Spectrophotometric determination of the main polyphenol groups in propolis samples from different regions of Croatia

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ABSTRACT

Spectrophotometric procedures for the rapid characterization of propolis have been performed on propolis samples from different regions of Croatia. In order to determine the major groups of bioactive compounds in propolis, the following optimised and validated spectrophotometric methods were carried out: the Folin-Ciocalteu method for the content of total phenolics (TPs) and two distinct methods for the content of total flavonoids: aluminium chloride ($AlCl_3$) complexation method for total flavones/flavonols (TFFs) and the 2.4-dinitrophenylhydrazine (2.4-DNPH) method for total flavanones/dihydroflavonols (TFDs). Validation parameters, including linearity, sensitivity, range, accuracy, limit of detection, limit of quantification, precision and robustness were implemented. The following polyphenol standards were used for the validation procedure: gallic acid (GA), pinocembrin (PC), galangin (GN), quercetin (QC) and a mixture of PC and GN. Validated methods were applied to analyse six samples of raw propolis from Croatian continental and Adriatic regions. The high qualitative/quantitative variability of the TP, TFF and TFD content was observed. Although the method of extraction (ultrasonic-assisted extraction or microwave assisted extraction) showed a non-significant effect on extraction yield (P>0.05) and the polyphenolic concentrations obtained of each sample in general, ultrasonic extraction was found to be more selective. Furthermore, the calibration compound used for constructing the calibration curve highly influenced the final concentrations of TPs and TFFs. The study showed good linearity, accuracy, repeatability, intermediate precision, LOD and LOQ for all three spectrophotometric methods, considering that these analyses are the basis for further research into the individual polyphenolic compounds in the propolis samples covered by this research.

Keywords: raw propolis; polyphenols; ultrasonic-assisted extraction; microwave-assisted extraction; spectrophotometric determination; validation

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Introduction

Propolis, a bee product, continues to fascinate many researchers around the world. Regardless of its complex and diverse composition, the increasingly frequent application of propolis in veterinary medicine could provide a pool of chemical substances that may have various bioactivities and fewer tendencies to resistance, since antibiotic resistance is a leading public health problem. The increasing use of propolis preparations in food, the cosmetic industry, medicine and pharmaceuticals, as well as in traditional medicine due to its wide range of biological activity indicates the need to find reliable and rapid analytical procedures for chemical characterization of propolis samples and routine determination of polyphenols, its bioactive components. Although its polyphenolic fraction makes propolis a valuable source of beneficial biological properties associated with human and animal health (CARDOSO et al., 2010; SFORCIN and BANKOVA, 2011; ORŠOLIĆ et al., 2019), the diversity of the chemical structures of polyphenols is at the same time a significant problem concerning the standardization and quality control of propolis-based products (CUNHA et al., 2004; BANKOVA, 2005; ESCRICHE and JUAN-BORRAS, 2018). Propolis in its native form cannot easily be consumed or used in cosmetic preparations, since it needs to be prepared as a solution. For this purpose, extraction of the lipophilic (poorly water soluble) bioactive components from the resinous matrix is usually performed, using different solvents, among which ethanol-water solutions (70% and 80%) have been found to be the most effective for producing low-wax propolis extracts rich in polyphenolic fraction and free from impurities (CVEK et al., 2007; MAŠEK et al., 2018). New technologies for extraction of raw propolis samples, ultrasonic-assisted extraction (UAE) and microwave-assisted extraction (MAE), have been introduced and have become concurrent due to their efficiency, time saving and lower solvent consumption (TRUSHEVA et al., 2007; POBIEGA et al., 2019). Propolis balsam is a term used for the complex mixture of compounds that remain in the solution after removing the wax and impurities by extraction techniques. This mixture also includes flavonoids, phenolic acids and sugars.

Spectrophotometric methods are simple, low cost and widely used for routine quality control of raw propolis samples. On the other hand, chromatographic methods are more suitable for compound identification and quantification, but also not appropriate for routine control because of the high cost of the instruments, higher solvent consumption, the use of many different standards, and longer analysis time. Due to their simplicity, accuracy and time-saving features, UV/Vis spectrophotometric techniques have been widely used as routine methods for the rapid characterization of various types of propolis, in order to estimate the total content of phenolics and flavonoids. This is especially because the bioactivity of propolis has been attributed to the mixture of compounds and not an individual one. These methods are based on the detection of polyphenolic compounds of similar structure, and as such can be divided according to the type of reaction with a specific reagent. The Folin-Ciocalteu oxido-reduction method is commonly used for detection of total phenolics (TPs) in various types of plants and other natural samples. The aluminium chloride ($AlCl_3$) complexation method is usually applied for the determination of total flavonoids i.e. the total flavones/flavonols (TFFs) in many different samples, due to the detectable complexes that aluminium ions form with carbonyl and hydroxyl groups of flavones and flavonols (PĘKAL and PYRZYNSKA, 2014). As propolis samples contain high amounts of flavanones/dihydroflavonols, it was considered appropriate to measure the content of total flavanones/dihydroflavonols (TFDs) separately, reacting with 2.4-dinitrophenylhydrazine (2,4-DNPH) as the reagent.

