticed that the samples under analysis express a very distinct range of physicochemical composition: sample 10 shows low wax values, around 10%, but with a balsamic content above 70%, reflecting a rich composition in phenolics and particularly flavanone/dihydroflavonol, while sample S05 displays an opposite composition, with high values for wax and medium/low for phenolic content. This high dispersion between the quality of the samples is a good condition to test the robustness of the methodologies at distinct ranges.

Variance contribution

The main goal of a collaborative trial is to assess the performance of a specific method by measuring the trueness (the differences between the average and the true value), and the precision (reflecting the fluctuation between results). This assessment is statistically achieved by the values of repeatability variance, S_r, and reproducibility variance, S_R. The first, is a deviation measure of the analytical procedure within a laboratory, where the method, the operator, the instruments and the materials are the same. The reproducibility describes the maximum variability, where the method is the same, but the operator, the laboratory environmental conditions, the equipment and materials may differ.

The individual variance components for every concentration (sample) and for each parameter (method) are given in the supplementary material Table S5 and S6, and resumed in Table 2. For ash, the impact of the factors *replicates* and *laboratory*, to the total variability, is similar with 41% and 59%, respectively, and seems to be directly affected by the concentration level, since higher variances were found for higher ash content, as in sample S06 and S11. For the evaluation of wax and balsamic content, the influence of the *laboratory* on those parameters becomes the variance dominant factor, reaching values two to three times higher. The contribution of differences in the equipment where the experiments were performed, but also some technical aspects taken by the operators on the sample manipulation and experimental set up, may be the causes under these output. For the spectrophotometric procedures the ratio between the reproducibility and repeatability standard deviation the impact of the laboratory variability is even higher, Table 2, but for these parameters, and since its implementation requires the previous extraction of the balsamic content from raw propolis, we must have in mind that this deviation may be a result of cumulative effect of variability from extraction and spectrophotometric analysis of the extracts.

Methods comparison.

The amount of wax present in raw propolis and its balsamic content are two critical parameters to define the quality of propolis and so its market value. These analytical procedures will be compulsory and routine in any laboratory, so the time taken to execute it is an important issue in defining the default method. The use of ultrasounds in routine analysis of wax and balsamic content in propolis, as an alternative to the common Soxhlet and maceration under mechanical agitation, could reduce dramatically the time spent under these procedures, as long as it produces reliable analytical results.

As previously pointed out, within this trial, the wax content for each sample did not vary significantly when Soxhlet or ultrasounds were used, Table S4, although a slight trend may be notice, since, in general, when the values differ for a sample they tend to be lower in ultrasounds extraction. The same was observed for the balsamic content procedure, however, the similarity between the results of the two methodologies are even closer than for wax. To attest the equivalence between procedures outputs is important to evaluate the reliability of the methods towards the variations within and between laboratories. It is clear from the *replicate* variance, Table 2, that the use of ultrasounds increases the variability for both wax and balsam content, duplicating the standard deviation of repeatability. The same qualitative reduction of the results is observed in respect to the factor *laboratory*, particularly significant for the balsam content, where the standard deviation of reproducibility increases three times using ultrasounds. A possible explanation for that increase in variability may rely once more in the differences between ultrasounds equipment available in each laboratory, and particularly the frequency and temperature control conditions, both not set in the protocol. Considering the time reduction when applying the ultrasounds, it is worth to keep exploring these procedures, however, and considering the decrease in repeatability and reproducibility,

they must be improved before recommended to the international community. Taken this in consideration, the following discussion will be made disregarding the phenolic quantification of the extracts obtained by ultrasounds.

Methods performance.

The standard deviation ratio (S_R/S_r) shown in Table 2, describe a good agreement between the means of each laboratory for ash, wax and balsamic content and a fair agreement for total phenolics and flavone, with values below 3 or in between 3 and 6. In the opposite side, the ratio output for flavanone is over the threshold for acceptable agreement between means, indicative that this analytical method is not providing satisfactory results.

