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Extraction kinetics and physicochemical characteristics of Colombian propolis

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Abstract

Propolis, a resinous matter collected by *Apis mellifera* bees from exudates of plants, exhibits different biological properties, such as antimicrobial, antifungal, antiviral, and antioxidant activities. Standardization of the extraction of their bioactive compounds is a complex issue in particular for rural regions in developing countries due to the need for specialized equipment and personnel. Herein, kinetic extractions by maceration over 7 days were compared with ultrasound and supercritical fluids extractions in terms of the polyphenolic content obtained. Ultrasound showed the fastest kinetic extraction for each propolis without affecting the antioxidant activity. Supercritical fluids showed the lowest polyphenolic content recovery and no selectivity for antioxidant activity, in agreement with the solubility predictions. The main difference among the extraction methods was the extraction time required to reach the equilibrium concentration. No significant differences in selectivity were observed for the different methods. The antioxidant activity remained almost constant with the extraction method and extraction time, indicating similar extraction kinetics for the extracted polyphenols. We found that the physicochemical characteristics and UV-vis spectra could be used as predictors of the polyphenolic contents for the studied samples.

Practical Application

Even though most of the beneficial properties to the human health of the propolis extracts depend largely on the compounds present in the matrix and their extractability, the evaluation of these compounds and their biological activities might be costly and time-consuming for beekeepers, particularly in developing countries. Also, the extraction method and the extraction time are important decisions that significantly affect the economics of the propolis production process. The main difference among extraction methods was the extraction time required to reach equilibrium and no significant differences in selectivity were observed for the different methods. Moreover, it was found that simple parameters such as ash and moisture can be used as predictors of the polyphenolic contents and antioxidant activity of the propolis, and they can provide important information about the handling during harvest, transport, and storage of the propolis.

KEYWORDS
extraction, kinetics, propolis

1 | INTRODUCTION

Over the last few decades, bee products especially propolis have been widely studied, due to their chemical composition and beneficial properties for human health (Król et al., 2013). Propolis or bee glue is a resinous substance produced by bees mixing wax with plant exudates gathered mainly from leaves, flower buds, and stems of plants and trees. Some of these are mucilages, resins, and gums that are secreted by plants to protect themselves from the weather and attacks by bacteria, viruses, and fungi.

Raw propolis is commonly composed of 50% of resins and balms, 30% of waxes and fatty acids, 10% of essential oils, 5% of pollen, and 5% of other substances (Anjum et al., 2019). Over 300 compounds have been discovered in the propolis matrix, most of them belonging to polyphenols and terpenoids (Escriche & Juan-Borrás, 2018). Propolis has a wide range of medicinal applications, such as antimicrobial (Pobiega et al., 2019), anti-inflammatory (Ezzat et al., 2019), antioxidant (Andrade et al., 2017), antitumoral (Popova et al., 2017), antifungal (Waller et al., 2017), antiviral (Silva-Beltrán et al., 2020), antimutagenic (Fernandes et al., 2015), and antihepatotoxic (Alm-Eldeen et al., 2017). These medical benefits are attributed to the phenolic compounds in the propolis matrix, specifically flavonoids and phenolic acids. Phenolic acids and flavonoids are plant secondary metabolites responsible for the growth, color, and aroma of plants. They are acknowledged as strong natural antioxidants having a key role in a wide range of biological and pharmacological properties (Kumar & Goel, 2019).

The chemical composition of propolis depends not only on the bee species but also on other external elements, such as the geographical location, since it is a product highly dependent on the characteristics of the surrounding vegetation (Bankova, 2005).

The extraction time is one of the most critical variables in the extraction process. Although there are reports of maceration extractions under an hour with constant agitation and high temperatures (Devequi-Nunes et al., 2018), most use sporadic agitation and room temperatures for at least 1 day (Mora et al., 2019), 7 days (Pobiega et al., 2019; Reis et al., 2019) or 30 days (Woisky & Salatino, 1998). da Silva Cunha et al. (2006) showed that increasing the extraction time from 20 to 365 days increased the extraction only by 7%. Ultrasound can speed up the mass exchange between the propolis and the solvent by (i) fragmentation of the extracted material increasing its exposure to the solvent, and (ii) increasing membrane permeability favoring the rate of solvent diffusion (Pobiega et al., 2019). Ultrasound extraction is reported to require lower contact time compared with maceration, with common reports between 20 and 50 min (Tao et al., 2014; Yuan et al., 2019).

Analysis of the extraction kinetics is a widely used tool for studying different extraction techniques. Extraction kinetics can provide useful information to design, optimize, and control processes by fitting the experimental data with mathematical models (Ruslan et al., 2014). For propolis ultrasound extraction, models such as second-order have been implemented to analyze the behavior of the recovery of phenols and flavonoids (Yusof et al., 2020).

Even though most of the beneficial properties to the human health of the propolis extracts depend largely on the phenolic compounds present in the matrix and their extractability. The evaluation of the phenolic compounds and biological activities might be costly and time-consuming for beekeepers, particularly in developing countries. Thus, it is important to develop guidelines relating common quality criteria such as moisture, wax content, ash content, and soluble matter to the extractable polyphenols and resulting biological activities as functions of the extraction kinetics. This work aimed to evaluate the kinetic behavior over a week for the extraction of propolis using three different extraction methods (ultrasound, maceration, and supercritical fluids extraction) from four different Colombian regions. The extraction method, extraction time, and propolis quality parameters were correlated to the extract characteristics, such as the concentration of flavonoids and phenolic acids, and the antioxidant activity.

