Received: 30 May 2022 | Revised: 22 November 2022 | Accepted: 23 November 2022

DOI: 10.1002/jssc.202200440

RESEARCH ARTICLE

JOURNAL OF SEPARATION SCIENCE

Ultra-high-performance liquid chromatography using a fused-core particle column for fast analysis of propolis phenolic compounds

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Propolis is a bee product with a complex chemical composition formed by several species from different geographical origins. The complex propolis composition requires an accurate and reproducible characterization of samples to standardize the quality of the material sold to consumers. This work developed an ultra-high-performance liquid chromatography with a photodiode array detector method to analyze propolis phenolic compounds based on the two key propolis biomarkers, Artepillin C and *p*-Coumaric acid. This choice was made due to the complexity of the sample with the presence of several compounds. The optimized method was hyphenated with mass spectrometry detection allowing the detection of 23 different compounds. A step-by-step strategy was used to optimize temperature, flow rate, mobile phase composition, and re-equilibration time. Reverse-phase separation was achieved with a C18 fused-core column packed with the commercially available smallest particles (1.3 nm). Using a fused-core column with ultra-high-performance liquid chromatography allows highly efficient, sensitive, accurate, and reproducible determination of compounds extracted from propolis with an outstanding sample throughput and resolution. Optimized conditions permitted the separation of the compounds in 5.50 min with a total analysis time (sample-to-sample) of 6.50 min.

KEYWORDS
Artepillin C, fast analysis, fused-core, *p*- Coumaric acid, propolis

1 | INTRODUCTION

Natural products are being increasingly studied to treat and prevent many health conditions, attracting biomedical and technological researchers. The use of propolis is widespread, and the interest in scientific research is increasing due to the potential source of various bioactive substances [1, 2]. Propolis comprises hydrophilic and hydrophobic molecules and is usually extracted with ethanol, the primary extraction solvent used by researchers and industry [3, 4]. The chemical composition of propolis depends on the geographic location, climate, edaphic conditions of the plants used by the bees (soil type, chemical composition, and microbiota) and by the bee species [5]. Therefore, many types of propolis, such as green and brown propolis, are produced.

Article Related abbreviations: PDA, photodiode array detection.

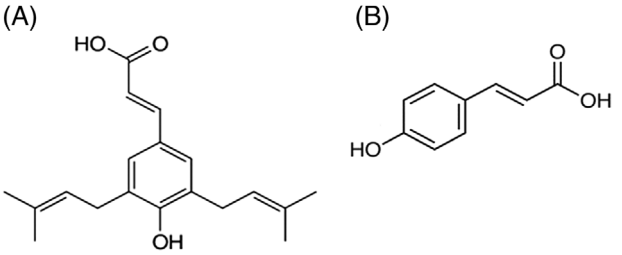

FIGURE 1 Chemical structure of biomarkers of Brazilian propolis Artepillin C (A), and *p*-Coumaric acid (B).

More than 500 compounds have been identified in different propolis samples, including terpenes, phenolic compounds (mainly flavonoids and acids), esters, sugars, hydrocarbons, and mineral elements [6, 7]. Since ancient times, phenolic compounds from propolis hydroethanolic extracts have been recognized to promote countless biological properties; namely, antioxidant, antimicrobial, antiviral, antitumor, antiparasitic, hepatoprotective, and immunomodulatory activities [8–10].

Some key markers have been identified in propolis samples, such as Artepillin C, and *p*-Coumaric acid (Figure 1), both with several biological properties [11, 12]. In addition, these compounds illustrate the variety of compound properties in propolis extracts. For example, *p*-Coumaric acid is predominantly polar and usually extracted with water or ethanol, and Artepillin C is a relatively apolar compound that cannot be extracted with water [13, 14].

This complexity implies that analyzing different types of propolis with the same method is challenging. Furthermore, different strategies for extracting natural compounds have been developed, impacting the sample composition [15]. Additionally, the complex composition of propolis requires an accurate and reproducible characterization of samples to standardize the quality of the material being sold to consumers [3, 16]. Finally, since not all propolis samples have the same chemical composition, they may not have similar biological potential.

Due to the complexity and variable composition, several methods have been developed to analyze the phenolic compounds from propolis, especially HPLC–MS and HPLC coupled with photodiode array detection (PDA) [17–22]. However, other techniques, such as GC–MS can be used [22, 23]. More recently, UPLC has emerged as an alternative to HPLC to decrease analysis time and solvent consumption [24–26] which is an essential feature in the context of precise and fast characterization (quantification and identification) of these complex samples [26].