The absence of propolis reference samples with true values and the lack of standard methods for propolis analysis prevents an effective evaluation of the bias uncertain for each laboratory, so the evaluation of the method performance is discussed on the basis of a consensus value and the interpretation of relative standard deviation for repeatability (RSD_r) and reproducibility (RSD_R). (Vander Heyden & Smeyers-Verbeke, 2007) Figure 2 presents the experimental values of RSD_r. In the majority, the levels are below 5%, with exception of the parameters ash, wax (US) and flavanones, where the repeatability performance is lower. The highest RSD_r values are noticed for ash, with 60% of the samples showing values above 10% in the within-laboratory deviation, what may be explained by the lower order of magnitude for this parameter, which is a common statistical behavior for RSD. (Horwitz & Albert, 2006)

Most method-performance studies rely on the more or less independence of the analyte, matrix, method and time, however, these conditions are not always fullfilled. (Horwitz & Albert, 2006; Linsinger & Josephs, 2006) Indeed, the specificities of this collaborative study

479 do not fit under those assumptions, due to the variability on composition of the propolis, an
480 empirical analyte which may lead to potential differentiated interaction with the method, but
481 also due to the particularities of the methods under evaluation, since they intent to access
482 properties (extractability) and quantify indefinite analytes (classes of compounds), rather
483 than specific compounds. In such conditions the performance of the methods often exhibit
484 low scaterring within a laboratory, but high variability among different laboratories.
485 (Horwitz & Albert, 2006; Linsinger & Josephs, 2006; Szewczak & Bondarzewski, 2016).
486 Indeed, the measured RSD_R, Figure 3, highlights the higher levels found for all the
487 parameters. In the context of method performance evaluation is particularly relevant to
488 observe that the flavanone method, widely used in propolis research, displayed an
489 unacceptable performance, with 60% of the samples showing RSD_R higher than 30%, and
490 only one sample with values below 20%. This variability discredits the comparison explored
491 in the literature between propolis samples, since there is no guarantee of the significance of
492 the values.

493 A similar low performance can be observed for the extraction procedure using ultrasounds,
494 with five out of fifteen samples displaying values of reproducibility variance above 30%. In
495 this particular case it is interesting to notice that there is an inversely proportional relation
496 between RSD_R and the concentration, not observed for RSD_r, which means that the
497 ultrasounds equipment used in the different labs did not show the same level of extraction
498 effectiveness for propolis samples with low balsamic content. An opposite behavior was
499 revealed for the extraction procedure at room temperature, Figure 3, with the lowest RSD_R
500 values between all the methods under evaluation (< 8%). Moreover, this method seems to
501 be independent of the quality of the raw propolis, since the performance was similar for both
502 high and low balsamic content samples. These outputs clearly lead us to propose the RT
503 extraction procedure as the recommended for propolis standardization, at least until

504 improvements are made in the use of ultrasounds procedure to enhance its statistical
505 performance.

506 For wax extraction, the performance of the ultrasounds methods does not reveal such
507 unfavourable behaviour, nevertheless, the inter-laboratory variance for 14 out of 15 samples
508 are over the 10% and sample S10 even surpasses 30%, while for Soxhlet wax extraction only
509 four sample shown a RSD_R above 10%, Figure 3. Again, the statistical outputs recommend
510 that the proposed ultrasounds procedure should be passed over the Soxhlet wax extraction
511 procedure.

512 For the other methods, ash, total phenolics and flavone/flavonol, the statistical performance
513 of the inter-laboratory relative variance, considering the limitation of the analyte and the
514 non-specificity of the methodologies, (Horwitz & Albert, 2006; Linsinger & Josephs, 2006)
515 evidenced an acceptable performance for almost all samples, with values below 20%.

516 In Figure 4 it is highlighted, in an aggregated mode, the behavior of all methods under
517 evaluation, identifying the mean and the range of variances for all the concentrations
518 (samples). The average performance for repeatability can be described as acceptable for
519 ashes, and good for all the remaining methods, with values below 13% and 10%,
520 respectively, Figure 4A. Although, for the two ultrasounds extraction procedures, the
521 average is slightly higher, the difference in repeatability is not significant comparing with
522 the standard procedures.

523 For reproducibility, the performance for wax and balsamic extraction under the common
524 procedures (Soxhlet and RT) remains at a good level, with values below 10%, but, the
525 remaining methods behave not as good, however with acceptable reproducibility
526 performance, RSD_R below 30%, Figure 4B. The flavanone/dihydroflavonol method is the

one that do not fall under those conditions, with reproducibility average above 30%, revealing a clear lack of consistency, and so it cannot be a recommended procedure for propolis analysis. Additionally, and because the performance of the ultrasounds extraction procedures is clearly lower than the other alternative tested, the choice should rely on the most consistent methods, wax Soxhlet extraction and balsamic extraction with mechanical agitation at room temperature.

