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ORIGINAL RESEARCH ARTICLE

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 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-5725 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.

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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. To date, more than 7700 documents can be found on the Scopus database with a clear increase year rate (above 670 publications/year), focusing on 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 et al., 2020; Mountford-McAuley et al., 2021; Popova, Trusheva, & Bankova, 2021; Salatino et al., 2021) quality and composition (Alvarenga et al., 2021; V. Bankova et al., 2021; Farag et al., 2021; Shahinozzaman et al., 2021; Shanahan & Spivak, 2021; Šuran et al., 2021), food technology (Irigoiti et al., 2021; Yong & Liu, 2021), pharmacologic interactions (Arentz et al., 2021; Asfaram et al., 2021; Ekeuku & Chin, 2021; Forma & Bryś, 2021; Masadah et al., 2021; Rivera-Yañez et al., 2020; Zulhendri et al., 2021) or even about propolis impact on SARS-CoV-2 (Ali & Kunugi, 2021; Elmahallawy et al., 2020; Merarchi et al., 2021).

This continues increment in scientific information, undoubtedly relevant, involve frequently systematic

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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 procedures, 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 comparing final results. Minor individual modifications of 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 performance of the methods, 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 searching 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 et al., 2017; Osés et al., 2020; Zaccaria et al., 2019).

The International Honey Commission, a worldwide voluntary network targeting the development and implementation of new analytical methods for quality control of bee products, within the Propolis Working Group defined as a priority to strengthen the scientific studies on propolis and establish a background for the future definition of quality standards for industry, producers, and laboratories. In the 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 were 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 levels of the parameters 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 was 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 subsamples, which were then distributed to each participant laboratory, so all the labs analyzed the same samples. Overall, each laboratory received 15 subsamples gathered 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 $-20\,^{\circ}C$ until further analysis.

Protocols establishment

The parameters to be tested were previously agreed upon 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 harmonize/evaluate should be 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 time consumption. The harmonized protocols are described below, step by step, to disseminate and potentiate its use in future research.

Ash content

- Heat a silica or platinum crucible to redness for 30 min, and allow to cool in a desiccator and weight (W1).
- Weight 1 g of raw propolis (W2) in the dried crucible previously weighted.
- Incinerate the sample in a muffle furnace at 600 °C during 3H, or until white or light cream colored ashes are obtained.
- Cool in a desiccator and weight (W3).
- Repeat the incineration process (additional 30 min), cooling and weighing until constant weight (W3).
- All the procedure must be performed in triplicate.

$$\text{Ash content was calculated as \% Ash} = [(W3 - W1)/(W2 - W1)] \times 100$$

Wax content

The wax protocol was performed with two options. Option 1 required Soxhlet extraction, while option 2 required ultrasounds.

Option 1 (soxhlet)

- Extract 2 g of propolis (W1) with petroleum ether in a Soxhlet apparatus for 6H.
- Evaporate the extract to dryness under reduced pressure.
- Leave the residue to cool in a desiccator until constant weight (W2).
- All the procedure must be performed in triplicate.

$$\text{Wax content was calculated as \% wax1} = (W2/W1) * 100$$

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 weight (W2).
- All the procedure must be performed in triplicate.

$$\text{Wax content was calculated as \% wax2} = (W2/W1) * 100$$

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 a 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 weight (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 a 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 (3 × 2 mL) and evaporate to dryness.
- Leave the residue to cool in a desiccator until constant weight (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 °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 describes, 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–Ciocalteùs 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), and 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 the samples, into which the results were entered in triplicate. A previous pre-evaluation of 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 by 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 removed based on Mendel's *k* statistics and Cochran's test, to guarantee the homogeneity of variances at certain levels, and using Mendel's *h* statistics and Grubb's test, to guarantee the consistency of the laboratories average (Dispas et al., 2018). Once gathered the statistically relevant values for each parameter and sample, the method was checked for its consistency based on mean, repeatability and reproducibility values (ISO, 1994). The ratio between the reproducibility and repeatability standard deviation (S_R/S_r) was used to evaluate whether means between laboratories results are in agreement, setting values below 3, between 3 and 6 and above 6 as good, fair of unsuitable performance. (Henderson et al., 2014) The Relative standard deviation values (RSD) were interpreted as tolerance levels for sample-to-sample and lab-to-lab variability. The performance of each laboratory was also assessed based on Z-scores (Vander Heyden & Smeyers-Verbeke, 2007). Statistical analysis was performed using R Software Version 3.2.4 and RStudio, applying *metRology* and *outliers* open access libraries.