On the other hand, one of the most recent advances in LC to optimize the separation process and reduce the impact of particle size on column backpressure was the development of fused-core particles. Due to their characteristics, fused-core particles generate a lower pressure, allowing smaller particles to improve the chromatographic resolution and speed up the analysis. Methods employing columns packed with fused-core particles can usually reduce analysis time by 3–4 times compared to conventional HPLC methods [27–30]. This particle technology addresses the slow mass transfer of solute molecules inside conventional particles by providing a small diffusion path into and out of the stationary phase, reducing the time molecules spend inside the particles. Their physical characteristics also allow increasing mobile phase flow rate to achieve faster separation by generating lower column backpressure [20].

Conversely, published UPLC methods for analyzing minor compounds in propolis require between 15 and 60 min for the separation, which is a lengthy analysis time for such an efficient technique [31]. Unfortunately, fused-core particle technology has not yet been explored to analyze propolis samples, which presents enormous potential to improve performance and speed. Thus, given propolis's biological potential and chemical complexity, it is challenging to create reliable and robust analytical methods allowing the precise quantification of the compounds in different extracts. Thus, this work aimed to explore the potential of combining the performance of UPLC-PDA with the advantages of fused core particles to develop a fast and reproducible method for analyzing extracts from different varieties of propolis, here testing with green and brown varieties.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Acetic acid, acetonitrile (ACN), and methanol UPLC grade were purchased from Sigma Aldrich. Ultra-pure water was supplied by a Milli-Q Advantage 8 water purifier system (Purelab Elga, UK). The reference standard of *p*-Coumaric acid (*trans*-4-hydroxycinnamic acid, ≥98.0%) and Artepillin C ((2*E*)-3-[4-Hydroxy-3,5-bis(3-methyl-2-butene-1-yl) phenyl]-2-propenoic acid, ≥90.0%), was purchased from Sigma-Aldrich Brazil Ltda (São Paulo, Brazil).

2.2 | Sample

Two green raw propolis were used, donated by two companies, Mn Propolis (Mogi das Cruzes, SP, Brazil) and Breyer (União da Vitória, PR, Brazil), to optimize the chromatographic method. The frozen samples were milled in a domestic blender (Model OSTER, 450W 220V, Sao Paulo,

Brazil) for a few seconds. Next, the milled propolis samples were sifted in a steel sieve (Model Bestifer, Limeira, Brazil), standardizing the particle size sample between 0.5 and 1 mm. Finally, the samples were stored in a dark container at -20°C until use. The company Vale Verde (Santos, SP, Brazil) donated commercial extracts of propolis.

2.3 | Sample preparation

To develop the chromatographic method, the raw samples were submitted to ultrasound-assisted extraction (ultrasonic bath P60h, Elmasonic, Schmidbauer GmbH, Germany) at 37 kHz, 135 W, solid-liquid-ratio (SLR) 0.05 g/mL, and 45°C for 2 hours (h) [32]. Pure water, ethanol, and hydroethanolic solutions (70; 50, 55, and 25% v/v) were used for extraction (according to the experiment). After the extraction, an aliquot of 5 mL was centrifuged at 14 000 rpm, 20°C for 15 minutes (min); the supernatant was collected and diluted in different solvents for analysis. The extracts were diluted using various dilution factors (DF) and solvents for the analysis described throughout the sessions. The diluted extract was filtered through a nylon syringe filter 0.22 μm x 25 mm (Analytical, São Paulo, Brazil) before the UPLC-PDA analysis.

2.4 | UPLC-PDA method development

The analysis was carried out in a UPLC-PDA system (Waters Corp, Acquity H-Class, Milford, Massachusetts, EUA). Compounds were separated in a fused-core type column (Kinetex C-18, 50×2.1 mm, particle size 1.3 μm: Phenomenex, Torrance, CA, USA). Different mobile phases (water, ACN, and methanol), acidification with acetic acid (0.1% and 1% v/v), temperatures (30–55°C), flow rates (0.5 and 1.0 mL/min), and equilibration times (1–10 min) were tested. Chromatograms were recorded in the 200–400 nm range, peaks were integrated at 260 nm, and the injection volume was 1 μL.

