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Research Article

Sustainable and Innovative Method for Real-Time Extraction and Analysis of Polyphenols from Green Propolis by Pressurized Liquid Extraction Coupled Inline with Photodiode Array Detection (PLE-PDA)

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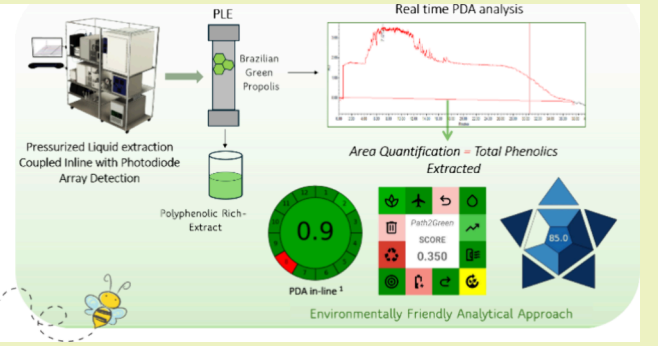
ABSTRACT: This article proposes an innovative process for extracting green propolis polyphenols, integrating real-time monitoring while adhering to green practices. This development is significant due to the complex propolis composition that requires extensive extraction and analytical methods, leading to an environmental impact. The study employed pressurized liquid extraction (PLE) combined with photodiode array detection (PDA) to simultaneously recover and quantify polyphenolic compounds from green propolis. The process was fully optimized regarding operational extraction variables, specifically the solvent-to-feed ratio, solvent composition, and temperature. An exhaustive extraction was achieved using ethanol as solvent, at 130 °C, 2 mL·min^{-1} solvent flow rate, and 1500 psi, resulting in a total polyphenolic content of 148 mg·g$_{propolis}^{-1}$ in 35 min. Additionally, the ability to quantify the signal obtained by the PDA represents a significant advancement in real-time monitoring processes, reducing the need for toxic solvents typically required in offline chromatographic analysis and shortening extraction times. The optimized method achieved a high green analytical score of 0.9 (AGREE) and a Path2Green score of 0.477, demonstrating strong adherence to the 12 principles of green analytical chemistry and green extraction, respectively. Also, high Blue Applicability Grade Index (BAGI) of 85.0 is present, demonstrating great practicality and applicability of the method. This work highlights an innovative technique, opening new perspectives for future innovations, even when complex matrices are studied.



KEYWORDS: *coupling of techniques, high-pressure extraction, real-time analysis, sustainable, polyphenols*

INTRODUCTION

Propolis, a natural resinous substance from honeybees, has become a pivotal point of extensive scientific investigation due to its diverse blend of bioactive compounds.[1] With over 800 identified substances, propolis boasts a rich chemical composition, including hydrophobic and hydrophilic compounds, namely, resins, waxes, essential oils, microelements, vitamins, and bioactive compounds, mainly phenolic compounds like flavonoids and acids.[2] The variety and abundance of those compounds establish this product as a powerful strength in biomedical uses, particularly renowned for its anti-inflammatory, antioxidant, and antimicrobial effects. This characteristic reflects the production of several propolis-based products, like sprays, syrups, mouthwash, capsules, and even functional food ingredients.[3−5]

Brazil is a significant global propolis producer, boasting a variety known as green propolis.[6] This unique type is abundant in cinnamic acid derivatives; notably, Artepillin C is recognized for its traditional biological benefits and powerful anticancer and anti-inflammatory properties.[7] Hence, there is a growing necessity for creating standardized green propolis extracts with high concentrations of polyphenolic compounds, particularly abundant in cinnamic acids already identified in this propolis variant and responsible for a large part of propolis' biological

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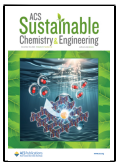