Results

A total of 3531 final values were gathered, however, not all the laboratories were able to perform the entire set of experiments, Table S2, either because they did not comply with technical requirements to perform the analytical procedures, or due to the lack of valid results. The higher participation was attained for ash, wax content (with Soxhlet) and extraction (using mechanical agitation at room temperature), with 9 laboratories presenting full valid results. The

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

		Wax		Balsam		Total Phenolics		Flavone/Flavonol		Flavanone/Dihydroflavonol	
Sample	Ash	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

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

application of ultrasounds was clearly the procedure where a lower number of laboratories fulfilled the technical requirements, and so, it can be regarded as a 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 et al., 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, which 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

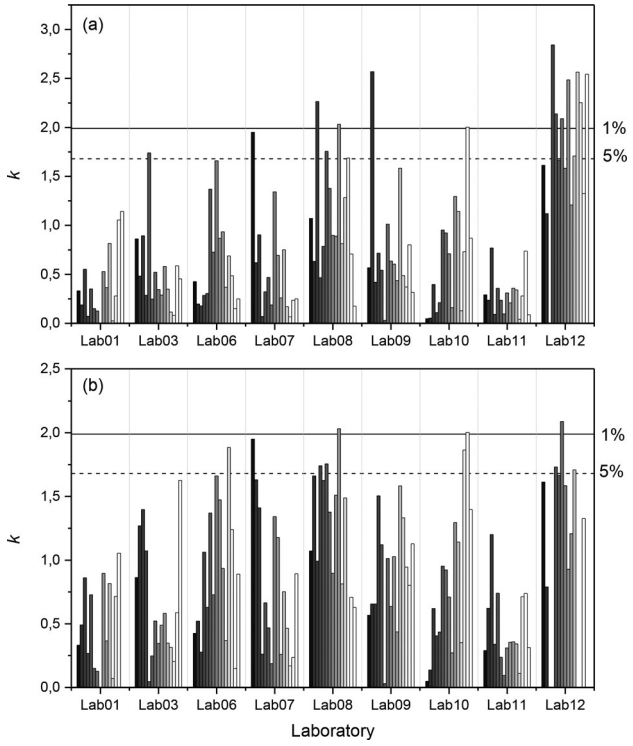

Figure 1. Mendel's *k* plot applied in the data set of the balsamic content of propolis using mechanical agitation at room temperature. Within each lab, the columns represent the 15 propolis samples. (a) Full data; (b) Second iteration after outlier's removal.

removal from the study, only if the outlier situation is also confirmed using the Cochran's test.

After the data were 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 corresponds 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 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

Table 2. Variance components average.

		Wax		Balsam		Total Phenolics	Flavone/ Flavonol	Flavanone/ Dihydroflavonol
Source of variability	Ash	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

RT: Room temperature.

laboratory environmental conditions, the equipment and materials may differ.

The individual variance components for every concentration (sample) and each parameter (method) are given in the supplementary material Tables 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 this output. For the spectrophotometric procedures 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 them 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 noticed, 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, it 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 on 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 it to keep exploring these procedures, however, and considering the decrease in repeatability and reproducibility, they must be improved before being recommended to the international community. Taking this into 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, describes 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. On 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