2.5 | Identification of compounds by UPLC-PDA-MS/MS

The optimized method was hyphenated with MS detection (UPLC-PDA-MS/MS) to identify the other peaks on the sample. Initially, to provide the fragmentation profile of the compounds extracted from green propolis, flow injection analysis was performed using a Thermo Fisher Scientific LTQ XL linear ion trap mass spectrometer (San Jose, Ca, USA) equipped with an electrospray ionization source. Afterward, MS and MS/MS analysis were performed in negative ionization, with the following operational conditions: standard solution of (+)-catechin (1 μg/mL in methanol), flow rate 0.5 mL/min capillary voltage -35 V, spray voltage 5 kV, tube lens offset 75 V, capillary temperature 250–300°C, sheath gas (N_2) flow rate 8 (arbitrary units). Negative ion mass spectra were recorded in *m/z* 100–2000 Da. Data were acquired and processed using Xcalibur software (version 2.2 SPI.48).

2.6 | Method validation

Method validation was performed according to the guidelines provided by the FDA (Food and drug administration, USA) and INMETRO (National Institute of Metrology, Quality, and Technology, Brazil) agencies. Sensitivity, limits of detection and quantification, linearity, accuracy, reproducibility, and robustness were evaluated. These parameters focused on the biomarkers *p*-Coumaric acid and Artepillin C, the molecule for which the method was validated. According to the USA, system suitability was used to monitor the main chromatographic parameters (Empower 3.0 software, Waters), namely resolution, retention factor (k), and symmetry. Pharmacopoeia must be higher than 1.5, $k > 2.0$, and between 1 and 1.20, respectively [33].

The selectivity of the UPLC method developed for propolis was evaluated by comparing the peaks, retention time, and absorption spectrum in the UV-Vis, of the pure standards of (*p*-Coumaric acid – 100 ppm and Artepillin C –1000 ppm) with a mixture of four different green propolis extracts (FD = 4, with EtOH 55% v/v), made with solvents, pure water, pure ethanol, and hydroethanolic solutions (70%, 50%, and 25% v/v).

The calibration curves of *p*-Coumaric acid were built by injecting the following different concentration levels of the standard: 100, 50, 25, 12.50, 6.25, 3.13, 1.56, 0.78 mg/L; and Artepillin C was built by injecting the following different concentration levels of the standard: 1000; 500; 250; 125; 100; 50; 25; 12.5; 6.25 and 3.13 mg/L) (Figure S1 and Table S1). The standard concentrations versus the area of the peaks were used to plot the calibration curve, which was statistically evaluated by the linear correlation coefficient (*r*), analysis of variance, and Cochran test to verify the homoscedasticity of the residuals. These data were calculated using Microsoft Excel 2010 software using the mean of the peak area of three different injections.

LODs and LOQs were calculated following the International Union of Pure and Applied Chemistry method [34]. LODs and LOQs were calculated for *p*-Coumaric acid and Artepillin C, determined by analyzing sample blanks, and estimated for the S/N of 3 and 10, respectively, following the following equations: LOD = 3×(Sb/m) and LOQ =

10×(Sb/m), where *Sb* corresponds to the standard deviation of the noises measured for each sample and *m* is the slope of the calibration curve.

Accuracy was evaluated with the standards of *p*-Coumaric acid and Artepillin C. They were added to the same mix used for selectivity analysis (a mixture of four different green propolis extracts,FD = 4, with EtOH 55% v/v), made with solvents, pure water, pure ethanol, and hydroethanolic solutions (70; 50, and 25% v/v), to obtain solutions whose additions follow the high, medium, and low analytical curve points of this biomarkers. *p*-Coumaric acid, high: 33.3 ppm; medium: 16.6 ppm and low: 1 ppm and Artepillin C, high: 332 ppm; medium: 166 ppm and low: 10 ppm. The recovery (%) of each sample was determined following the equation: Recovery (%) = [(CF—OC)/AC]×100. CF, OC, and AC are the concentration found by the calibration curve, original concentration, and concentration of the added solution, respectively.

The repeatability (intra-day) and intermediate precision (inter-day) of the analytical method were assessed by the RSD of the *p*-Coumaric acid and Artepillin C of three repeated injections of the same high, medium, and low analytical curve points previously described. RSD lower than 3% was expected as the threshold to validate this parameter. For comparison, a test of variance (analysis of variance) was applied, followed by Student's T analysis to compare the test between the days and the levels of the same day significant difference of selected solvents was determined at 95% ($p < 0.05$, $n = 3$), using the JAMOVI software.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of method conditions

The objective of the method was to achieve, in a short time, a high-resolution separation for propolis samples using the UPLC-PDA instrument. The method conditions were optimized by evaluating the chromatographic separation efficiency using different solvents in the mobile phase, column temperatures, and flow rates. The criterion used was based on the separation of the main compounds and the number of peaks detected. A comparative analysis of each chromatogram obtained with variations in conditions was performed. Although the trial-and-error strategy is not sophisticated, the experience and short analysis time made it possible to optimize the conditions relatively quickly. The use of DOE (design of experiments) is a handy tool for this application. Still, in this case, it was possible to develop the method relatively quickly without the need for this type of strategy.