2 | MATERIALS AND METHODS

2.1 | Propolis samples

Samples from four different Colombian regions were provided by apiculture associations in San Rafael (SR) 1000 m above the sea level (masl), Salgar (SL) 1250 masl, Necoclí (NI) 8 masl, and Bagre (BG) 50 masl. Each of those regions with two apiaries corresponding to propolis collected from January to February. A suffix after the region name is used to indicate the apiary used in each case, for instance, SL1 and SL2 correspond to two different apiaries in the Salgar region. The samples were frozen and ground with an electric coffee grinder (Krups, F203) and stored at −80°C until extraction.

2.2 | Characterization

Moisture and ash content were determined following the AOAC methods of analysis (Horwitz et al., 1970). Moisture content was determined by drying 5 g at 105°C over 5 h (AOAC 930.04). Ash content was determined from 2.5 g of propolis incinerated at 550°C overnight.

Dry residue and wax content were determined by extracting 0.3 g of propolis with 30 ml of 96% vol/vol ethanol (Bell Chem International), under constant agitation (110 rpm) for 24 h. The supernatant and residues were separated by centrifugation (Hettich Universal 32R). The residues were heated at 60°C overnight, and the supernatants were kept at 4°C for 24 h and separated by centrifugation. The precipitate was separated and dried at 60°C, and the ratio of its weight to the propolis weight sample was reported as the wax content.

The soluble matter in ethanol was adapted from Bankova et al. (2019). Two milliliters of each extract were concentrated in vacuum at 45°C. The soluble matter was calculated as the ratio of the solids' concentration of the extract and the initial propolis sample.

The absorption spectrum of the ethanolic extracts was measured in the range of 200–600 nm with 96% ethanol as blank in a UV-vis spectrometer (Thermo Scientific Genesys 10 S UV-Vis) (Abdullah et al., 2019). Flavonoids and phenol contents were also measured with spectrometric techniques (Thaipong et al., 2006).

2.3 | Propolis extraction

For maceration extraction, a solution was prepared with 3 g of propolis and 300 ml of 96% vol/vol ethanol. The concentration of ethanol was determined from the Hansen solubility parameters, as presented in Section S1. The solution was placed under constant agitation (110 rpm) in the dark. For the kinetic experiments, samples were taken at 10, 30, 100, 180, 315, 560, 1440, 2880, 4320, and 10080 min (7 days).

Ultrasound-assisted extractions were carried out in an ultrasound system (Branson 5210) at 40 kHz. The extractions were performed at room temperature for 0.3 g samples of each propolis for 15, 20, and 30 min with 96% EtOH (1:100, wt:vol).

Supercritical fluid extractions were performed with a Helix Supercritical fluids system (Applied Separations, Inc) at 50°C and 350 bar using supercritical CO_2 and ethanol as co-solvent, with 4.5 g of propolis sample mixed homogeneously with glass beads of 4 mm in the extraction cell. The flow of CO_2 was 3.9 g/min and the total time of extraction was 120 min. Samples were collected in vials of 15 ml at 12.84 psi and 25°C at 4, 8,12, 20, 30, 40, 50, 60, 70, 80, 90, and 120 min. The effect of the miscibility of the polyphenols with the co-solvent was evaluated by (i) pumping 5% of ethanol as part of the CO_2-ethanol mixture, (ii) pumping 10% of ethanol as part of the CO_2-ethanol mixture, and (iii) adding ethanol to the propolis extraction vessel directly before the addition of CO_2.

In all cases (maceration, ultrasound, and supercritical fluids extraction) the ethanolic propolis extracts were centrifuged at 6000 rpm at 4°C for 10 min (Graikini et al., 2019). The supernatant was kept at 4°C for 24 h to remove waxes by decantation and was stored in the dark at 4°C.

2.4 | Phenols and flavonoids

Total phenols were quantified by mixing 150 µl of the extract with 2.4 ml distilled water and 150 µl of a Folin-Ciocalteau (Panreac AppliChem) 1 N solution by vortexing (Daigger Genie 2). After 5 min, 300 µl of a 1 N sodium carbonate (Merck) solution was added, then this mixture was vortexed and allowed to stand for 60 min in the dark at room temperature. The absorbance of samples was read at 725 nm using a UV spectrophotometer (Thermo Scientific Genesys 10 S UV-Vis) and compared with a calibration curve of gallic acid (Alfa Aesar). The total phenols are expressed as gallic acid equivalent (GAE) in milligrams per gram of propolis. (Thaipong et al., 2006) Total flavonoids were estimated by mixing 0.5 ml of extract, 4.3 ml UHPLC methanol (Panreac AppliChem), 0.1 ml 10% of $AlCl_3$ solution (Sigma-Aldrich), and 0.1 ml of a 10% (wt/vol) potassium acetate solution (Carlo Erba Reagents SRL) by vortexing. The absorbance of the solutions was measured at 415 nm. The results were calculated using a standard curve for known concentrations of quercetin (Sigma-Aldrich), and the results are expressed as quercetin equivalent (QE) in milligrams per gram of propolis (Pham et al., 2017).