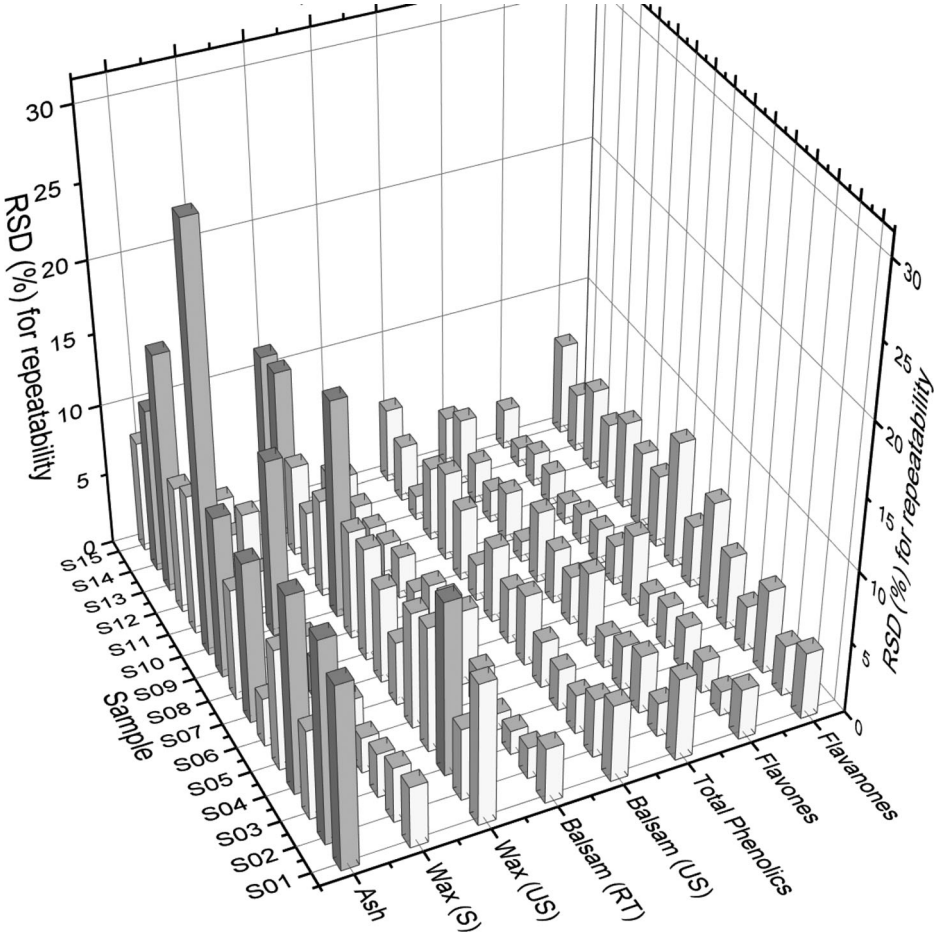

Figure 2. Within-laboratory variability, for each propolis sample, accordingly to the analytical procedure. RSD = Relative standard deviation: US: ultrasounds; RT: Room temperature.

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

A similar low performance can be observed for the extraction procedure using ultrasounds, with five out of fifteen samples displaying values of reproducibility variance above 30%. In this particular case it is interesting to notice that there is an inversely proportional relation between RSD_R and the concentration, not observed for RSD_r, which means that the