Proficiency evaluation

Although the goal of the study is not a proficiency test but rather to assess the performance of the analytical procedures, it is also possible to measure, for each parameter, the ability of each individual laboratory by comparing their measurement with the average obtained from the other laboratories, considering the true value is not available. The indicator most commonly used to classify is the z-score, which compare the individual value with the average. So, if $|Z| \leq 2$ the performance of the laboratory is satisfactory, but if $|Z| \geq 3$ the analytical procedure within the laboratory must be reviewed, since the confidence on the result cannot be guaranteed. In order to avoid a systematic masking of individual tendencies, the scores were analyzed separately for each parameter, rather than combined. (Powell, Collins, Cussens, MacLeod, & Penkman, 2013; Vander Heyden & Smeyers-Verbeke, 2007) Of the 10 laboratories with valid results, 6 did not had any result requiring action ($Z<3$) with 2 showing excellent performance with all results below the threshold of $Z=2$, Table S7. Laboratory 1 revealed some fragilities on the extraction procedures (wax and balsamic content) presenting 7% of samples with unacceptable results in each situation, slightly above the expectations. The same difficulty was observed for laboratory 10 but in this case the percentage of unacceptable results was even higher and additional outside results were observed for ash with 20% of samples with unacceptable results. The worst performance

was however being observed for laboratory 12, and specifically for the evaluation of flavone/flavonol with the majority of the results outside the warning limit of Z>2 and even with 47% of the results above the level of action. For this laboratory its clearly recommended the evaluation of the procedure/material/equipment since a systematic error is the most probable cause for discrepancy.

Conclusions

Fifteen samples of propolis from around the globe, with distinct characteristics, were used to assess the performance of common methodologies usually applied in the quality evaluation or propolis through an international collaborative study. The first stage of the study, and after the identification of the protocols used in each participant laboratory, allowed the definition of common protocols for evaluation of ash, wax and balsamic content in raw propolis and the quantification of the total phenolic, flavone/flavonol and flavanone/dihydroflavonol content of the extract. Additionally, two alternative methods were set up for the evaluation of wax and balsamic content, aiming to minimize the execution time requirements. Although not all participant laboratories were able to perform the entire set of protocols, a total of 3531 final values were gather and subject to statistical validation for within-laboratory and between-laboratory consistency base on ISO 5275-2 approach. A total of 103 results were classified as outliers, which corresponds to an average of 3% data exclusion, with maximum for ashes, wax and flavone content, with 5, 6 and 7%, respectively. The validated data confirm the diversity of the propolis under study, with very distinct parameters combination that results from the different botanical origin of the resins collected by the bees.

The performance of the methods was statistically evaluated through the repeatability and reproducibility variance, measuring the within-laboratories and between-laboratories

scattering, respectively. The precision within the laboratories, expressed as relative standard deviation, revealed good levels of agreement, below 5%, with exception of flavanones, wax (by ultrasounds) and ash, where the repeatability performance was slightly higher. Nevertheless, only for the latter method the value of RSD_r was above 10%, what may be explained by the lower order of magnitude for this parameter. The inter-laboratorial variability was, as expected, higher for the generality of the methods, however, the wax (by Soxhlet) and the balsamic content, at room temperature, kept the same good performance with RSD_R below 10%. For the other methods, although lower, the performance can be considered acceptable taking into consideration that the relative standard deviation of reproducibility was below 30% and the fact that we are dealing with non-specific analytical methodologies and a complex matrix that may interfere with method performance. The exception to the acceptable behavior is the flavanone/dihydroflavonol method, that do not fall under reproducibility conditions, with RSD_R above 30%, revealing a clear lack of consistency, and so it cannot be a recommended procedure for propolis analysis. Additionally, and because the performance of the ultrasounds extractions is clearly lower than the other tested methods, the recommended method for wax is the Soxhlet extraction and for balsamic content is the extraction with mechanical agitation at room temperature, at least until improvements are made to the procedure explored under this collaborative study.

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support by national funds FCT/MCTES to CIMO (UIDB/00690/2020). Thanks to the Programa Apícola Nacional 2020-2022 (National Beekeeping Program) for funding the project "Standardization of production procedures and quality parameters of bee products" and to Project PDR2020-1.0.1-FEADER-031734: “DivInA-Diversification and Innovation

on Beekeeping Production". National funding by FCT- Foundation for Science and Technology, through the institutional scientific employment program-contract with Soraia I. Falcão. A special thanks is given to Hartmut Scheiter and Allwex Food Trading GmbH, Bremen, Germany, for providing, handling and delivering the propolis blind samples.

Disclosure statement

No potential conflict of interest was reported by the authors.

612 **Figure Captions**

613

614 Figure 1. Mendel's *k* plot applied in the data set of the balsamic content of propolis using
615 mechanical agitation at room temperature. Within each lab, the columns represent the 15
616 propolis samples. (a) Full data; (b) Second iteration after outlier's removal.