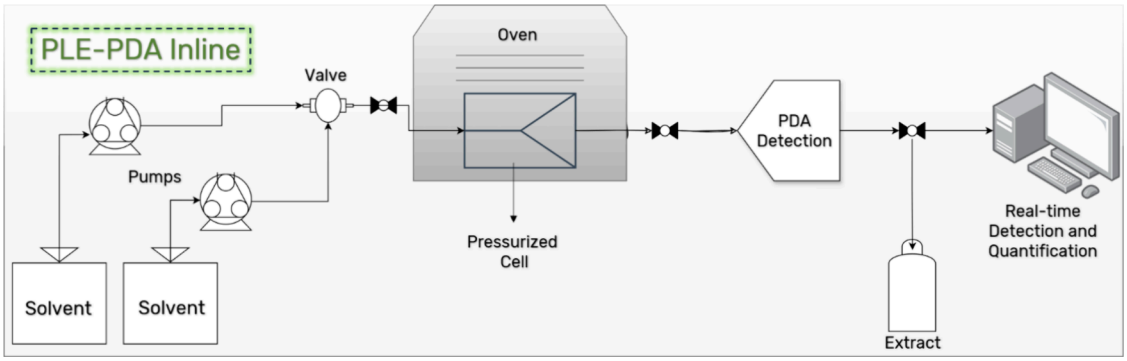

Figure 1. Schematic diagram of integrated pressurized liquid extraction system coupled inline with photodiode array detection (PLE−PDA).

advantages.[8] Ensuring the accurate and safe production of green propolis extracts is crucial, given that some commercial extracts are erroneously marketed as green propolis when they stem from different propolis types, like brown variants or other unrelated sources.[9]

Furthermore, it is necessary to ensure that green propolis extracts have a high composition of phenolic compounds. Due to the complexity of the sample, to achieve this, a precise match between the solvent and extraction technique is essential.[10] The conventional solvent involves using ethanol, which is both safe and efficient. While water has emerged as a cleaner substitute for ethanol, it does not consistently match the extraction performance of ethanol-based processes. The water also performs poorly in extracting cinnamic acids and derivatives.[1,11]

Regarding extraction techniques, pressurized liquid extraction (PLE) has experienced a notable rise in use over recent decades. This method has been successfully applied to extract major classes of compounds from a wide range of sample types.[12,13] Overall, PLE stands out as a robust and versatile technique that uses high temperature and pressure to alter solvent properties such as viscosity, surface tension, and the dielectric constant. These factors are pivotal to improving the mass-transfer coefficient, resulting in highly efficient extraction processes.[14] In addition, PLE also has a high degree of automation and can be combined with other extraction techniques, such as ultrasound.[15] In the case of propolis, PLE has been successfully used to obtain high concentrations of phenolic compounds in different samples. For example, de Carvalho et al.[16] extracted low-polarity compounds from red propolis using the solvent hexane in a PLE system, and Erdogan et al.[17] extracted phenolic compounds from Anatolian propolis using ethanol:water:HCl (70:25:5, v/v/v) containing 0.1% *tert*-butylhydroquinone (tBHQ) as solvent. However, the safety and environmental impact of these solvent systems are significant concerns, underscoring the urgent need for greener and more sustainable extraction alternatives. This is particularly important for reducing solvent consumption and minimizing reliance on offline analytical methods, which can be time-consuming and environmentally demanding. Emphasizing green extraction techniques, such as the use of biobased solvents and integrated, inline analysis, can help mitigate these issues, promoting safer, more efficient, and environmentally friendly processes.[18]