Although widely used, these methods are not specific and may be calibrated according to different standards, resulting in different polyphenol concentrations in the same sample (MIGUEL et al., 2010; FALCÃO et al., 2013; MATIĆ et al., 2017).

The aim of our study was to optimise and validate methods for estimation of the TPs, TFFs and TFDs in ethanolic extracts of Croatian propolis samples, according to different calibration standards, that include gallic acid (GA), pinocembrin (PC), galangin (GN), quercetin (QC) and a mixture of

pinocembrin and galangin (PC-GN). Through this work, the effectiveness of two extraction techniques, UAE and MAE and their impact on yield was evaluated. To the best of our knowledge, very few papers have reported TPs, TFFs and TFDs results on the basis of balsam content, thus our study improved the presentation of data in a more precise mode.

Materials and methods

Propolis samples. Six samples of raw propolis were collected from six different regions of Croatia, presented in Fig. 1: Valpovo (Osijek-Baranja County, 45°40′N 18°25′E), Požega (Požega-Slavonia County, 45°18′40″N 17°44′24″E), Senj (Lika-Senj County, 44°42′25″N 15°10′27″E), Dubrovnik (Dubrovnik-Neretva County, 42°39′13″N 18°05′41″E), Pešćenica (Sisak-Moslavina County, 45°13′15″N 16°15′5″E), and the area around Zagreb (City of Zagreb, 45°49′0″N 15°59′0″E). All samples were obtained from local beekeepers and collected during 2019 and 2020. Sensory properties, including consistency, colour and odour, were investigated by direct observation. The samples were placed in sealed plastic bags and stored at -10 to -20 °C until analysis.

Fig.1. Map of Croatia's counties with the locations of propolis samples' collection indicated

Reagents and standards. The following reagents and standards were used for the spectrophotometric assays: GN (Sigma Aldrich, China), PC (Sigma Aldrich, Uzbekistan), Folin-Ciocalteu reagent (a mixture of phosphotungstates and molybdates) and GA (Sigma-Aldrich, Buchs, Switzerland), sodium carbonate, $AlCl_3$ and sulphuric acid (Sigma-Aldrich, Steinheim, Germany), 2,4-DNPH and QC (Sigma-Aldrich, India), and potassium hydroxide (Kemika, Zagreb, Croatia). Absolute ethanol, for the analysis, was purchased from Supelco (Darmstadt, Germany), methanol and water (HPLC grade) were purchased from Honeywell (Seelze, Germany).

Instrumentation. Spectrophotometric measurements were performed on a Jenway Double Beam 6800 UV-Vis spectrophotometer with Flight Deck software version 1.0 (Bibby Scientific Ltd., Staffordshire, United Kingdom), equipped with 1 cm quartz cuvettes (HellmaOptik, Jena, Germany). For determination of the intermediate precision and robustness of the methods, 1 cm quartz cuvettes (Hach Co, Loveland, Colorado) were used. For preparation of the propolis extracts an analytical balance (AND, HR 202, Frankfurt, Germany), homogenizer (La Moulinette XXL, 1000 W, Moulinex, France), ultrasonic bath (RK-100 H, 230 V, Bandelin Sonorex, Berlin, Germany), microwave oven (Samsung, MW73E-WB, 800 W, Malaysia), centrifuge (322A, Tehtnica Železniki d.o.o., Železniki, Slovenia) and rotary evaporator (RV 10 control, IKA-Werke GmbH & Co. KG, Staufen, Germany) were used. A density meter (DE40, Mettler Toledo GmbH, Greifensee, Switzerland) and a pH-meter (PH 843P, Schott-Gerätte GmbH, Mainz, Germany) were used for determination of the physical and chemical properties of the extracts.

Extraction procedure. Each sample of still frozen raw propolis was grounded to fine powder in the homogenizer and subsequently milled through a laboratory sieve. Fifty milligrams of powdered propolis was weighed and 5 mL of 80% ethanol-water solution added. The extraction process was carried out in an ultrasonic bath at room temperature for 15 min, or in a microwave oven for a duration of 2 x 10 seconds. After that, 10 min centrifugation at 3500 rpm was carried out, the supernatant was quantitatively separated from the residue by filtration through regenerated cellulose membranes (RC, 0.45 µm pore size) into a volumetric flask, and the volume was made up to 10 mL. The extracts were stored at 2-8 °C until analysis. Additional dilution of extracts was employed to fit the calibration curves for determination of the TPs and TFFs.