The UPLC-PDA method development was based on the two key propolis biomarkers, Artepillin C and *p*-Coumaric acid. This choice was made due to the complexity of the sample with the presence of several compounds. Moreover, these compounds have different polarities while *p*-Coumaric acid is predominantly polar, with lower retention in C18 columns, and Artepillin C is much less polar, with higher retention time. Therefore, an optimization of analysis of these two compounds guarantees a clean and comprehensive chromatographic profile

For mobile phase selection, the flow rate was set at 0.5 mL/min, and the column temperature was set at 30°C, half the maximum column operating temperature of the UPLC-PDA instrument, 15 000 psi at 1.0 mL/min and 60°C, respectively. The mobile phase selection was based on a previous series of experiments using water (solvent A) and methanol or ACN (solvent B), both acidified with different concentrations of acetic acid (0%–1% v/v). A series of runs using a linear gradient of these other solvents from 0% to 100% B were used. The maximum analysis time was initially fixed at 26 min. In our analysis, we noticed that lower back pressure was generated using ACN with 0.1% acetic acid, so this solvent was selected for mobile phase B.

To optimize the column temperature is essential not to exceed the maximum column operating temperature (60°C) as it can significantly reduce the expected column life [24]. Higher temperatures reduce the analysis time since the diffusion coefficient is directly proportional to the absolute temperature and inversely proportional to the mobile phase viscosity. Thus, the lower viscosity and higher diffusivity of a mobile phase at high temperature promote a much lower mass transfer resistance, thus decreasing peak width and leading to flatter Deemter curves. This explains that fused-core columns allow for increased flow (i.e., linear mobile phase velocity) without the loss of efficiency characteristic of traditional fully porous particles.

Based on this, the column temperature was gradually increased from 30 to 55°C (setting the flow rate at 0.5 mL/min). It was observed that increasing the column temperature from 30 to 55°C produced a narrowing of the peak's width (-47%), an increase in the peak height (+14%), and better resolution in the separation of the peaks, especially *p*-Coumaric acid and Artepillin C (Figure S2). Also, increasing the temperature to 55°C decreased the peak retention time (Figure S2), because the use of higher temperatures reduces the analysis time, which is helpful to lower the viscosity and high the diffusivity of the mobile phase [35]. Thus, 55°C was defined as the optimum working temperature.

This analysis observed that the lowest flow rate (0.5 mL/min) allowed good separation while minimizing

TABLE 1 Conditions used in the UHPLC-PDA system for the green propolis extract analysis

Parameter	Time (min)	Concentration of B (% v/v)
Gradient	0	12
	0.5	15
	1.0	20
	1.50	25
	2.0	29
	2.50	32
	3.00	50
	3.50	65
	4.50	70
	5.00	70
Return to initial conditions	5.50	12
Equilibration period	6.50	12
Phase A	Ultra-pure water with 0.1% (v/v) of acetic acid	
Phase B	ACN with 0.1% (v/v) of acetic acid	
A+B flow rate	0.5 mL/min	
Injection volume	1 μL	
Column temperature	55°C	

solvent consumption. Therefore, the flow was set at 0.5 mL/min). Furthermore, keeping a shortened analysis time and an optimal separation of the chromatographic peaks was possible with the temperature increases. Thus, the shorter analysis time was achieved using the highest temperature (55°C) and the lowest tested flow rate (0.5 mL/min)

After this optimization, we focus on the time required to ensure that the column environment returns to an initial stable condition (re-equilibration time) because reducing column re-equilibration time helps reduce column usage time, increasing column life [24]. Equilibrium times between 1 and 10 min were tested, and no significant differences were observed (Figure S3 – supporting information). Therefore, an equilibration time of 1 min between the injections was selected.