Moreover, one underexplored advantage of the PLE technique is its potential for coupling with other techniques, such as sample preparation and analysis, in a single step.[12] Some authors have already reported this use in different couplings, including PLE coupled with chromatographic systems (PLE-HPLC) and with solid phase extraction, as a purification first approach (PLE-SPE-HPLC).[12,19−23] Monitoring a PLE process using a UV−vis detector (PLE-SPE-UV) has also been successful for phenolics and alkaloids from mate leaves (*Ilex paraguariensis*).[24] However, a fixed wavelength in the UV−vis detector is a crucial limitation. The photodiode array (PDA) detector is an analytical instrument that enables real-time measurement of the complete wavelength spectrum, which is crucial for facilitating rapid and accurate monitoring of compounds during extraction processes. This capability enhances the efficiency of inline analysis, allowing for immediate adjustments and optimization of extraction conditions and ultimately contributing to more sustainable and precise methodologies. The signal recorded by the PDA detector indicates the progression of an extraction process, especially when compounds exhibit absorption at a specific wavelength.[25] So, it is possible to infer that the extraction process has been completed when the signal is no longer detected. In this regard, coupling PLE with PDA (PLE−PDA) can enable real-time monitoring of the extraction process.[20] Furthermore, monitoring can be evaluated at different wavelengths, exploring the different UV−vis spectra of the extracted compounds. This helps maintain product consistency and compliance with industry standards, ultimately enhancing consumer safety and trust. Implementing such advanced analytical techniques (especially combined with the extraction approach) is particularly important in commercial settings where product quality and regulatory adherence are critical.[26]

While PLE alone, in addition to its notable credentials, is not a new technique, the inline coupling for real-time process monitoring represents a novel advancement. Particularly within complex biological matrices, this becomes a hot topic toward complete valorization of the extracted biomass. This precisely meets the Sustainable Development Goal 12 (Responsible Consumption and Production).[27] In this context, the present article employs a novel approach by integrating pressured extraction coupled directly inline with photodiode array detection (PLE−PDA). The main objective is to evaluate the possibility of precisely monitoring and quantifying the Brazilian green propolis polyphenol extraction ($mg_{polyphenols} \cdot g_{propolis}^{-1}$). This innovative technique meets green extraction practices and opens a new perspective to further studies using different raw materials.

EXPERIMENTAL SECTION

Materials and Samples. The company "Mn Propolis" (Mogi das Cruzes, SP, Brazil) kindly donated the green raw propolis sample used

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in this study. The frozen samples were blended briefly using a household blender (Model OSTER, 450W 220 V, São Paulo, Brazil). The resulting milled propolis samples were sifted through a steel sieve (Model Bestifer, Limeira, Brazil) to ensure a 0.5−1 mm standardized particle size. The samples were stored in a dark container at −20 °C until use. Acetic acid, acetonitrile, and methanol of LC grade were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Ethanol absolute was purchased from Synth (Diadema, SP, Brazil). The ultrapure water was provided by a Purelab Flex 3 purifying system (ELGA Veolia, High Wycombe, United Kingdom). The reference standard (green propolis biomarkers) 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, Ltd. (St. Louis, Missouri, USA).

Pressurized Liquid Extraction Coupled In Line with Photodiode Array Apparatus (PLE−PDA). The experiments were carried out in a system coupling PLE in line with a chromatographic system (HPLC-PDA), patent BR 1320210187220. The system is conceptually divided into two modules, one based on PLE and the other dedicated to the HPLC-PDA. In this article, we use the PLE in line with the PDA detector, as represented on Figure 1. This was made possible by the device's high versatility, which features valves that control the system, allowing the modules to operate independently or simultaneously. The main components of the system are PDA detector (Waters, Milford, Massachusetts, USA), oven for extraction cells (Memmert GmbH, UF55, Buechenbach, Germany), PLE stainless-steel extraction cells (15 mm × 100 mm, Waters Corporation, Milford, Massachusetts, USA), and valves for selection, depressurization, automatic pressurization, and samples collection. The PLE is performed in semicontinuous flow. All systems are controlled through Waters Empower 3 software. The 3D representation of the prototype is depicted in Figure S1 of the Supporting Information.