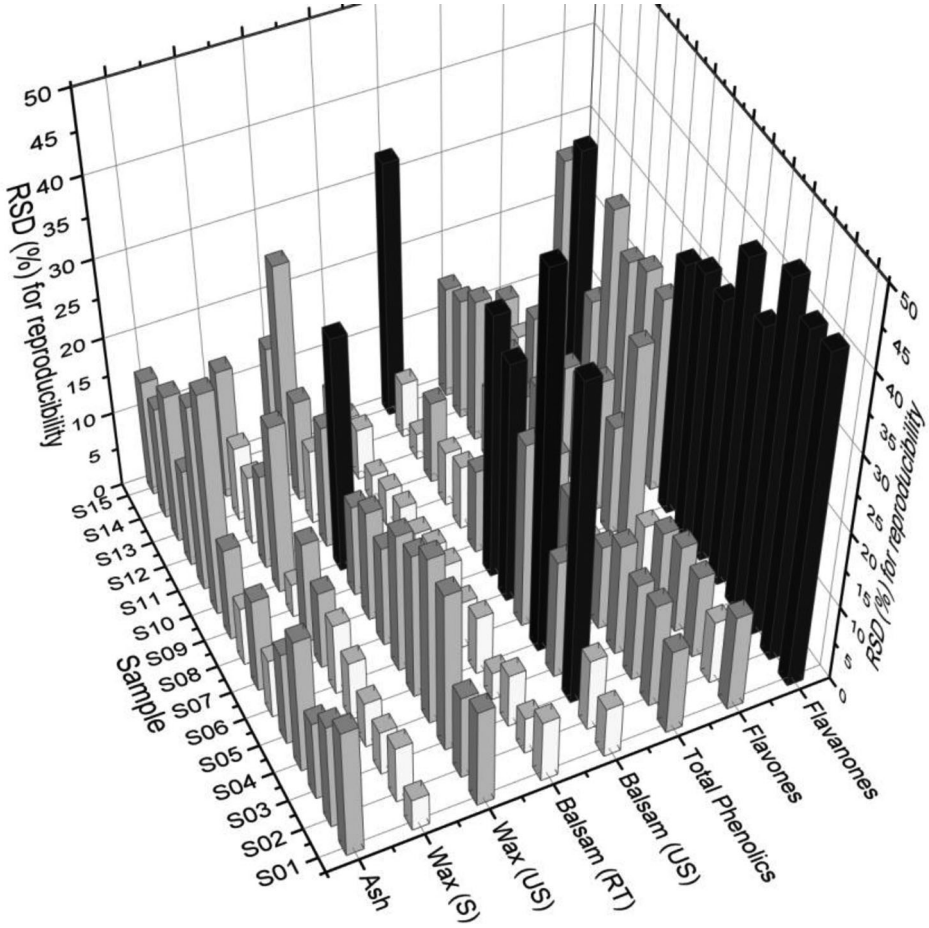

Figure 3. Between-laboratory variability, for each propolis sample, accordingly to the analytical procedure. RSD = Relative standard deviation: US: ultrasounds; RT: Room temperature.

ultrasounds equipment used in the different labs did not show the same level of extraction effectiveness for propolis samples with low balsamic content. An opposite behavior was revealed for the extraction procedure at room temperature, Figure 3, with the lowest RSD_R values between all the methods under evaluation (<8%). Moreover, this method seems to be independent of the quality of the raw propolis, since the performance was similar for both high and low balsamic content samples. These outputs clearly lead us to propose the RT extraction procedure as the recommended for propolis standardization, at least until improvements are made in the use of ultrasounds procedure to enhance its statistical performance.

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

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

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

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

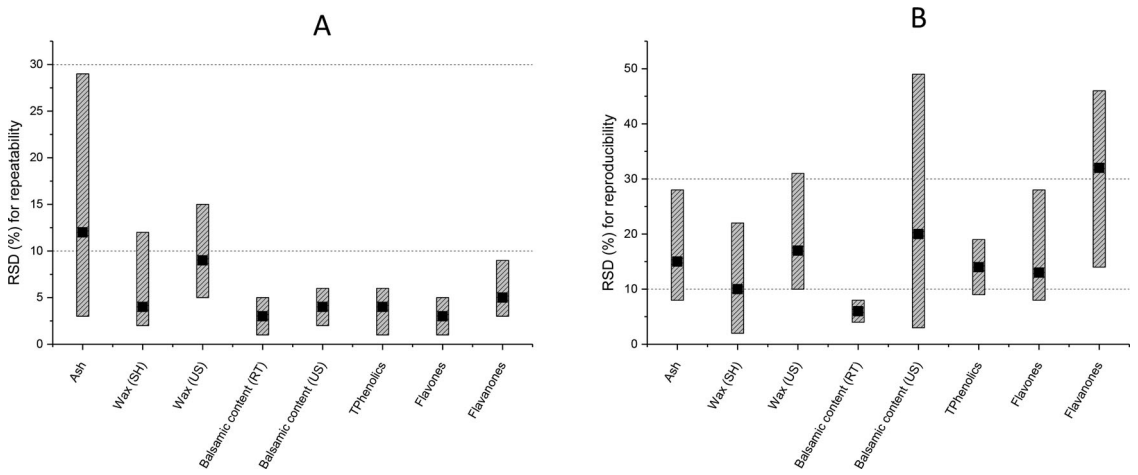

Figure 4. Average statistical performance accordingly to the analytical procedure: (a) repeatability; (b) reproducibility. (■) – Mean value. US: ultrasounds; RT: Room temperature.