The optimized conditions are shown in Table 1, and a mix of the extracts obtained with different solvents (pure water, pure ethanol, and hydroethanolic solutions with 70; 50; and 25% v/v), were analyzed with the optimized conditions (representative chromatogram is shown in Figure 2). This extract (mix) was diluted in ethanol at 55% (v/v). Table 2 shows the system suitability parameters obtained from the optimal chromatogram, being a separation close to the baseline is a relevant parameter to guarantee precise quantification. The resolution was higher than 1.5, which is considered a baseline separation. The peak symmetry factor was excellent for *p*-Coumaric (1.11) and acceptable

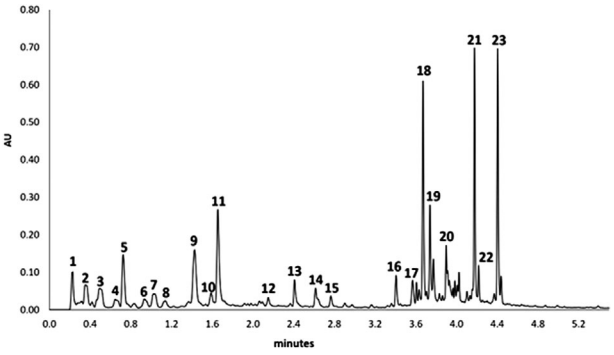

FIGURE 2 Chromatogram recovered at 260 nm using the optimized method conditions for the mix of green propolis extract obtained with pure water, pure ethanol, and hydroethanolic solutions (70%, 50%, and 25% v/v), by ultrasound bath. The mix was diluted in ethanol 55% (v/v), FD = 4. Separation was performed at 55°C, 0.5 mL/min, and 1 min for column re-equilibration.

for Artepillin C (1.25), indicating a slight tail caused by a partial overlap of Artepillin C with the subsequent peak. Finally, the retention factor (k) shows how much the column retains the compound. Ideally, it should be lower than 20; in the developed method, both compounds are in the ideal range, 7.21 for *p*-Coumaric acid and 6.07 for Artepillin C.

The influence of extracts' injection volume and dilution on the chromatographic parameters (retention time, retention factor (k), resolution, and symmetry factor) was also evaluated, as shown in Tables S2 and S3 and Figures S4 and S5. We observed that the injection volume affected the first part of the chromatogram, where the most polar compounds are presented. However, there was no significant difference in the optimized results in the second part of the chromatogram, represented by Artepillin C and other flavonoids and cinnamic acids. This trend can be expected as injecting a large volume will create a broad sample band with an extra column effect that does not affect the later eluted compounds which are properly refocused on C18 due to their lipophilicity while polar compounds do not get properly refocused and appear as broadened substance zones.

Therefore, optimal results are obtained with an injection volume set at 1 μL. However, this does not mean larger injection volumes cannot be used when low-concentration extracts are analyzed. Still, the compounds' quantification at the chromatogram's beginning may be impaired.

3.2 | UPLC-photodiode array detection-MS/MS

With the UPLC- PDA optimized method, it was possible to see the separation of 23 different peaks. To identify each

TABLE 2 System suitability parameters obtained from the optimal chromatogram (Figure 2) of the mixed green propolis extract obtained with pure water and hydroethanolic solutions (100; 70; 50; and 25% v/v), by ultrasound bath. The mix was diluted in ethanol 55% (v/v), FD = 4. The repeatability of the retention time was expressed in %RSD (variation coefficient), calculated in relation to the mean of the triplicate performed on two different days ($n = 6$)

Compound	RT Day 1	RT Day 2	Repeatability (% RSD)	Retention factor (k)	Resolution	Symmetry factor
p-Coumaric	0.731 (±0.015)	0.714 (±0.003)	0.80	7.21	3.55	1.11
Artepillin C	4.163 (±0.004)	4.59 (±0.005)	0.10	6.07	8.38	1.25

RT: retention time (min).

peak, the technique was hyphenated with MS detection (UPLC-PDA-MS/MS). The identified compounds, retention times, UV-vis spectra (λ_{max}), *m/z* $[M\text{-}H]^-$, and MS-MS are presented in Table 3. The mass spectra of the identified peaks are presented in Figures S6–S24. It is possible to observe different phenolic classes like hydroxycinnamic acids, flavonols, flavones, and prenylated derivatives of *p*-Coumaric acid-like Artepillin C and Baccharin.

Thus, the present method can quickly and efficiently separate compounds in a high-complexity propolis sample. Given the importance and complexity of propolis, the method's performance indicates that it is possible to overcome the challenge of creating a reliable and robust analytical approach that allows accurate quantification of the most relevant compounds. It is worth mentioning that to complement the optimization of the analysis method, the influence of the extraction solvent on the chromatographic performance was analyzed, see section "Influence of the extraction solvent on the chromatographic performance", Table S6 and Figures S27–S29 present in the supporting information.