2.5 | Antioxidant activity by the DPPH method

Antioxidant activity was determined using the 2,2-diphenyl-picrylhydrazyl (DPPH, Sigma-Aldrich) free radical scavenging assay (Blois, 1958). Propolis extracts were dried in vacuum and dissolved in methanol. A 10 µl of each methanol solution was then mixed with 990 µl of methanolic DPPH solution of 200 µg ml^{-1} and the mixtures were vortexed. The solutions were left at room temperature for 1 h in the dark. The decrease of DPPH radical in the mixture, as indicated by the reduction of its purple color, was quantified by measuring the

TABLE 1 Kinetic models evaluated for extraction of polyphenols from the studied propolis samples.

Model	Equation
First-order expression$_{(1)}$	$$ln\left(\frac{C_{e_1}}{C_{e_1}-C}\right)=k_1 t \quad (1)$$
First-order + intercept model$_{(1r)}$	$$ln\left(\frac{C_{e_{1r}}}{C_{e_{1r}}-C}\right)=k_{1r} t+a_1 \quad (2)$$
Second-order model$_{(2)}$	$$C=\frac{C_{e_2}^2 k_2 t}{1+C_{e_2} k_2 t} \quad (3)$$
Parabolic diffusion model$_{(PD)}$	$$C=a_{PD}+k_{PD} t^{0.5} \quad (4)$$
Power law model$_{(Pl)}$	$$C=k_{Pl} t^n \quad (5)$$
Weibull's equation$_{(W)}$	$$\frac{C}{C_{e_w}}=1-exp\left[-(k_W t)^m\right] \quad (6)$$
Elovich's equation$_{(E)}$	$$C=a_E+k_E ln(t) \quad (7)$$

TABLE 2 Characterization of each propolis sample and total phenols and total flavonoids of the ethanol-soluble extracts for each sample after 24 h of maceration with a ratio of 1:100 of 96%ethanol.

Sample	Moisture (%)	Ash (%)	Residue (%)	Wax (%)	Soluble matter (%)	Phenols (mg GAE/g)	Flavonoids (mg QE/g)
SL1	8.76_{5}	1.57_{3}	38.30_{368}	11.13_{18}	36.75_{35}	11.68_{8}	6.96_{74}
SL2	6.57_{71}	0.71_{24}	42.37_{207}	15.57_{23}	56.25_{742}	42.06_{113}	8.67_{49}
NI1	1.51_{58}	0.74_{2}	25.95_{21}	10.39_{12}	70.25_{247}	130.04_{100}	12.98_{67}
NI2	2.78_{64}	0.89_{22}	30.77_{62}	16.27_{17}	53.75_{106}	11.07_{35}	6.49_{37}
BG1	6.41_{62}	0.79_{21}	52.75_{191}	16.27_{22}	38.50_{212}	2.59_{1}	0.05_{1}
BG2	16.46_{30}	0.54_{1}	33.75_{35}	23.73_{19}	39.00_{354}	5.61_{1}	0.01_{1}
SR1	2.46_{2}	0.87_{17}	21.80_{99}	11.77_{15}	76.75_{318}	46.42_{342}	6.00_{2}
SR2	5.49_{89}	1.27_{14}	37.70_{14}	14.57_{17}	59.25_{742}	35.10_{93}	4.02_{14}

Note: Numbers in subscripts indicate the uncertainty of the last digit(s).
Abbreviations: BG, Bagre, GAE, gallic acid equivalent, NI, Necoclí, SL, Salgar, SR, San Rafael, QE: quercetin equivalent.

absorbance at 517 nm using methanol as blank. The half-maximal effective concentration (EC_{50}) was reported as the concentration of propolis extracts required for a 50% reduction of DPPH.

2.6 | Kinetic models

Seven different kinetic models for solid–liquid extraction were evaluated. Namely, first order, second order, parabolic diffusion, power law, Weibull, and Elovich models were studied to find the best fit for the experimental data of the extraction process of phenols and flavonoids from propolis. The models assessed in this work and their corresponding equations are presented in Table 1. In general, all the models have the following assumptions: (i) all solid particles are spherical with uniform size, (ii) the extractable component is evenly distributed in the solid, (iii) the diffusion coefficient of the extractable component is constant, and (iv) the solid particles are well dispersed in the extracting solvent. See Sections S2 and S3 for further details of the fitting procedure and the corresponding parameters for maceration and supercritical fluid extraction.

In Table 1 C_e corresponds to the equilibrium concentration, C to the concentration at time t during the extraction process, k, a, m, and n are free parameters in the kinetic models. The subscripts 1, 1r, 2, PD, Pl, W, and E correspond to the first-order, first-order + intercept, second-order, parabolic diffusion, power law, Weibull, and Elovich models, respectively. A more detailed description of the free parameters in each of the kinetic models is given in Section S2.

3 | RESULTS

3.1 | Propolis characterization

The complex interaction of the solvent with the propolis matrix requires a careful evaluation of the extraction parameters based on the characteristics and composition of the raw propolis. Propolis properties such as moisture, wax content, ash content, and amount of matter soluble in ethanol can be used to characterize the propolis condition (Bankova et al., 2019). For instance, high concentrations of biologically inert components (waxes) and other physical impurities (loam, pieces of wood) are related to low amounts of bioactive compounds in propolis (Sawaya et al., 2011), and high humidities reflect poor conditions during the storage and/or harvest stages.