Preparation of standard and blank solutions. The calibration curves were constructed by preparing appropriate dilutions of standard stock solutions. For the TPs method, GA, or a mixture of PC and GN at a ratio 2:1 in methanol, was used to prepare the standard stock solutions (1.00 mg/mL). Working standard solutions were prepared at concentration levels ranging from 16.5 to 330 µg/mL for each calibration standard. The preparation of calibration curves for the TFFs method with GN or QC as the reference compounds was carried out using six different concentrations in a range of 4-64 µg/mL. A methanolic solution of PC was used as the standard stock solution for preparation of the calibration curve for TFDs ranging from 90 to 900 µg/mL. Blank solutions were prepared by using 80% ethanol instead of the reference or test solution following the assay procedure. Each determination was analysed in triplicate.

TPs, TFFs and TFDs determination. TPs, TFFs and TFDs were quantified using the standard methods proposed by BANKOVA et al. (2016) with minor modifications regarding the use of GA and QC for TPs and TFFs, respectively, as additional standards. Propolis samples and working standard solutions for all three methods were prepared in 80% ethanol instead of methanol. The 2,4-DNPH method was additionally modified by centrifugation of the reaction mixture before analysis. TPs were measured at 760 nm, TFFs were detected at 415 nm and TFDs at 495 nm.

Extraction yield. The extraction yield of the propolis samples was expressed as balsam content, and calculated using the equation:

Balsam content = [(weight of the dry ethanolic extract (g)) / (weight of the raw propolis (g))] x 100%

Each ethanolic extract was evaporated to dryness in a rotary evaporator under reduced pressure at 60 °C. Every determination was performed in triplicate.

Validation parameters. All three spectrophotometric methods were validated (TAVERNIERS et al., 2004; ICH, 2005) by determining the following validation parameters: linearity, sensitivity, range, limit of detection (LOD), limit of quantification (LOQ), precision (intra-day precision and intermediate precision), accuracy (through recovery) and robustness.

The linearity of the methods was determined by generating two replicate calibration curves for each standard. The linear equation and correlation coefficient (R^2) were determined by linear regression analysis calculation. LOD and LOQ were calculated from the calibration curves of each standard using the standard deviation of the intercept and the slope of the curve. For evaluation of the precision and accuracy of the methods, three concentrations of standard solutions were prepared as two replicates, each measured three times over one day by one analyst in one laboratory. The concentrations of each calibration standard for the TPs were 40, 100 and 150 µg/mL, for the TFFs 10, 20 and 50 µg/mL, and for the TFDs, 200, 250, 750 µg/mL. Precision was additionally evaluated by the repeatability of sample preparation carried out by preparing six replicate propolis extracts and by one person measuring each one three times in one laboratory over one day. Intermediate precision was determined by preparing six independent propolis extracts, each measured three times on another day using different equipment, i.e. quivettes and different solvent lots (for water and ethanol).

The accuracy of the methods was expressed via percentage recovery (MATIĆ et al., 2017) using the following equation:

$$\text{recovery} = (\gamma_{\text{measured}} / \gamma_{\text{prepared}}) \times 100\%$$

The robustness of the methods was evaluated through determination of the intermediate precision and the stability of the test solutions. The stability of the standard and sample solutions was assessed by storing test solutions at 2-8 °C for 24 hours.

Statistical analysis. Data analysis was performed in Microsoft Office Excel (Microsoft Office 2010 Tools) using linear regression. Differences between TPs, TFFs and TFDs and between extraction techniques were determined according to statistical analysis by Student's t-test. P values of 0.05 and below were considered significant for all statistical analyses.

Results

Methods validation. For validation purposes, different calibration standards were employed to allow comparison between results obtained by the same method: GA and the mixture of PC and GN in a ratio of 2:1 for TPs, QC and GN for TFFs, and for TFDs PC was used as the representative of flavanones, since this flavanone is predominantly found in continental propolis samples. The linearity of the calibration curves was determined by regression analysis. The calculated calibration equations, LOD and LOQ were acceptable, as reported in Table 1. Together with LOD and LOQ, the slope of each constructed calibration curve was used to determine the sensitivity of the specific method.

Table 1. Validation parameters of the spectrophotometric methods used for quantification of flavonoids and phenolics in propolis

Standard	Range (µg/mL)	Calibration equation	R^2	LOD (µg/mL)	LOQ (µg/mL)
Folin-Ciocalteu					
PC-GN	16.5 - 330	$y = 1.6715x - 0.0063$	0.9997	2.37	7.17
GA		$y = 2.5333x + 0.0034$	0.999	3.97	12.04
Aluminium chloride					

Table 1. Validation parameters of the spectrophotometric methods used for quantification of flavonoids and phenolics in propolis (continued)

Standard	Range (µg/mL)	Calibration equation	R^2	LOD (µg/mL)	LOQ (µg/mL)
GN	4 - 64	$y = 2.7567x + 0.0003$	0.9998	0.38	1.16
QC		$y = 2.3715x - 0.0036$	0.9991	0.82	2.48
2,4-DNPH					
PC	90 - 900	$y = 0.1406x - 0.0118$	0.9995	9.38	28.43