3.3 | Validation of the optimized method

The method's repeatability was evaluated by comparing the peaks, retention time repeatability, and absorption spectrum in the UV-Vis of a mixture of four different green propolis extracts (FD = 4 with EtOH 55% v/v), made with solvents (EtOH 100; EtOH 70% and EtOH 50%). We compared with the authentic standards of (*p*-Coumaric acid – 100 ppm and Artepillin C 1000 ppm). According to the results of retention time repeatability, the method is adequate for quantitative analysis of these compounds in the studied matrices since the variation between the sample peaks in the chromatogram did not exceed 0.1 min (Figure S25). Moreover, the peak purity was checked by DAD, as shown in Figure S26. We verified the absorption spectrum in the UV-Vis at different points of the interest compound peaks (*p*-Coumaric acid and Artepillin C), and no changes were detected in the spectrum profile that could indicate contamination by other compounds.

Linearity corresponds to the ability of the method to provide results directly proportional to the concentration of the analyte in a range of applications. An excellent correlation coefficient was obtained for the reference compounds: the r^2 were 0.9993 and 0.9982 for *p*-Coumaric acid and Artepillin C, respectively. LOD and LOQ were respectively 0.09 ppm and 0.19 ppm for *p*-Coumaric acid and 2.621 and 5.73 ppm for Artepillin C.

The developed method's accuracy was validated by the standards of *p*-Coumaric acid and Artepillin C, which was 83%–94% for *p*-Coumaric acid and 81.9%–94.93% for Artepillin C (Table S4) within the acceptable range for the concentrations studied (80%–110%).

The repeatability (intra-day) and intermediate precision (inter-day) of the analytical method were assessed by the RSD of the *p*-Coumaric acid and Artepillin C of three repeated injections of the same high, medium, and low analytical curve points previously described. The RSD for intra-day and inter-day, Table S5 was lower than 3%, which was expected as the threshold to validate this parameter, which agrees with the values suggested by specialized guidelines.

3.4 | Applications of the optimized method to commercial propolis samples

To assess its applicability, the method developed was used to analyze two samples of commercial propolis extract, one green and one brown, and a crude green propolis extract obtained using 50% ethanol (v/v) as solvent (Figure 3). Brown propolis has similar bioactive profile composition compared to green propolis, but the compounds are quantitatively different. Brown propolis shows a higher content of flavonols. dyhydroflavonols flavones compared to green propolis. However, green propolis concentrations of Artepillin C and *p*-Coumaric acid are higher [36, 37]. So, with few variations, the same profile is portrayed, differing only in concentration in both propolis.

TABLE 3 UHPLC–PDA–MS/MS fragmentations performed during the chromatographic analysis of the compounds extracted from green propolis in the negative ionization mode

Peak	RT	Compound	UV/vis λ_{max} (nm)	*m/z* $[M—H]^-$	MS/MS	Representative Figure
1	0.20	Ferulic acid	230, 300, 326	193	-	S6
2	0.34	Feruloylquinic acid	246, 320	367	-	S7
3	0.48	Quinic acid	234. 304, 326	191	172, 163, 110	S8
4	0.64	Caffeoylquinic acid (a)	298,325	353	191	S9
5	0.72	*p*-Coumaric acid	224.8, 309	163	-	S10
6	0.92	n.i	238,323	519	-	S11
7	1.02	Caffeoylquinic acid (b)	246, 335	353	MS^2: 191, 173	S12
8	1.12	Dicaffeoylquinic acid (a)	244, 327	515	MS^2: 353 MS^3 [515 → 353]: 191, 173	S13
9	1.42	Dicaffeoylquinic acid (b)	244, 327	515	MS^2: 353 MS^3 [515 → 353]: 191, 173	S13
10	1.58	Dicaffeoylquinic acid (c)	244, 327	515	MS^2: 353 MS^3 [515 → 353]: 191, 173	S13
11	1.66	Dicaffeoylquinic acid (d)	244, 327	515	MS^2: 353 MS^3 [515 → 353]: 191, 173	S13
12	2.14	Tricaffeoylquinic acid (a)	244, 327	677	MS^2: 515 MS^3 [677 → 515]:353 MS^4 [677 → 515 → 353]: 191, 173	S14
13	2.40	Tricaffeoyquinic acid (b)	244,327	677	MS^2: 515 MS^3 [677 → 515]:353 MS^4 [677 → 515 → 353]: 191, 173	S14
14	2.62	3,4-dihydroxy-5-prenyl-cinnamic acid	335	247	MS^2: 257, 229,185, 179, 151	S15
15	2.76	Dihydrokaempferide	265, 365	301	MS^2: 284, 152	S16
16	3.40	Quercetin	255, 355	301	MS^2: 179, 151, 125	S17
17	3.55	Dihydrokaempferide derivative	-	475	MS^2: 301, 284, 255, 173	S18
18	3.65	n.i.	240, 310	459	MS^2: 431, 415, 387 MS^3 [459 → 415]: 387, 346	S19
19	3.74	Chrysin	268, 346, 239	253	MS^2: 181, 209, 253, 165	S20
20	3.90	Kaempferide	265, 365	299	MS^2: 284, 271, 255	S21
21	4.18	Artepillin C	313, 218	299	MS^2: 284, 255 MS^3 [299 → 284]: 256, 228, 164, 151	S22
22	4.20	Kaempferol-methyl-ether	288, 338	299	-	S23
23	4.40	Baccarin	280	561	-	S24