617

618

619 Figure 2. Within-laboratory variability, for each propolis sample, accordingly to the
620 analytical procedure. RSD = Relative standard deviation: US: ultrasounds; RT: Room
621 temperature.

622

623 Figure 3. Between-laboratory variability, for each propolis sample, accordingly to the
624 analytical procedure. RSD = Relative standard deviation: US: ultrasounds; RT: Room
625 temperature.

626

627 Figure 4. Average statistical performance accordingly to the analytical procedure: (a)
628 repeatability; (b) reproducibility. (■)- Mean value. US: ultrasounds; RT: Room temperature.

629

630 Table 1. Untreated average values (in percentage of raw propolis) and standard deviation for the analytical parameters under evaluation.

Sample	Ash	Wax		Balsam		Total Phenolics		Flavone/Flavonol		Flavanone/Dihydroflavonol	
		Soxhlet	Ultrasounds	RT	Ultrasounds	RT	Ultrasounds	RT	Ultrasounds	RT	Ultrasounds
S01	0,9±0,1	23±2	21±4	68±6	69±5	15±2	15±2	2,2±0,3	2,4±0,5	7±3	9±1
S02	1,4±0,3	21±2	19±3	71±7	73±5	19±3	17±3	5,4±1,1	5,5±1,0	9±4	11±2
S03	1,2±0,1	35±2	35±11	58±5	51±8	13±2	12±2	3,6±0,5	3,4±0,4	7±3	8±1
S04	0,8±0,2	30±2	30±6	63±6	58±9	12±2	12±1	2,6±0,4	2,6±0,5	7±3	8±1
S05	1,4±0,2	47±4	49±9	48±5	52±11	14±2	13±2	4,5±0,9	4,6±0,8	7±3	8±1
S06	3,0±0,5	19±2	16±3	58±5	58±7	12±2	11±2	3,1±0,6	3,3±0,7	6±3	7±2
S07	1,8±0,3	28±3	25±3	53±5	54±6	7±1	7±1	1,2±0,3	1,3±0,4	9±4	9±2
S08	2,2±0,2	40±5	36±5	56±4	59±12	11±2	11±2	3,0±0,7	3,0±0,9	7±3	8±2
S09	1,0±0,2	31±4	31±4	64±5	64±5	16±3	17±1	6,1±1,1	6,7±1,1	10±3	11±2
S10	0,4±0,2	11±3	11±4	72±5	67±5	18±3	16±2	6,6±0,9	6,6±1,1	10±4	11±2
S11	2,4±0,7	20±3	18±1	69±4	68±8	20±5	18±2	7,8±1,2	7,7±1,1	11±3	11±2
S12	1,3±0,2	28±3	27±3	65±4	64±4	16±3	16±2	6,8±1,3	6,6±1,1	9±3	9±3
S13	1,0±0,9	21±2	21±3	71±7	67±6	22±7	18±3	8,0±1,4	7,9±1,2	11±3	10±3
S14	0,7±1,1	18±3	20±6	73±4	70±7	26±8	23±3	8,9±1,9	9,4±1,5	13±4	13±3
S15	0,7±0,1	44±2	42±6	51±9	50±10	16±5	14±2	4,0±0,9	4,2±0,7	8±3	8±3
$\bar{X} \pm SD$	1,3±0,7	28±10	27±10	63±8	62±8	16±5	15±4	4,9±2,4	5,0±2,4	9±2	9±1

631 RT: Room temperature. Average values and standard deviation was calculated using the full set of raw data from all the laboratories.

632

633 Table 2. Variance components average

Source of variability	Ash	Wax		Balsam		Total Phenolics	Flavone/Flavonol	Flavanone/Dihydroflavonol
		Soxhlet	Ultrasounds	RT	Ultrasounds	RT	RT	RT
Replicate variance (S^2_{rep})	0,020	1,1	6,5	3,6	7,7	0.34	0,014	0,25
Laboratories variance (S^2_{Lab})	0,029	5,7	17,2	11,5	159,2	5,2	0,45	8,1
Repeatability sd (S_r)	0,13	1,0	2,3	1,8	2.6	0,53	0,11	0,49
Reproducibility sd (S_R)	0,19	2,4	4,4	3,8	11,1	2,2	0,61	2,8
Ratio (S_R/S_r)	1,4	2,6	2,1	2,4	5,2	4,4	5,2	6,5
Repeatability RSD (%)	11,6	4,0	8,5	2,8	4,2	3,6	2,6	5,5
Reproducibility RSD (%)	15,3	9,5	17,3	6,1	19,6	14,0	13,2	32,5

634 RT: Room temperature.

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