The results for precision and accuracy shown in Table 2 are expressed as a percentage of the relative standard deviation (RSD). Precision was tested through determination of intra-day precision by one analyst over one day (analysis of three standard solutions measured three times each, and six replicated sample preparations measured three times each) and intermediate precision by one analyst on different days (analysis of six replicated samples). Standard solutions used for precision evaluation were also employed for accuracy testing. Accuracy was estimated by measuring the recovery of three different standard concentrations three times. For TPs, recovery was 86.6-109.3%. Precision was expressed through the RSDs that ranged from 0.10 to 3.21% for the standard preparation and 2.35-2.52% for the sample preparation, while the intermediate precision was 1.93-2.27%. For the TFFs, recovery was between 77.8 and 111.2%. The RSDs for standard preparation were 0.73-10.31%, for sample preparations they were 2.10% and intermediate precision was 1.80%. The recovery for the TFDs method was between 88.9 and 106.6%, while the RSDs for standard preparation were 0.18-9.88% and for sample preparation 5.55%. Intermediate precision for TFDs was 3.56%.

Table 2. Precision and accuracy of the spectrophotometric methods used for quantification of TPs, TFFs and TFDs in propolis samples

Standard	Accuracy [a]			Precision [b]		
	Prepared conc. (µg/mL)	Measured conc. (µg/mL)	Recovery (%)	Intra-day precision (%)		Intermediate precision (%)
				Standard RSD, n=6	Sample RSD, n=18	Another day, RSD, n=18
Folin-Ciocalteu						
PC-GN	39.6	34.3	86.6	0.66	2.06	2.22
	99.0	90.5	91.4	0.18		
	148.5	139.6	94.0	0.16		
GA	39.0	42.6	109.3	0.38	2.32	2.03
	98.8	91.5	92.6	3.21		
	145.6	137.4	94.3	0.10		

Table 2. Precision and accuracy of the spectrophotometric methods used for quantification of TPs, TFFs and TFDs in propolis samples (continued)

Standard	Accuracy [a]			Precision [b]		
	Prepared conc. (µg/mL)	Measured conc. (µg/mL)	Recovery (%)	Intra-day precision (%)		Intermediate precision (%) Another day, RSD, n=18
				Standard RSD, n=6	Sample RSD, n=18	
Aluminium chloride						
GN	9.6	8.9	93.3	10.31	1.81	1.71
	19.2	14.9	77.8	4.14		
	48.0	40.5	84.3	0.73		
QC	10.2	9.6	94.1	1.44	1.77	2.64
	20.4	22.5	110.3	1.75		
	51.0	56.7	111.2	0.81		
2,4-DNPH						
PC	195.1	171.5	88.9	9.88	6.62	3.76
	264.0	247.6	93.8	1.95		
	745.9	794.9	106.6	0.18		

[a] Accuracy is expressed as % recovery;
[b] Precision is tested through determination of: intra-day precision, sample preparation and intermediate precision.

The testing of the robustness of the methods was assessed through the intermediate precision and the stability of the test solutions. Intermediate precision showed that the methods are applicable to use regardless of variations in ambient conditions on different days, or the use of several different lots of solvents and quivettes. The stability studies of the standard and sample solutions showed results within 5% of the initial values.

Methanol and 80% ethanol were used as solvents during the optimisation of the methods. Since the absorbances detected were very similar and no significant differences were found between the two solvents, 80% ethanol was chosen as it is less toxic and very efficient in producing propolis extracts without waxes. Furthermore, optimization of the method's procedures was carried out during sample preparation, using MAE and UAE as extraction techniques, in order to ensure the good reproducibility of the methods. The Folin-

Ciocalteu and $AlCl_3$ methods were additionally optimized by employing two different standards for constructing the calibration curve to select more appropriate parameters for quantification (LOD, LOQ and sensitivity). On the basis of the results of optimization testing, the best analytical procedure for determination of TPs, TFFs and TFDs in propolis extracts was chosen and validated. A sample from Valpovo was used for validation purposes.

Physical and chemical properties and extraction yield. Raw propolis samples, originated from different locations, presented different physical properties. By visual observation, the samples appeared to be heterogeneous mixtures of variable consistency, from rigid and sticky, to loose and brittle. The colour ranged from yellow-orange to light brown for continental samples, and dark brown for Adriatic samples. The odour varied from mild pleasant to intense aromatic. The relative densities of extracts ranged from 0.8580 to 0.8622

and pH values were from 4.99 to 5.40. These physical-chemical properties were the first signs of the differences in chemical composition between the samples.

In this study, two advanced extraction techniques were used to produce ethanolic solutions of propolis, UAE and MAE. Fig. 2 shows the results for balsam content obtained by these two extraction procedures. Taking into account the balsam contents obtained from the same sample, extracted by different extraction techniques, the means were not statistically significant (P>0.05).