Basic propolis characteristics, such as moisture, ash, residue, wax, and soluble matter, are reported in Table 2. The propolis color for all samples was brown, except for NI, with red color in NI1 and red traces in NI2. The extracts of each sample varied from red (NI1), orange (NI2), brown (SL2, SR1, and SR2), yellow (SL1), and transparent (BG1 and BG2). For the moisture content, BG2 propolis exceeded 10%, which might be related to poor manipulation conditions in harvesting and storage. The residue after extraction with ethanol was higher than 40% for SL2 and BG1. This shows either that the matrix comes from a region where the bees could not obtain a satisfactory amount of resins in neighboring plants or that it may have been admixed with external components to adulterate the product (Sawaya et al., 2011). However, since the soluble matter for all propolis samples was higher than 30% this supports the former rather than the latter.

The soluble matter represents the extractable components of propolis. When this parameter increases it is expected that the polyphenolic content also increases. For instance, the samples with the highest soluble matter (NI1 and SR1) presented a large concentration of total phenols (130 and 46 mg GAE/g). In contrast, BG1 and BG2 had the lowest soluble matter (∼39%) and the lowest phenolic content (∼5 mg GAE/g and 0 mg QE/g).

In general, moisture and wax in propolis should be lower than 10% and 25%, respectively (Bankova et al., 2019). We found that if the sum of the moisture, ash, and residue were greater than the soluble matter the corresponding concentration of polyphenols was low. That is, BG1, BG2, and NI2 present a much lower concentration of polyphenols than SR1, SR2, and SL2, which have a higher concentration of soluble matter.

The concentration of polyphenols can vary widely depending on the type of propolis and the origin region (Bankova, 2005). Phenolic contents of Brazilian brown propolis have been reported to range between 31 and 63 mg GAE/g and flavonoids content between 6 and 25 mg QE/g (Bittencourt et al., 2015; Olegário et al., 2019), whereas

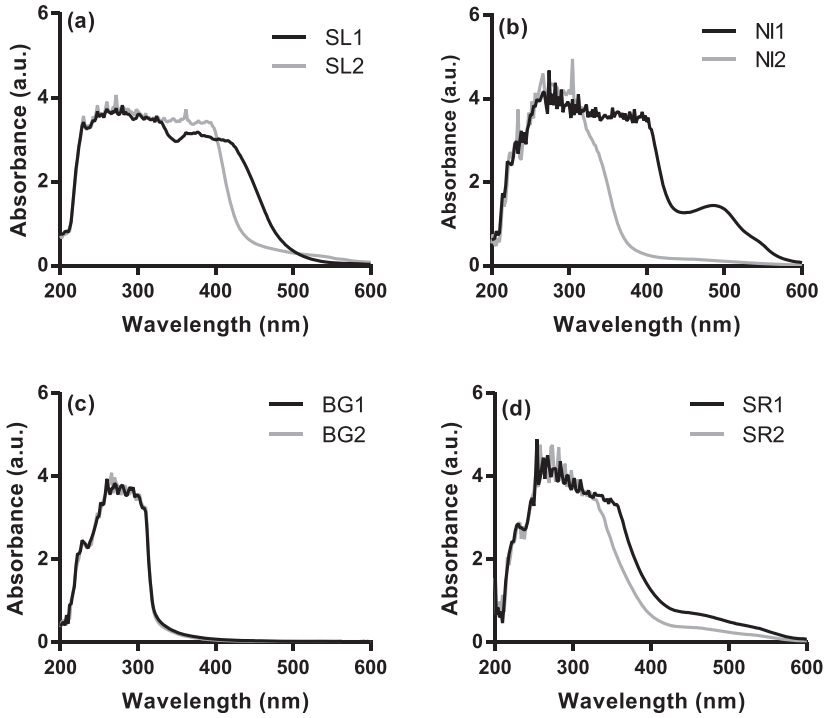

FIGURE 1 UV-vis absorption spectrum of the propolis extracts after 24 h of maceration with a ratio 1:100 of 96% ethanol in the range of 200–600 nm for (a) Salgar (SL), (b) Necoclí (NI), (c) Bagre (BG), and (d) San Rafael (SR)

Brazilian red propolis flavonoids range between 27 and 43 mg QE/g (Andrade et al., 2017) and phenols between 90 and 250 mg GAE/g (da Silva Frozza et al., 2013). In this case, the composition of Colombian propolis ranged between 3 and 130 mg GAE/g for phenols and between 0 and 13 mg QE/g for flavonoids.