flavanone/dihydroflavonol method is the one that does 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 in 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 et al., 2013; Vander Heyden & Smeyers-Verbeke, 2007) Of the 10 laboratories with valid results, 6 did not have 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 it 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 gathered and subject to statistical validation for within-laboratory and between-laboratory consistency based 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%, which 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 does 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.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Ali, A. M., & Kunugi, H. (2021). Propolis, bee honey, and their components protect against coronavirus disease 2019 (Covid-19): A review of in silico, in vitro, and clinical studies. *Molecules*, *26*(5), 1232. https://doi.org/10.3390/molecules26051232

Alvarenga, L., Cardozo, L. F. M. F., Borges, N. A., Chermut, T. R., Ribeiro, M., Leite, M., Shiels, P. G., Stenvinkel, P., & Mafra, D. (2021). To bee or not to bee? The bee extract propolis as a bioactive compound in the burden of lifestyle diseases. *Nutrition*, *83*, 111094. https://doi.org/10.1016/j.nut.2020.111094

Arentz, S., Hunter, J., Khamba, B., Mravunac, M., Lee, Z., Alexander, K., Lauche, R., Goldenberg, J., & Myers, S. P. (2021). Honeybee products for the treatment and recovery from viral respiratory infections including SARS-COV-2: A rapid systematic review. *Integrative Medicine Research*, *10*. https://doi.org/10.1016/j.imr.2021.100779

Asfaram, S., Fakhar, M., Keighobadi, M., & Akhtari, J. (2021). promising anti-protozoan activities of propolis (bee glue) as natural product: A review. *Acta Parasitologica*, *66*(1), 1–12. https://doi.org/10.1007/s11686-020-00254-7

Bankova, V., Bertelli, D., Borba, R., Conti, B. J., da Silva Cunha, I. B., Danert, C., Eberlin, M. N., I Falcão, S., Isla, M. I., Moreno, M. I. N., Papotti, G., Popova, M., Santiago, K. B., Salas, A., Sawaya, A. C. H. F., Schwab, N. V., Sforcin, J. M., Simone-Finstrom, M., Spivak, M., … Zampini, C. (2019). Standard methods for Apis mellifera propolis research. *Journal of Apicultural Research*, *58*(2), 1–49. https://doi.org/10.1080/00218839.2016.1222661

Bankova, V., Trusheva, B., & Popova, M. (2021). Propolis extraction methods: a review. *Journal of Apicultural Research*, *60*(5), 734–743. https://doi.org/10.1080/00218839.2021.1901426

Bogdanov, S. B., V. (2017). Propolis: Origin, production, compostion. In S. Bogdanov (Ed.), *The Propolis Book*.

Cunha, I. B. S., Sawaya, A. C. H. F., Caetano, F. M., Shimizu, M. T., Marcucci, M. C., Drezza, F. T., Povia, G. S., & Carvalho, P. d O. (2004). Factors that influence the yield and composition of Brazilian propolis extracts. *Journal of the Brazilian Chemical Society*, *15*(6), 964–970. https://doi.org/10.1590/S0103-50532004000600026

Dezmirean, D. S., Paşca, C., Moise, A. R., & Bobiş, O. (2020). Plant sources responsible for the chemical composition and main bioactive properties of poplar-type propolis. *Plants*, *10*(1), 22–20. https://doi.org/10.3390/plants10010022

Dispas, A., Marini, R., Desfontaine, V., Veuthey, J.-L., Kotoni, D., Losacco, L. G., Clarke, A., Muscat Galea, C., Mangelings, D., Jocher, B. M., Regalado, E. L., Plachká, K., Nováková, L., Wuyts, B., François, I., Gray, M., Aubin, A. J., Tarafder, A., Cazes, M., … Hubert, P. (2018). First inter-laboratory study of a Supercritical Fluid

Chromatography method for the determination of pharmaceutical impurities. *Journal of Pharmaceutical and Biomedical Analysis*, *161*, 414–424. https://doi.org/10.1016/j.jpba.2018.08.042