Offline Propolis Polyphenol Quantification (UHPLC-PDA-MS/MS). A reference offline method proposed by Contieri et al.[8] was used to quantify the propolis polyphenols on the extracts. The samples were analyzed using ultrahigh-performance liquid chromatography and a photodiode array detector (UHPLC-PDA, Waters Corp, ACQUITY H-Class, Milford, Massachusetts, USA). The chromatographic separation was accomplished utilizing a fused-core column (Kinetex C-18, 50 × 2.1 mm, 1.3 μm: Phenomenex, Torrance, California, USA). No dilution was applied to the samples, and before injection, the extracts were thoroughly mixed through vortexing and filtered using a nylon syringe filter (particle diameter of 0.22 μm, Analitica, São Paulo, Brazil). As recommended for the proposed method, two reference molecules, namely, *p*-coumaric acid and Artepillin C (recognized as the green propolis biomarkers), were employed to quantify the phenolic content of the extracts. Quantification was done by integrating the peak areas at 290 nm; 320 and 360 nm using an external standard calibration curve (Table S1 of the Supporting Information). The *p*-coumaric standard curves were prepared by plotting the concentration (100; 50;25; 12.50; 6.25; 3.13;1.56, 0.78 ppm) against the area of the peak, which was used to quantify ferulic acid and derivatives, *p*-coumaric acid, and quinic acid derivatives. The Artepillin C calibration curve was achieved by plotting the concentration (1000; 500;250;125;100;50;25;12.5;6.25, and 3.13 ppm) against the peak area to quantify cinnamic acids and flavonoids. The results were expressed in $mg_{polyphenols}{\cdot}g_{propolis}^{-1}$. Offline quantification was performed in this study as a standard reference, allowing the results obtained from the PLE−PDA quantification to be compared to the offline data. This comparison aims to validate the reliability and accuracy of the inline approach.

Pressurized Liquid Extraction and Inline PDA Detection. The stainless-steel extraction cell was filled as follows: 0.3 g of glass wool and 16 g of glass beads (2 mm) as proposed by de Souza Mesquita et al.[12] The glass wool was employed for filtering the extract and eliminating solid particles from the biomass, and glass beads were utilized to minimize the dead volume within the extraction cell. The extraction solvents used were pure water (100%) and ethanol (100%) and a mixture of water:ethanol (50:50 v/v). The amount of propolis (grams of biomass used in the extraction) was optimized considering the signal in the PDA detection. The solvents continuously flowed at 2 $mL_{solvent}{\cdot}min^{-1}$ at the desired time. Then, the oven of the extraction cell was heated to the experimental temperature (using the appropriate preheating time to guarantee that the desired temperature in the interior of the extraction cell is precise), and the extraction pressure was fixed at 1500 psi, as proposed by Maciel-Silva et al.[20] Initially, the extraction was conducted offline (without PDA coupling) to study the PLE variables and the optimization of the extraction procedure, i.e., maximize the recovery of the target compounds from green propolis. To optimize the PLE conditions, the analyzed variables were temperature, ranging from 40 to 160°, and solvent-to-feed (SF) ratios of 120, 150, 240, and 460 $mL_{solvent}{\cdot}g_{propolis}^{-1}$. The response was the yield of extracted propolis polyphenols extracted ($mg_{polyphenols}{\cdot}g_{propolis}^{-1}$). For each PLE experiment, 30 mL of extracts was collected in a container and stored in a freezer until analysis. After the PLE optimization, part of the extract to be collected was directed to the PDA to monitor the extraction process. The flow was the same as the extraction, with 2 $mL_{solvent}{\cdot}min^{-1}$. The absorbance was monitored at 290, 320, and 360 nm, to help in monitoring propolis polyphenol extraction.