The UV-Vis absorption spectra of the extracts are shown in Figure 1. UV-vis absorption spectra have been previously studied as qualitative evaluations in propolis (Abdullah et al., 2019). Miyataka et al. (1997) showed that absorption bands at 270–330 nm indicate the presence of flavonoids and phenols in propolis. Gregoris and Stevanato (2010) found that pure compounds like kaempferol, quercetin, and galangin have absorbance peaks at ~350 nm and caffeic acid derivatives and apigenin show absorbance between 300 and 350 nm. For all samples, the UV-vis spectrum began at 200 nm with the largest peaks around 260 nm, except for NI1 (270 nm) and NI2 (304 nm). The main difference among the spectra was the range of the adsorption bands. For instance, for SL1 the absorption band started to decay around 420 nm (the largest spectral area for the absorption band) and for BG1 and BG2 it started to decay around 300 nm (the smallest spectral area). The spectral areas of the absorption bands followed the order SL1 > NI1 > SL2 > SR1 > SR2 > NI2 > BG1 > BG2. The spectral area showed a strong correlation with the phenolic and flavonoid content in the extracts ($r = 0.70$ for phenols and $r = 0.92$ for flavonoids, see details of the correlation in Section S4).

3.2 | Maceration and ultrasound extraction

The kinetics of the extraction of phenols and flavonoids was analyzed and compared among the apiaries of each region. Figure 2 shows the time evolution of the phenolic content extracted for each propolis. For maceration the kinetics are described with the superscript "m" and for ultrasound with the superscript "u".

Large differences in the extractable phenols can be observed among the samples, even for the same regions. The quality criteria suggest that when contrasting propolis of the same region, a higher concentration of polyphenols can be extracted from samples with lower moisture, residue, ash, and wax contents. For instance, NI1 had the highest concentration of phenolic compounds (130 mg GAE/g) whereas NI2 had <10% of that (11 mg GAE/g), and these results are consistent for maceration and ultrasound. This might be caused by (i) the presence of extraneous components in the propolis due to its handling, as indicated by the differences in the soluble matter between NI1 and NI2; or (ii) differences in the plant species surrounding each apiary.

The lowest phenolic content was observed for both BG samples with ~4 mg GAE/g even after 7 days of extraction or the use of ultrasound-assisted extraction. This is related to their high moisture, ashes, waxes, and residues; low soluble matter; and their small spectral area, which might be the result of poor floral accessibility and/or propolis' poor handling conditions.

Extraction kinetics for total phenols equilibrated approximately between 300 and 600 min for maceration. Indicating that extractions beyond 10 h are not necessary to obtain a propolis extract for the studied samples. SR propolis showed no change during the sampled period, implying the fastest extraction kinetics for all the studied propolis. This behavior was also observed by Tsibranska et al. (2012) where the mass transfer was completed quickly even with no stirring and it was attributed to the bioactive components located on the surface of the matrix.

For ultrasound extraction, the phenolic content extracted after 30 min was within 85%–115% of the equilibrium concentration in

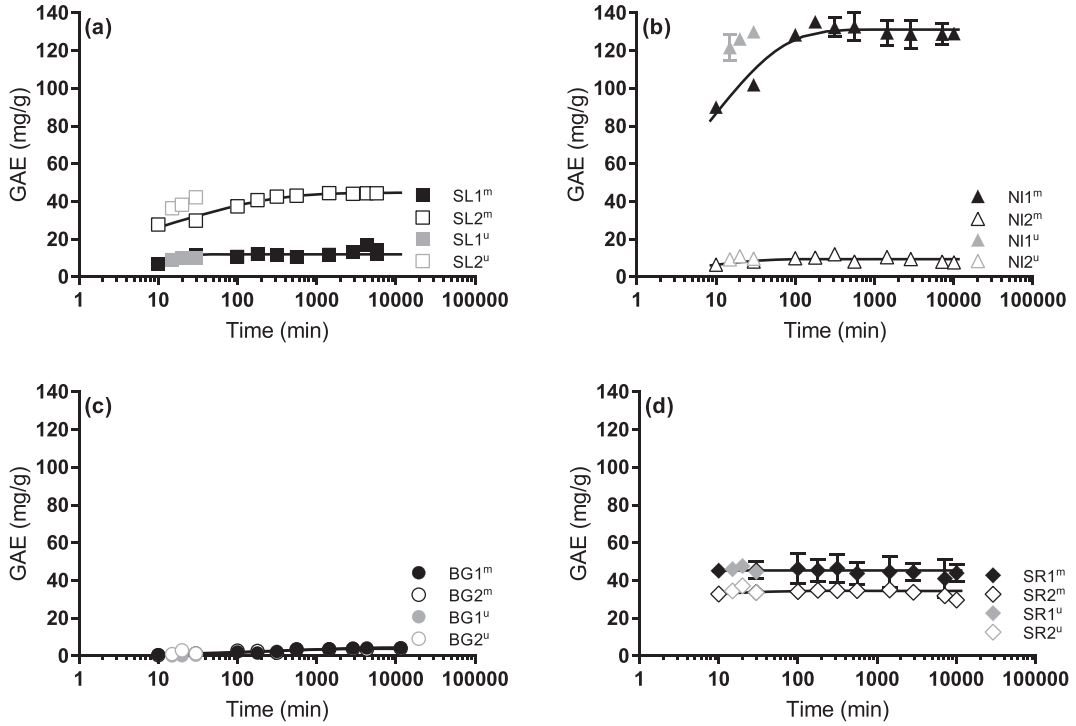

FIGURE 2 Extraction kinetics for total phenolic content during maceration (black symbols) and ultrasound-assisted extraction (gray symbols) for (a) Salgar, (b) Necoclí, (c) Bagre, and (d) San Rafael. The solid lines correspond to Weibull's kinetic model fit for the maceration extractions