Efsa Panel on Dietetic Products & Allergies. (2010). Scientific opinion on the substantiation of health claims related to propolis (ID 1242, 1245, 1246, 1247, 1248, 3184) and flavonoids in propolis (ID 1244, 1644, 1645, 3526, 3527, 3798, 3799) pursuant to Article 13(1) of regulation (EC) No 1924/2006. *EFSA Journal*, *8*(10), 1810. https://doi.org/10.2903/j.efsa.2010.1810

Ekeuku, S. O., & Chin, K. Y. (2021). Application of propolis in protecting skeletal and periodontal health—A systematic review. *Molecules*, *26*(11), 3156. https://doi.org/10.3390/molecules26113156

Elmahallawy, E. K., Mohamed, Y., Abdo, W., El-Gohary, F. A., Ahmed Awad Ali, S., & Yanai, T. (2020). New insights into potential benefits of bioactive compounds of bee products on COVID-19: A review and assessment of recent research. *Frontiers in Molecular Biosciences*, *7*, 618318. https://doi.org/10.3389/fmolb.2020.618318

Falcão, S. I., Freire, C., & Vilas-Boas, M. (2013). A proposal for physicochemical standards and antioxidant activity of Portuguese propolis. *Journal of the American Oil Chemists' Society*, *90*(11), 1729–1741. https://doi.org/10.1007/s11746-013-2324-y

Farag, M. R., Abdelnour, S. A., Patra, A. K., Dhama, K., Dawood, M. A. O., Elnesr, S. S., & Alagawany, M. (2021). Propolis: Properties and composition, health benefits and applications in fish nutrition. *Fish & Shellfish Immunology*, *115*, 179–188. https://doi.org/10.1016/j.fsi.2021.06.010

Forma, E., & Bryś, M. (2021). Anticancer activity of propolis and its compounds. *Nutrients*, *13*(8), 2594. https://doi.org/10.3390/nu13082594

Henderson, R. G., Verougstraete, V., Anderson, K., Arbildua, J. J., Brock, T. O., Brouwers, T., Cappellini, D., Delbeke, K., Herting, G., Hixon, G., Odnevall Wallinder, I., Rodriguez, P. H., Van Assche, F., Wilrich, P., & Oller, A. R. (2014). Inter-laboratory validation of bioaccessibility testing for metals. *Regulatory Toxicology and Pharmacology : RTP*, *70*(1), 170–181. https://doi.org/10.1016/j.yrtph.2014.06.021

Horwitz, W., & Albert, R. (2006). The Horwitz ratio (HorRat): A useful index of method performance with respect to precision. *Journal of AOAC INTERNATIONAL*, *89*(4), 1095–1109. Retrieved from https://www.scopus.com/inward/record.uri?eid=2-s2.0-33750686965&partnerID=40&md5=980f9b915e2264b1276e512484244839 https://doi.org/10.1093/jaoac/89.4.1095

Irigoiti, Y., Navarro, A., Yamul, D., Libonatti, C., Tabera, A., & Basualdo, M. (2021). The use of propolis as a functional food ingredient: A review. *Trends in Food Science & Technology*, *115*, 297–306. https://doi.org/10.1016/j.tifs.2021.06.041

ISO. (1994). Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. *International Standard ISO*, 5725–2.

Linsinger, T. P. J., & Josephs, R. D. (2006). Limitations of the application of the Horwitz equation. *TrAC Trends in Analytical Chemistry*, *25*(11), 1125–1130. https://doi.org/10.1016/j.trac.2006.11.002

Lopes, M., Nunes, N., L. F., Falcão, S. I., & Vilas-Boas, M. (2017). Harmonização de metodologias de análise da própolis. *Revista de Ciências Agrárias*, *40*(SP), 208–215. https://doi.org/10.19084/RCA16225

Masadah, R., Ikram, D., & Rauf, S. (2021). Effects of propolis and its bioactive components on breast cancer cell pathways and the molecular mechanisms involved. *Breast Disease*, *40*(S1), S15–S25. https://doi.org/10.3233/BD-219003

Merarchi, M., Dudha, N., Das, B. C., & Garg, M. (2021). Natural products and phytochemicals as potential anti-SARS-CoV-2 drugs. *Phytotherapy Research*, *35*(10), 5384–5396. https://doi.org/10.1002/ptr.7151