Propolis Polyphenol Quantification Inline (PLE−PDA). To develop an inline quantification method, calibration lines were constructed with different wavelengths, 290 nm; 320 and 360 nm, in which the *x*-axis corresponds to the total area obtained through PDA in real time, after exhaustive extraction by PLE (using the optimized extraction conditions) using different initial amounts of propolis (0.25 0.20, 0.125, 0.065, and 0.0325 g) and the *y*-axis corresponds to the total quantification of phenolic compounds extracted by PLE, analyzed offline by UHPLC-PDA. After constructing the straight line, an acceptable R^2 was obtained. To validate the calibration lines and understand their application, the PLE−PDA extraction method was carried out on the same sample of green propolis using a different solvent (ethanol:water 50% v/v). After extraction, the total area presented by the PDA reading was recovered and the amount of total propolis phenolic compounds was calculated using the curves (inline quantification); then, the amount of total phenolics from this same extract was read using the offline method (offline quantification). Finally, the results of inline and offline quantifications were compared, and the error percentage (%) was evaluated (calculated regarding the % variation between offline and inline quantification).

Greenness Assessment Criteria by the Analytical GREEnness (AGREE). The greenness assessment of the PLE−PDA process was compared with three other analysis processes using propolis, one by HPLC online analysis,[28] one by UHPLC offline analysis,[8] and one by HPLC offline analysis.[29] This impact was evaluated using the Analytical GREEnness (AGREE) calculator, the most robust green index.[30] The AGREE circle is segmented into 12 parts, each representing a green principle, and the corresponding AGREE value is depicted in the circle's center. The AGREE scores range between 0 and 1, with a score closer to 1 indicating better adherence to green principles. The final score was calculated based on principles using the most recent version of the Python code obtained from the repository maintained (git.pg.edu.pl/p174235/AGREE). The result pictogram indicates the final score and the performance of the analytical procedure.

Greenness Assessment by the Path2Green Metric. The greenness assessment of the PLE−PDA process was evaluated by the *Path2Green* metric proposed by de Souza Mesquita et al.[18] This metric was formulated following the 12 new principles of green extraction. In this metric, parameters were established by evaluating attributes throughout the pre- and postextraction procedures: (1) biomass, (2) transport, (3) pretreatment, (4) solvents, (5) scaling, (6) purification, (7) yield, (8) post-treatment, (9) energy; (10) application, (11) repurposing, and (12) waste management. Each principle was scored between −1 and 1, with a score closer to 1 indicating better adherence to the green principle. Also, each principle was evaluated individually, with the environmental aspect carrying a more pronounced weight (weight 3), followed by society (weight 2)

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Table 1. Results of the Extraction by PLE at 1500 psi, 2 $mL_{solvent}\cdot min^{-1}$, 120 $mL_{solvent}\cdot g_{propolis}^{-1}$ at 15 min, Using Water (100%) as Solvent at Different Extraction Temperatures (40, 70, 100, 130, and 160 °C)[a]

extraction temperature (°C)	recovery compounds ($mg_{compound}\cdot g_{propolis}^{-1}$)		
	ferrulic, *p*-coumaric, caffeoyl quinic acids, and derivatives ($mg_{p\text{-coumaricacidEq}}\cdot g_{propolis}^{-1}$ ± SD[b])	artepillin C, quercetin, kaempferide and baccarin, cinnamic acids, and derivatives ($mg_{ArtepilinCEq}\cdot g_{propolis}$ ± SD[b])	total *n* ($mg_{polyphenols}\cdot g_{propolis}^{-1}$)
40	0.524 (± 0.012)C	0	0.524 (± 0.012)D
70	0.786 (± 0.032)B	0.087 (± 0.030)D	0.873 (± 0.032)C
100	1.310 (± 0.164)B	0.787 (± 0.236)B	2.097 (± 0.160)B
130	1.950 (± 0.008)A	1.474 (± 0.045)A	3.424 (± 0.082)A
160	0.894 (± 0.065)B	0.348 (± 0.095)C	1.242 (± 0.065)C