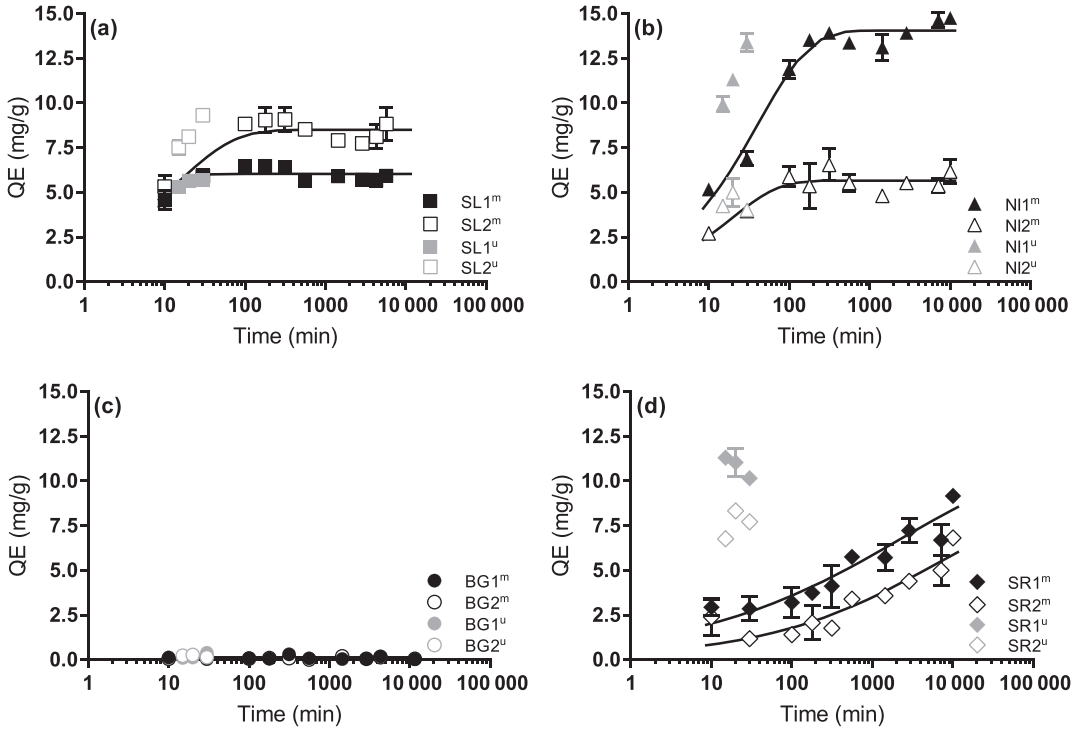

FIGURE 3 Extraction kinetics for total flavonoid content during maceration (black symbols) and ultrasound-assisted extraction (gray symbols) for (a) Salgar, (b) Necoclí, (c) Bagre, and (d) San Rafael. The solid lines correspond to Weibull's kinetic model fit for each sample.

maceration for all the samples studied. Ultrasound increased the extraction kinetics due to acoustic cavitation, which generates mechanical agitation between particles increasing their contact area.

The extraction kinetics of flavonoids for maceration and ultrasound are presented in Figure 3. Although the content extracted in maceration reached its maximum growth for most of the samples

between 300 and 600 min as in phenolic extraction, samples SR1 and SR2 showed a slow and steady increase in the concentration during the extraction (7 days). This suggests that, predominantly, flavonoids are located in internal pores, and thus, have slower diffusion kinetics (Rosa et al., 2019; Torun et al., 2015).

In ultrasound extraction, the highest concentration of flavonoids was between 90% and 135% of the highest extractable content in maceration, that is, 92% for NI, 103% for SL, and 135% for SR. Thus, for NI1 and NI2, the maximum extraction yield for polyphenols was obtained by maceration; however, for SL1, SL2, SR1, and SR2 ultrasound was a more effective extraction method.

The biological activity of propolis is related to the antioxidant activity of its phenolic compounds (Silva et al., 2019). Flavonoids and some phenolic compounds act as potent inhibitors of oxidative stress, protecting cells against the damage caused by oxidation (Zaccaria et al., 2017). Thus, the interaction of the propolis extracts with a free radical can indicate their antioxidant activity. Herein, the antioxidant activity was measured with the inhibition of the DPPH radical, which consists of the donation of electrons or hydrogen radicals by the biological compounds present in the extract (da Silva Frozza et al., 2013). This was carried out by the interaction of a 10 ppm methanolic solution of each propolis sample and 200 ppm of the DPPH radical (details are presented in Section S5). Table 3 presents the antioxidant activity determined using the DDPH method for all the propolis with non-negligible extraction of polyphenols.

TABLE 3 Antioxidant activities of the propolis samples for the extracts after 24 h of maceration with a ratio of 1:100 of 96% ethanol.

Sample	EC_{50} (mg/L)
SL1	35.2_{6}
SL2	12.2_{14}
NI1	14.4_{3}
NI2	105.3_{13}
SR1	12.3_{1}
SR2	17.0_{21}

Note: Numbers in subscript indicate the uncertainty of the last digit(s).
Abbreviations: BG, Bagre; NI, Necoclí; SL, Salgar; SR, San Rafael.