Mountford-McAuley, R., Prior, J., & Clavijo McCormick, A. (2021). Factors affecting propolis production. *Journal of Apicultural Research*, 1–9. https://doi.org/10.1080/00218839.2021.1938456

Osés, S. M., Marcos, P., Azofra, P., de Pabl, A., Fernández-Muíño, M. Á., & Sancho, M. T. (2020). Phenolic profile, antioxidant capacities and enzymatic inhibitory activities of propolis from different geographical areas: Needs for analytical harmonization. *Antioxidants*, *9*(1), 75. https://doi.org/10.3390/antiox9010075

Popova, M., Trusheva, B., & Bankova, V. (2021). Propolis of stingless bees: A phytochemist's guide through the jungle of tropical biodiversity. *Phytomedicine : international Journal of Phytotherapy and Phytopharmacology*, *86*, 153098. https://doi.org/10.1016/j.phymed.2019.153098

Powell, J., Collins, M. J., Cussens, J., MacLeod, N., & Penkman, K. E. H. (2013). Results from an amino acid racemization inter-laboratory proficiency study; design and performance evaluation. *Quaternary Geochronology*, *16*, 183–197. https://doi.org/10.1016/j.quageo.2012.11.001

Rivera-Yañez, N., Rivera-Yañez, C. R., Pozo-Molina, G., Méndez-Catalá, C. F., Méndez-Cruz, A. R., & Nieto-Yañez, O. (2020). Biomedical properties of propolis on diverse chronic diseases and its potential applications and health benefits. *Nutrients*, *13*(1), 78–31. https://doi.org/10.3390/nu13010078

Salatino, A., Salatino, M. L. F., & Negri, G. (2021). How diverse is the chemistry and plant origin of Brazilian propolis? *Apidologie*, *52*(6), 1075–1097. https://doi.org/10.1007/s13592-021-00889-z

Shahinozzaman, M., Obanda, D. N., & Tawata, S. (2021). Chemical composition and pharmacological properties of Macaranga-type Pacific propolis: A review. *Phytotherapy Research : PTR*, *35*(1), 207–222. https://doi.org/10.1002/ptr.6819

Shanahan, M., & Spivak, M. (2021). Resin use by stingless bees: A review. *Insects*, *12*(8), 719. https://doi.org/10.3390/insects12080719

Šuran, J., Cepanec, I., Mašek, T., Radić, B., Radić, S., Gajger, I. T., & Vlainić, J. (2021). Propolis extract and its bioactive compounds—From traditional to modern extraction technologies. *Molecules*, *26*(10), 2930. https://doi.org/10.3390/molecules26102930

Szewczak, E., & Bondarzewski, A. (2016). Is the assessment of interlaboratory comparison results for a small number of tests and limited number of participants reliable and rational? *Accreditation and Quality Assurance*, *21*(2), 91–100. https://doi.org/10.1007/s00769-016-1195-y

Vander Heyden, Y., & Smeyers-Verbeke, J. (2007). Set-up and evaluation of interlaboratory studies. *Journal of Chromatography. A*, *1158*(1–2), 158–167. https://doi.org/10.1016/j.chroma.2007.02.053

Yong, H., & Liu, J. (2021). Active packaging films and edible coatings based on polyphenol-rich propolis extract: A review. *Comprehensive Reviews in Food Science and Food*

Safety, *20*(2), 2106–2145. https://doi.org/10.1111/1541-4337.12697

Zaccaria, V., Garzarella, E. U., Di Giovanni, C., Galeotti, F., Gisone, L., Campoccia, D., Volpi, N., Arciola, C. R., & Daglia, M. (2019). Multi dynamic extraction: An innovative method to obtain a standardized chemically and biologically reproducible polyphenol extract from poplar-type propolis to be used for its anti-infective properties. *Materials*, *12*(22), 3746. https://doi.org/10.3390/ma12223746

Zulhendri, F., Felitti, R., Fearnley, J., & Ravalia, M. (2021). The use of propolis in dentistry, oral health, and medicine: A review. *Journal of Oral Biosciences*, *63*(1), 23–34. https://doi.org/10.1016/j.job.2021.01.001