RT: retention time (min).
n.i.: not identified.

Thus, with this test, we observe that the developed chromatographic method works for different commercial and raw propolis samples, which are the most common types (green and brown propolis), as shown in Figure 3. For natural compounds, it is known that currently having fast and efficient detection methods is one of the leading research focuses of the pharmaceutical and agrochemical industries. This aspect is due to the increasingly frequent commercialization of products made with natural compounds, the search for the quality of the marketed product, and a reduction in production costs [38]. In this sense, the UPLC technique becomes a critical analysis tool. Furthermore, it reduces column dimensions and expenses with mobile phases and allows the development of faster analytical methods.

Some works in the literature show propolis is analyzed by UPLC using UV-Vis-MS detection, as shown in Table 4. The methods presented use mobile phases like the cur-

TABLE 4 Representative chromatographic methods reported in the literature

Sample	Column type	Time (min)	Column temperature (°C)	Mobile phase	Main components identified	Reference
Brazilian green propolis	Fused-core (1.3 µm C-18, 2.1×50 mm)	5.50	55	A: Water 0.1% acid acetic (v/v%) / B: ACN 0.1% acid acetic (v/v %)	p-Coumaric acid, ferulic acid, quinic acid, caffeoylquinic acid and derivates, quercetin and derivates, chrysin, Artepillin C and baccharin	Present work
European propolis	BEH (1.7 µm, C-18, 2.1×150 mm)	11	40	A: Water 0.5% acid methanoic (v/v %) / B: ACN 0.5% acid methanoic)	3-methyl-2-butenyl-(3M2B), 3-methyl-3-butenyl-(3M3B), 2-methyl-2-butenyl-(2M2B), benzyl- (CABE), caffeic acid and derivates	[19]
Chinese propolis	BEH (1.7 µm, C-18, 2.1×150 mm)	20	45	A: Water 0.1% formic acid (v/v%) / B: ACN	P-coumaric acid, ferulic acid, ferulic acid, 3,4-dimethylcaffeic acid, pinobanksin, chrysin, pinocembrin, galangin, pinobanksin 3-acetate, and caffeic acid	[20]
South Africa and Brazilian propolis	BEH (1.7 µm, C-18, 2.1×150 mm)	14	35	A: Water 0.1% formic acid (v/v%) / B: ACN	Caffeic acid, p-Coumaric acid, quercetin, apigenin, pinocebrim, chrysin and galangin	[31]
Brazilian red propolis	Reversed-phase (1.8 µm, 2.1×10 mm)	16	45	A: Water 0.1% formic acid (v/v%) / B: Methanol	Guttiferone and xanthochymol	[17]
Truly green propolis	RHD (1.8 µm, C-18, 2.1×150 mm)	50	60	A: Water 0.1% acid acetic (v/v%) / B: Ethanol	n.e	[16]
Korean propolis	BEH (1.7 µm, C-18, 2.1×50 mm)	15	40	A: ACN / B: Water 0.1% phosphoric acid (v/v%)	Chrysin and pinocebrin	[39]
Polish propolis	HSS T3 (1.8 µm, 2.1×150 mm)	40	25	A: Water 0.1 % formic acid (v/v%) / B: ACN 0,1% formic acid (v/v%)	Apigenin, quercetin, chrysin, myricetin, galangin, kaempferol, rutin, naringenin, pinobanksin, pinocembrin, epicatechin, genistein, pinostrobin, caffeic acid, coumaric acid, sinapinic acid, ferulic acid, p-hydroxybenzoic acid, vanillic acid, and syringic acid	[40]
Egyptian propolis	BEH (1.7 µm, C-18, 2.1×50 mm)	30	30	A: Water 0.1% formic acid (v/v%) / B: Methanol 0.1% (v/v%).	Kaempferide, coumaroyl acetyl glycerol, galangin, palmitic acid and pinobanksin	[21]