A very broad range of balsam content was obtained from different samples, from 32.35% to 96.6% for extracts obtained by UAE, and from 31.12 to 95.45% using MAE, (Fig. 2). The differences between average balsam content, 61.48% for UAE and 63.63% for MAE, were considered statistically non-significant (P>0.05). The content of balsam was found to be higher in the continental samples than in the coastal samples. The lowest, and very similar, values for balsam content were found in samples from Senj and Dubrovnik, i.e. 32.35% and 35.81% for UAE, 31.12% and 33.01% for MAE, respectively. The highest values of balsam content were found in samples from Croatia's eastern region, Valpovo and Požega.
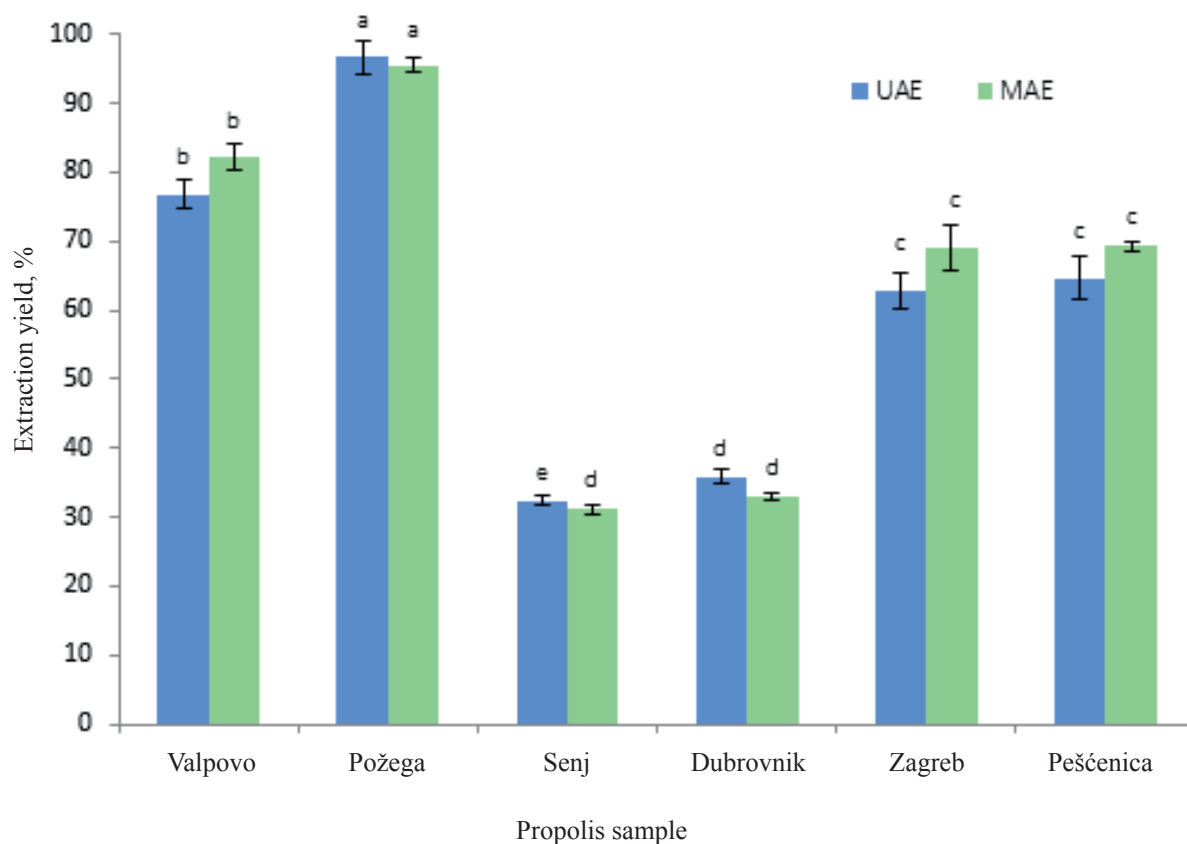

Fig. 2. Balsam content in raw propolis extracts obtained by UAE and MAE, presented as mean value (n=3) ± SD
*a-e Different letters indicate that means are statistically significant (P<0.05) comparing the means of each sample obtained by same extraction technique

In our study, the results were shown in relation to the content of extracted polyphenolic fraction, not the total amount of propolis weighed. As it is known that raw propolis contains variable amounts of insoluble impurities and wax, the content of balsam varied widely between samples. This affected the TPs, TFFs and TFDs as they differed significantly between the continental and coastal samples, but also between different continental samples (Table 3 and 4).

TFFs and TFDs. The results in Table 3 present the contents of TFFs and TFDs obtained by $AlCl_3$ and 2,4-DNPH methods, respectively. The content of total flavonoids in propolis extracts can be calculated by adding together the content of TFFs and TFDs which was between 90 and 350 mg/g balsam.

Using the PC as a standard, the highest content of TFDs was found in samples from Zagreb and Valpovo. These results were in accordance with the balsamic content in those samples, which confirmed that a higher balsam content means a higher content of polyphenols.