[a]The table presents extraction yields relative to the first part of the chromatogram calculated using the *p*-coumaric acid curve ($mg_{p\text{-coumaricacidEq}}\cdot mg_{propolis}^{-1}$) and relative to the second part of the chromatogram, calculated using the Artepillin C curve ($mg_{ArtepilinCEq}\cdot mg_{propolis}^{-1}$).The standard deviations are relative to the triplicate of the HPLC-PDA injection. Different letters between the columns indicate significant statistical results ($p < 0.05$, Bonferroni post hoc test). [b]SD: standard deviation.

and economic (weight 1). The evaluation was made by the *Path2Green* cellphone app. The resulting pictogram indicates the final score and the performance of the extraction process and allows the perception where the red flags are in which the developed process can be improved in terms of sustainability. The final score is translated in $g_{CO2}/g_{biomass}$ equivalent by the regression: The final score is translated in $g_{CO2}/g_{biomass}$ equivalent by the regression, as shown in the following equation.

$$y_{\left(\frac{g_{CO2}}{g_{biomass}}\right)} = -1079.3\text{Score}_{(path2green)} + 721.3$$

Method Practicality Assessment by the Blue Applicability Grade Index (BAGI). The practicality and applicability of the PLE−PDA process were evaluated by BAGI proposed by Manousi et al.[31] The BAGI focused on practical aspects of white analytical chemistry. This tool evaluates 10 attributes: (1) type of analysis, (2) number of analytes that are simultaneously determined, (3) analytical technique and required analytical instrumentation, (4) number of samples that can be simultaneously treated; (5) sample preparation; (6) number of samples that can be analyzed per hour; (7) type of reagents and materials used in the analytical method; (8) requirement for preconcentration; (9) automation degree; and (10) amount of sample. In each attribute, four discrete scores of equal weights are used in the assessment: 10, 7.5, 5.0, and 2.5 points correspond to dark blue (no. 0c305b), blue (no. 3a89c1), light blue (no. adcffd), and white (#FFFFFF), respectively. Through the evaluation of these attributes, an asteroid pictogram is generated together with the respective score. The evaluation was made using a web application.

Statistical Analysis. All the statistical analyses were performed using the Jamovi program, version 1.6.6. Data were submitted to one-way ANOVA followed by Bonferroni post hoc, with a significance level fixed at 95% ($p < 0.05$). All the results were expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

Water Pressurized Liquid Extraction. Water was initially considered as an extraction solvent, given its frequent use as an ethanol alternative and its high safety credentials. However, it often proved less effective, extracting lower phenolic content than ethanol.[1,32] Thus, we hypothesized that employing a water high-pressure extraction method might amplify its efficiency as a solvent. Under high-pressure conditions, water undergoes several physical changes that can increase its efficiency as a solvent, especially for extracting phenolic compounds.[33,34] These changes can be an increase in its solubility and density and a modification of its water hydrogen bonds. Furthermore, if the pressure reaches a certain point (supercritical pressure), water behaves differently than that in the liquid state. In the supercritical state, water has a unique combination of liquid density and gas-like viscosity, which increases its solvency power and allows it to extract a wider range of phenolic compounds.[34,35]

Various factors are considered when optimizing PLE conditions, like solvent, temperature, time, and pressure.[36] For this step, a brief extraction time of 15 min was opted for, as it not only conserves energy but also minimizes solvent usage,[37] and the pressure was fixed at 1500 psi, and the flow was fixed at 2 $mL_{solvent}\cdot min^{-1}$, as proposed by Maciel-Silva et al.[20] So, emphasis was placed on temperature and solvent-to-feed ratio (SF), with the other parameters held constant.