The best antioxidant activities were observed for SL2, NI1, and SR1, which also correspond to the samples with the highest extraction of polyphenols. Even though SR1 corresponds to the propolis with the highest soluble matter (Table 2) it had a much lower percentage of extracted phenolics and flavonoids than NI1, this indicates that the polar compounds extracted as soluble matter corresponded to other substances in addition to the polyphenolic compounds studied, as it is also reflected in the inhibition of DPPH.

The extraction of phenols and flavonoids increased over time as part of the extraction kinetics, however the DPPH inhibition of these extracts remained almost constant with the extraction time (see Section S5). This indicates that the composition of the specific molecules responsible for the inhibition of DPPH remained almost constant during the extraction.

Note that the samples from the same region with large differences in the extraction of phenols and flavonoids (SL1 and SL2 and NI1 and NI2) also presented large differences in the antioxidant activities. Whereas similar results in the antioxidant activities were observed for SR1 and SR2 in agreement with their extraction results.

3.3 | Kinetic models

Seven kinetic models were evaluated with the experimental data of maceration for both phenols and flavonoids extraction of each propolis sample. The Akaike information criterion (AIC) was used to classify the best models for the studied dataset (Akaike, 1974). Further details of the error minimization using AIC and the maximum absolute error are presented in detail in Section S2. Even though the first-order plus intercept model, the second-order model, and Weibull's model were the best fit models, Weibull's equation was chosen to represent the behavior of the experimental extraction data because in this model the initial points of the extraction were fitted to the origin. The kinetic parameters of Weibull's model (Equation 6) for phenols and flavonoids for each propolis sample are presented in Table 4. Detailed information on the other kinetic models evaluated, and their parameters can be found in Section S2.

TABLE 4 Weibull's kinetic parameters of phenols and flavonoids for maceration extraction of the propolis samples.

Kinetic extraction of phenols				Kinetic extraction of flavonoids			
Sample	Ce_w (mg GAE/g)	m	k_w (10^{-4}/s)	Sample	Ce_w (mg QE/g)	m	k_w (10^{-4}/s)
SL1	12.66	1.37	14.62	SL1	6.04	1.08	22.89
SL2	45.17	0.32	11.64	SL2	8.50	0.58	12.63
NI1	131.26	0.47	19.55	NI1	14.05	0.67	4.21
NI2	9.52	0.61	20.99	NI2	5.67	0.75	8.45
BG1	4.65	0.47	0.33	BG1	0.10	1.43	123.63
BG2	3.76	0.48	1.05	BG2	0.09	1.00	7.34
SR1	44.69	0.83	539.91	SR1	11.05	0.29	0.07
SR2	33.64	1.27	46.52	SR2	8.59	0.35	0.03

Abbreviations: BG, Bagre, GAE, gallic acid equivalent, NI, Necoclí, SL, Salgar, SR, San Rafael, QE: quercetin equivalent.

Weibull's model presents three phases (depending on the combination of exponent m and k_w value) which are: fast growth with a large slope, a slow flattening of the curve, and a stable trend once the equilibrium (Ce_w) is reached. The parameter m represents the shape of the extraction kinetics, namely an exponential ($m = 1$), sigmoid ($m > 1$), or parabolic curve ($m < 1$), whereas the parameter k_w defines the scaling time of the process. Although the growth of low values of m is greater in the early stages, as it approaches the equilibrium it becomes slower, while for sigmoid growth it reaches it in a faster period; this might be related to the availability and accessibility of the chemical content in the matrix. For NI1, phenols and flavonoids reached equilibrium at 500 min, phenols presented a fast growth ($m = 0.47$ and $k_w = 1.46{\cdot}10^{-3}$), whereas flavonoids took longer to reach the equilibrium concentration ($m = 0.67$ and $k_w = 0.40{\cdot}10^{-3}$). In contrast for NI2, phenols and flavonoids reached faster their equilibrium in 250 min due to m (0.61 and 0.75) and k_w (2.10 and 0.84×10^{-3}) being larger, however in that case their equilibrium concentration was much lower.

SR2 had the smallest m and k_w values (0.29 and 3×10^{-6}, respectively) for flavonoid extraction, which indicates a very slow extraction rate, reflected in only 80% of the amount extracted by ultrasound obtained after 7 days of maceration.

3.4 | Supercritical fluids extraction

Extraction by supercritical fluids has been studied as an alternative to conventional extractions for biological matrices because of its flexibility and the possibility of adjusting the supercritical fluid polarity (Machado et al., 2015). For propolis extraction, the phenolic compounds present high polarity and are insoluble in supercritical CO_2 with extraction yields of 3%–7% (Fianco et al., 2018), but are sufficiently soluble in CO_2-ethanol mixture reaching yields of 51% (Monroy et al., 2018). The use of ethanol as a co-solvent can triple the extraction yield, compared only with supercritical CO_2 extraction (Biscaia & Ferreira, 2009).