Table 3 also presents the results for TFFs, depending on the standard and extraction technique used. The contents of TFFs were significantly higher with QC than GN for all samples. Considering UAE, the highest contents of TFFs were found in samples from Zagreb and Valpovo, and the lowest in samples from Senj and Dubrovnik, regardless of the standard used. Furthermore, when comparing the differences between UAE and MAE, the means of the same samples (three replicate samples each measured three times, n=9) showed statistical significance, P<0.05 for all analysed samples.

Table 3. Results of quantification of TFFs and TFDs in propolis extracts obtained by UE and MAE

| Sample | $AlCl_3$* | | | | 2,4-DNPH** | |
| | GN | | QC | | PC | |
	UAE	MAE	UAE	MAE	UAE	MAE
Valpovo	134.76±0.72[b]	116.64±2.33[b]	164.96±0.75[b]	142.75±2.69[b]	139.83±7.06[b]	157.38±5.32[a]
Požega	22.02±0.55[d]	41.60±3.15[c]	31.88±0.91[d]	54.46±3.83[c]	90.69±3.99[e]	96.80±2.99[e]
Senj	18.44±0.1[e]	17.82±0.57[e]	21.61±0.73[e]	22.83±1.24[d]	93.36±6.02[d]	130.74±4.88[b]
Dubrovnik	5.03±0.22[f]	6.22±0.26[f]	6.70±0.22[f]	8.21±0.34[e]	89.37±4.87[e]	75.02±4.06[f]
Zagreb	182.47±4.30[a]	159.64±4.70[a]	222.22±5.20[a]	194.32±5.36[a]	169.41±9.11[a]	123.43±8.87[c]
Pešćenica	32.43±2.21[c]	38.22±1.53[d]	47.06±2.85[c]	52.96±1.74[c]	105.61±1.76[c]	98.23±0.27[d]

* Results are expressed as mean values ± SD (mg of galangin/g balsam or as mg of quercetin/g balsam).
** Results are expressed as mean values ± SD (mg of pinocembrin/g balsam).
a-f comparing means of different propolis samples within the same column (three replicate samples each measured three times, n=9), different letters (a, b, c, d, e, f) indicate that means differ significantly, P<0.05 according to Student's t-test

TPs. The highest amount of TPs was found in a sample from Zagreb county, and the lowest in a sample from Dubrovnik, regardless of the standard used. Concentrations of TPs were much lower with GA than PC-GN, as can be seen in Table 4. When comparing the differences between extraction techniques, the means of the same samples extracted by different techniques showed statistical significance (P<0.05), except the sample from Požega which was statistically non-significant, P>0.05.

Table 4. Results of quantification of TPs in propolis extracts obtained by the UE and MAE Folin-Ciocalteu methods

| Sample | Folin-Ciocalteu* | | | |
| | PC-GN | | GA | |
	UAE	MAE	UAE	MAE
Valpovo	539.53±6.24[b]	603.47±10.56[c]	336.53±4.31[b]	312.37±5.36[c]
Požega	395.82±9.75[d]	479.39±11.,25[e]	245.74±6.95[d]	247.68±6.34[d]
Senj	333.05±4.17[e]	505.48±14.98[d]	174.60±1.09[e]	196.89±6.04[e]
Dubrovnik	3.16±0.20[f]	49.90±1.17[f]	0.07±0.11[f]	23.94±0.58[f]
Zagreb	671.76±5.06[a]	736.16±9.39[a]	419.54±2.60[a]	382.98±5.77[a]
Pešćenica	468.40±2.24[c]	648.88±12.54[b]	287.19±1.47[c]	334.01±6.77[b]

* Results are expressed as mean values±SD (mg pinocembrin:galangin (2:1)/g balsam, or mg of gallic acid/g balsam)
a-f comparing means of different propolis samples within the same column (three replicate samples each measured three times, n=9) different letters (a, b, c, d, e, f) indicate means that are statistically different, $P<0.05$ according to Student's t-test

Discussion

The complex nature of propolis demands purification of samples before analyses. UAE was carried out at room temperature for 15 minutes at a power of 230 W, and even though the ultrasonic bath operated at room temperature (higher than 20 °C), no heating of the sample solution caused by the ultrasonic waves was noticed, implying that the polyphenolic fraction was preserved. MAE was performed for 2 x 10 seconds at 800 W. Boiling and overflow of the extraction solution from the test tube were caused by longer periods of microwave extraction (15 s). Therefore, we applied conditions that were acceptable in relation to the results for balsam content presented in Fig. 2. Maceration was not used in our research as recent studies have reported lower or approximately equal extraction yields when compared to UAE, only the maceration was much slower (TRUSHEVA et al., 2007; ESCRICHE and JUAN-BORRAS, 2018).