(Continues)

TABLE 4 (Continued)

Sample	Column type	Time (min)	Column temperature (°C)	Mobile phase	Main components identified	Reference
Brazilian brown propolis	Octadecylsilane column	80	40	A: 0.1% acetic acid in water, B: 0.% ACN	Six main diterpenes	[22]

Abbreviations: BEH, ethylene bridged hybrid; HSS, high strength silica; RRHD, rapid resolution high definition.

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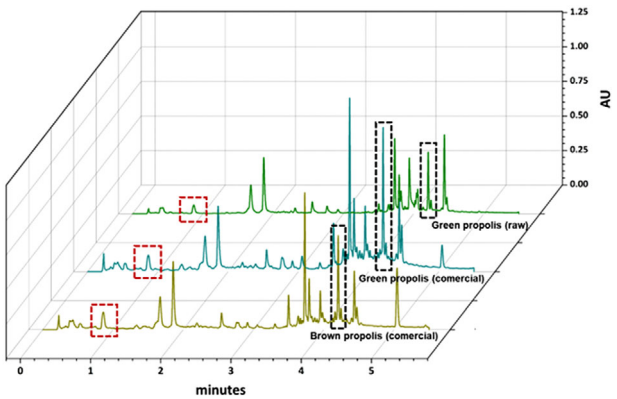

FIGURE 3 Application of the developed method to different samples (commercial and raw green propolis). The target compounds *p*-Coumaric acid and Artepillin C are labeled red and black, respectively.

rent method. Phase A usually composed of acidified water with different acids in a specific percentage and B ACN or methanol (pure or acidified). However, all these methods take longer than described in this article (more than 5.5 min of analysis + 1 re-equilibration time necessary with the reported method). This can be explained by the fact that the temperatures range from 25°C to 45°C, lower than the 55°C used in the current study. Moreover, our method uses a higher column temperature and solvents with low viscosity in combination with low injection volume and low flow.

4 | CONCLUDING REMARKS

Propolis compounds were separated in 5.50 min using a fused core (1.3 μm C-18, 2.1 × 50 mm). It was possible by applying a step-by-step optimization approach of optimizing the chromatographic parameters (mobile phase gradient and flow rate of the column temperature and re-equilibration time) to develop a simple, selective, reliable, and robust method for fast analysis of propolis. The UPLC-PDA method development was based on the two key propolis biomarkers, Artepillin C and *p*-Coumaric. Due to the complexity of the sample with the presence of several compounds. The total analysis time was only 6.50 min, including a re-equilibration period. The current method showed excellent chromatographic resolution, retention factor (k), and symmetry. With the optimization of method conditions, it was possible to separate 23 peaks with different phenolic classes like hydroxycinnamic acids, flavonols, flavones, and prenylated derivatives of *p*-Coumaric acid, like Artepillin C and Baccharin, which are important markers of the Brazilian green propolis. In addition, suitable peak shapes were achieved using fused-core technology, indicating that this method has great poten-

tial for determining some bioactive substances in different propolis samples.

ACKNOWLEDGMENTS

The work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (Letícia S. Contieri 2020/03626-1, Leonardo M. de Souza Mesquita 2020/08421-9, and Juliane Viganó 2020/15774-5) for their financial support. Maurício A. Rostagno thanks FAPESP for the Young Researcher assistantship (2018/14582-5) and for the Multi-User Equipment Program (2019/13496-0). The authors also thank Wagner Vilegas and Renan Canute Kamikawachi for helping in the MS analyses. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Financial code 001.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Contieri LS, de Souza Mesquita LM, Sanches VL, Viganó J, Kamikawachi RC, Vilegas W, Rostagno MA. Ultra-high-performance liquid chromatography using a fused-core particle column for fast analysis of propolis phenolic compounds. J Sep Sci. 2023;46:2200440. https://doi.org/10.1002/jssc.202200440