The conditions used for supercritical CO_2 extraction were 300 bar and 50°C, the fluid's density and viscosity at those conditions are 0.87 g/ml and 84.75 μPa/s, respectively. For this study, a 96% ethanol solution was used as a co-solvent. Three different cosolvent conditions were evaluated (i) a flow of 5% of ethanol during the extraction (SC-co5%), (ii) a flow of 10% of ethanol (SC-co10%), and (iii) the addition of a mix of 1:100 of propolis (g):ethanol (ml) to the extraction cell before the addition of CO_2 (SC-mix).

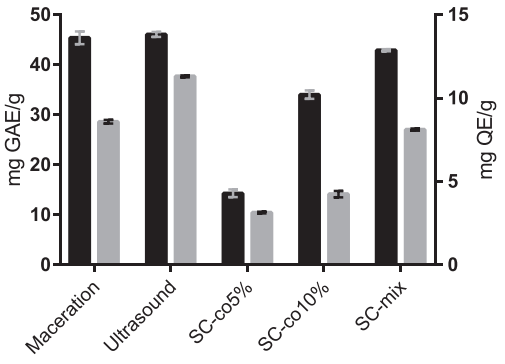

FIGURE 4 Comparison of the phenolic (black bars) and flavonoid (gray bars) contents of SR1 for different extraction methods. GAE, gallic acid equivalent, QE, quercetin equivalent.

Due to its high antioxidant activity SR1 was used as the model extract to evaluate the supercritical fluids extraction. A comparison of the total phenols and flavonoids obtained using the maceration, ultrasound, and supercritical fluids extraction methods is shown in Figure 4. Note that when ethanol was added as a co-solvent to the extraction vessel during supercritical extraction (SC-co5% and SC-co10%), the yield compared with the ultrasound extraction was <75% and 40%, respectively for phenols and flavonoids. However, when ethanol was mixed initially in the extraction vessel (SC-mix) those yields increased up to 93% and 72%.

The solvent consumption in SC-co5% and SC-co10% were equivalent to solvent:solute ratios of 9:1 and 26:1 for ethanol, respectively. These values are much lower than the 100:1 ratios used in ultrasound, maceration, and *SC-mix*. Indicating the strong affinity of the extracted phenols and flavonoids to this ethanolic mixture. This is in agreement with the solubility predictions given by the Hansen solubility parameters, presented in Section S1, which showed a clear increment in the solubility for the bioactive compounds studied as the ethanol concentration in the solvent increased. Solubility parameters are numerical estimates that indicate the interactions between a solvent and a solute and can be used to predict the solubility of a mixture in a solvent. This strong affinity with the ethanolic mixture is also reflected in the extraction rates, where the extraction kinetics for all the supercritical fluids extractions are much slower than ultrasound extractions (see details of the kinetics models for phenols and flavonoids for samples SR1, SL2, and NI1 in Section S3).

Although the yield of supercritical fluids extraction is similar to that of other extraction techniques, due to their slow kinetics, supercritical fluids extraction should be used to complement conventional extraction methods in propolis extraction. Previous studies have suggested that the best application of supercritical fluids in the extraction of propolis is to obtain lipophilic fractions enriched in specific constituents or as pretreatment of the crude matrix to facilitate further conventional extraction (De Zordi et al., 2014).

4 | CONCLUSION

In this study, the physicochemical properties and polyphenolic contents of propolis from four regions of Colombia were evaluated. The differences in color, consistency, UV-vis spectrum, and polyphenolic content show the high variability of propolis even in the same region. The physicochemical characteristics show important information about the handling during harvest, transport, and storage of the propolis and can also be used as predictors of the polyphenolic contents.

Maceration, ultrasound, and supercritical fluids extractions were investigated and evaluated in terms of the kinetic polyphenolic content obtained. In general, extraction by maceration takes the longest

to stabilize. However, this method can yield similar concentrations of polyphenols to ultrasound extraction. On the other hand, supercritical fluid extraction has, in general, slow kinetics and it is unable to efficiently extract the phenolic contents present in the propolis matrix unless large concentrations of ethanol are added to the extract. Ultrasound-assisted extractions were able to extract the highest content of polyphenols with the fastest kinetics, with <30 min required for equilibration. The Weibull model satisfactorily described the extraction kinetics for the total phenols and flavonoids for the studied samples. Moreover, it was found that the antioxidant activity remained almost constant with the extraction method and extraction time indicating similar extraction kinetics for the extracted polyphenols and the specific compounds responsible for the DPPH inhibition.

The extractions by maceration showed fast extraction kinetics under constant agitation indicating that extractions beyond 10 h were not necessary. High antioxidant activity was observed for the samples with the highest yields for each region, on average 13 mg/L for EC50, and this remained almost constant during the extraction kinetics. The composition of the studied propolis extracts ranged between 3 and 130 mg GAE/g for phenols and between 0 and 13 mg QE/g for flavonoids with a strong correlation to the quality criteria.

AUTHOR CONTRIBUTIONS

Juan C. Barrientos-Lezcano: Data curation; formal analysis; investigation; writing – original draft. **Jacobo Gallo-Machado:** Formal analysis; investigation. **Luz D. Marin-Palacio:** Formal analysis; funding acquisition; methodology; project administration; supervision; writing – review and editing. **Santiago Builes:** Conceptualization; formal analysis; funding acquisition; supervision; writing – review and editing.

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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest concerned with this publication.

DATA AVAILABILITY STATEMENT

All data included in this study are available upon reasonable request by contact with the corresponding author.

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