The balsam content obtained using MAE (2 x 10 s) was higher in a few samples when compared with samples extracted by ultrasound, but the differences were not statistically significant (P>0.05). This was noticed for samples that contained a higher mass of the dry ethanolic extract (from Valpovo, Požega, Zagreb and Pešćenica). Furthermore, MAE caused overheating of the extraction solution and provided clear extracts with a high amount of extracted, that is, dissolved wax, while UAE produced opaque extracts without wax extracted in a solution. This finding could suggest that even though the MAE was very fast and provided approximately equal or higher yields (Fig. 2), and consequently TPs, TFFs and TFDs (Table 3 and 4), UAE proved to be more effective in terms of higher selectivity.

Polyphenol analysis. Different geographical locations and plant sources around apiaries directly influence the chemical characteristics of the propolis samples in a specific area (BANKOVA et al., 2002; HUANG et al., 2014; GRAIKOU et al., 2016). Despite the different raw substances that bees collect, the primary role of propolis, as building and defence material, is equivalent all over the world. Croatia is a country positioned in a temperate zone, for which continental European or the "poplar" type of propolis is common, although recently JERKOVIĆ et al. (2016) and SAFTIĆ et al. (2019) reported another propolis type along the Adriatic coast and on the islands, with low polyphenolic content but rich in diterpenes, known as the Mediterranean type. Four of our samples originated from the temperate continental zone, one sample was from a "transitional" climate zone

between a mountainous area and the Mediterranean part of Croatia, and one was from a typical Mediterranean area.

In the literature, different reference compounds have been used to validate colorimetric spectrophotometric methods and to express the results, each specific for a specific group of polyphenols, including GA, caffeic acid, ferulic acid, a mixture of PC-GA for TPs and QC, GN, naringenin (NG), PC and catechin for total flavonoids (FALCAO et al., 2013; HERNANDEZ ZARANTE et al., 2018; EL MENYIY et al., 2021). The most appropriate calibration standard would be the one that occurs in the samples at a high level. This was the case with the 2,4-DNPH method and the use of PC for expression results for TFDs, a very important group of flavonoids in propolis. TFDs together with TFFs contribute to the content of total flavonoids, i.e. TFs. Despite the fact that no additional standard was used to compare the results obtained by 2,4-DNPH, the results presented in Table 3 may be considered reliable due to the fact that PC is one of the dominant flavanones in propolis samples, and was also used to calculate TFDs in many previous studies (MIGUEL et al., 2010; BANKOVA et al., 2016; POPOVA et al., 2017).

The calibration curves for GA and PC-GN in the Folin-Ciocalteu method, and also for QC and GN in the $AlCl_3$ method, were used to demonstrate the dependence of the calibration standard used on the polyphenolic concentrations, but at the same time to allow comparison with published reports. Many researchers use GA as a reference compound for determination of the TPs in propolis from different parts of the world, but according to MIGUEL et al. (2010), this phenolic acid is mostly found in tropical samples and is not a good representative of propolis from a temperate zone, thus it may exhibit lower results when compared with PC-GN. Although ESCRICHE and JUAN-BORRAS (2018) reported similar concentration ranges of TPs for GA and PC-GN, our results for PC-GN, presented in Table 4, were approximately double when compared with GA, regardless of the type of sample. On the other hand, the higher concentrations obtained using a mixture of PC-GN do not necessarily mean they are

reliable results. In fact, as the spectrophotometric methods described are not specific, and due to the very complex composition of propolis, many other constituents, such as sugars, ascorbic acid, amino acids and organic acids present in the sample, may interfere with the reagents used for the assays, resulting in increased concentrations of polyphenols measured, giving false-positive results (SÁNCHEZ-RANGEL et al., 2013). Having in mind that gallic acid is frequently absent in propolis samples, which may be confirmed by the significantly lower concentrations of TPs, the PC-GN mixture seemed to be the preferable option.

A similar pattern was observed for determination of TFFs when the calibration standard was QC in comparison with GN. Many researchers use QC as equivalent to express total flavonoids, although, according to POPOVA et al. (2004) and FALCÃO et al. (2013), GN is more suitable for the European type of propolis. Our study demonstrated that QC was a better choice for the $AlCl_3$ method, obtaining higher absorbances, and consequently higher concentrations of TFFs.

The physical characteristics of Croatian propolis, its colour, odour and consistency, showed wide differences which were reflected in the chemical diversity between the analysed samples, depending on the botanical diversity of plant sources and the climatic conditions characteristic for a specific geographical area. Due to the geographical differences, the season of collection, extraction technique, solvent used and the presentation of results, it is very difficult to compare reported studies. The concentrations of polyphenols in various propolis samples from different studies can only be compared if the same standard is used for their characterization. For example, KOSALEC et al. (2004) and CHANG et al. (2002) reported the concentrations of TFFs as QC equivalent and the concentration of TFDs as NG equivalent. Our results converted to % mg/g balsam (TPs 0.3-67.2%, TFFs 0.5-18.3% and TFDs 8.9-16.9%, balsam content 32-96%) were in the same range as previously reported studies by POPOVA et al. (2007) where TPs were 7.9-46%, TFFs 1.3-17.9%, TFDs 1.5-15.2% and balsam content was 18-82%. On the same basis, POPOVA et al. (2017) reported

TPs 11.2-42%, TFFs 2.9-13.5%, TFDs 3.5-9.4% and balsam content from 33 to 88%.

Taking into account the calibration with GA and QC, the mean values of our results in Tables 3 and 4 for TPs (0.07-336.5 mg/g) and TFFs (6.7-222.2 mg/g) could be comparable to a recent report by PAVLOVIĆ et al. (2020), where TPs were 236.32-242.42 mg/g, TFFs were 26.91-32.14 mg/g and balsam content ranged from 63.94 to 75.92%. JUG et al. (2014) reported TPs ranged from 205.8 to 219.7 mg GAE/g and TFs 115.9-119.2 mg /g.

Samples from the continental temperate region were richer in polyphenols than samples from the coast, confirming previously reported studies (JERKOVIĆ et al., 2016; SVEČNJAK et al., 2020). It is evident that hilly terrain vegetation and the influence of the Adriatic Sea have a major impact on the chemical composition of samples from Dubrovnik and Senj, showing the lowest concentrations of polyphenols, when compared with continental samples, regardless of the polyphenol standard used (Table 3 and 4). This study demonstrated that the choice of calibration standard significantly affected the final result, and that the most appropriate standards for quality control investigations of various propolis types, based on spectrophotometric methods, should be the dominant polyphenols in the sample.

Conclusion

In this study, three spectrophotometric methods were optimized and completely validated. It was shown that the methods were sensitive and applicable for evaluation of the wide concentration ranges of polyphenols in propolis extracts. Furthermore, we described the dependence of the total polyphenolic content on the reference compound used to express the results. TFDs were quantified by PC as a reference, considering its domination in propolis composition. Concentrations of TPs and TFFs were higher with PC-GN and QC, respectively, therefore those standards were considered more suitable for quantification than GA or GN. The contents of polyphenols varied significantly between samples. Samples from the continental region were richer in polyphenols than Adriatic samples. The polyphenol contents in samples from Senj and Dubrovnik were the lowest regardless of the standard used.

Low concentrations may be related to the absence of polyphenols in these samples and the specific vegetation of a particular geographical area, suggesting that the quality control analysis of these samples should be based on other compounds, characteristic for a specific area. The results obtained through this research are the basis for further chromatographic analysis of the individual polyphenolic components in propolis samples that were covered by this study.

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SAŽETAK

U ovom su radu provedene spektrofotometrijske metode za brzu analizu uzoraka propolisa iz različitih područja Republike Hrvatske. S ciljem određivanja glavnih skupina bioaktivnih spojeva u propolisu, optimizirane i validirane su tri spektrofotometrijske metode: Folin-Ciocalteuova metoda za određivanje ukupnih fenola i dvije različite metode za sadržaj ukupnih flavonoida - metoda kompleksiranja s aluminijevim kloridom za ukupne flavone/flavonole te 2,4-DNPH metoda za ukupne flavanone/dihidroflavonole. Validacijom su određeni sljedeći parametri: linearnost, osjetljivost, raspon, točnost, granica detekcije, granica kvantifikacije, preciznost i robusnost. Za provedbu validacijskog protokola korišteni su standardi polifenola, uključujući galnu kiselinu (GA), pinocembrin (PC), galangin (GN), kvercetin (QC) te smjesu pinocembrina i galangina (PC-GN). Validiranim metodama analizirano je šest uzoraka sirovog propolisa iz kontinentalne i jadranske regije Hrvatske. Primijećena je velika raznolikost u sadržaju ukupnih fenola, ukupnih flavona/flavonola te ukupnih flavanona/dihidroflavonola među uzorcima iz dviju hrvatskih regija. Iako metode ekstrakcije (ultrazvučna ekstrakcija ili ekstrakcija potpomognuta mikrovalovima) nisu pokazale statistički znakovitu razliku u obliku prinosa ekstrakcije (P > 0,05), te gledajući koncentracije polifenola cjelokupno, ultrazvučna je ekstrakcija bila selektivnija. Nadalje, velik utjecaj na konačne koncentracije ukupnih fenola i ukupnih flavona/flavonola imao je standard korišten za izradu kalibracijske krivulje. Dobivena je dobra linearnost, točnost, ponovljivost, srednja preciznost, granica detekcije i granica određivanja u svim trima spektrofotometrijskim metodama te su ovi rezultati temelj za daljnja istraživanja pojedinačnih polifenolnih spojeva u uzorcima propolisa.

Ključne riječi: sirovi propolis; polifenoli; ultrazvučna ekstrakcija; ekstrakcija potpomognuta mikrovalovima; spektrofotometrijsko određivanje; validacija

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