Initially, the optimal temperature was evaluated by keeping a constant solvent-to-feed ratio of 120 $mL_{solvent}\cdot g_{propolis}^{-1}$, with temperatures ranging from 40 to 160 °C. This range was selected based on conditions established in previous studies using the same extraction apparatus.[20] Additionally, higher temperatures were avoided to prevent the potential degradation of the propolis phenolic compounds. Similarly, temperatures below 40 °C were excluded, as previous research suggested that they may not achieve optimal extraction efficiencies under high-pressure conditions.[10,36] Table 1 presents the results of the extraction yield using the different temperatures after the analysis by the UHPLC-PDA method offline. Figure S2 presents the chromatogram recovery at each temperature, and Table S2 depicts the compounds extracted by water and their respective maximum absorbance at UV−vis (nm).

Table 1 presents the extraction yield relative to the first part of the chromatogram (peaks 1 to 8 in Figure S2 of the Supporting Information), calculated using the *p*-coumaric acid curve ($mg_{p\text{-coumaricacidEq}}\cdot g_{propolis}^{-1}$). These compounds refer to ferrulic, *p*-coumaric, and caffeoyl quinic acids and derivatives. The table also presents the yield relative to the second part of the chromatogram (peaks 9 to 14 in Figure S2 of the Supporting Information), relative to the Artepillin C curve ($mg_{ArpilllinCEq}\cdot g_{propolis}^{-1}$). These compounds refer to cinnamic acids and flavonoids (Artepillin C, quercetin, kaempferide, and baccarin). The total of polyphenols extracted ($mg_{polyphenols}\cdot g_{propolis}^{-1}$) are also exhibited in Table 1. Notably, the temperature of 130 °C resulted in the maximum phenolic extraction yield (3.424 ± 0.082 $mg_{polyphenol}\cdot g_{propolis}^{-1}$). In comparison, the lowest extraction yield (0.524 ± 0.012 $mg_{polyphenol}\cdot g_{propolis}^{-1}$) was obtained using the lowest temperature (40 °C). This trend was maintained in both parts of the chromatogram. It is important to note that at the lowest temperature (40 °C), cinnamic acids and flavonoids were not extracted, and these were not detected in the second part of

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Table 2. Results of the Extraction by PLE 1500 psi, 2 $mL_{solvent}{\cdot}min^{-1}$, 120 $mL_{solvent}{\cdot}g_{propolis}^{-1}$ at 15 min, 130°C Using Water (100%) as Solvent at Solid-to-Feed Ratio, $mL_{solvent}{\cdot}g_{propolis}^{-1}$ (120, 150, 240, 460)[a]

	recovery compounds ($mg_{compound}{\cdot}g_{propolis}^{-1}$)		
solid to feed ratio ($mL_{solvent}{\cdot}g_{propolis}^{-1}$)	ferrulic, *p*-coumaric, and caffeoyl quinic acids and derivatives ($mg_{p\text{-coumaricacidEq}}{\cdot}g_{propolis}^{-1}$ ± SD[b])	artepillin C, quercetin, kaempferide, baccarin, and cinnamic acids and derivatives ($mg_{ArtepilinCEq}{\cdot}g_{propolis}^{-1}$ ± SD[b])	total ($mg_{polyphenols}{\cdot}g_{propolis}^{-1}$)
120	1.950 (±0.020)B	1.474 (±0.040)B	3.424 (±0.035)B
150	2.430 (±0.016)A	1.850 (±0.032)A	4.282 (±0.192)A
240	0.975 (±0.082)C	0.737 (±0.164)C	1.712 (±0.188)C
460	0.324 (±0.041)D	0	0.324 (±0.041)D

[a]The table presents extraction yields relative to the first part of the chromatogram calculated using the *p*-coumaric acid curve ($mg_{p\text{-coumaricacidEq}}{\cdot}mg_{propolis}^{-1}$) and relative to the second part of the chromatogram, calculated using the Artepillin C curve ($mg_{ArtepilinCEq}{\cdot}mg_{propolis}^{-1}$).The standard deviations are relative to the triplicate of the HPLC-PDA injection. Different letters between the columns indicate significant statistical results ($p < 0.05$, Bonferroni post hoc test). [b]SD: standard deviation.

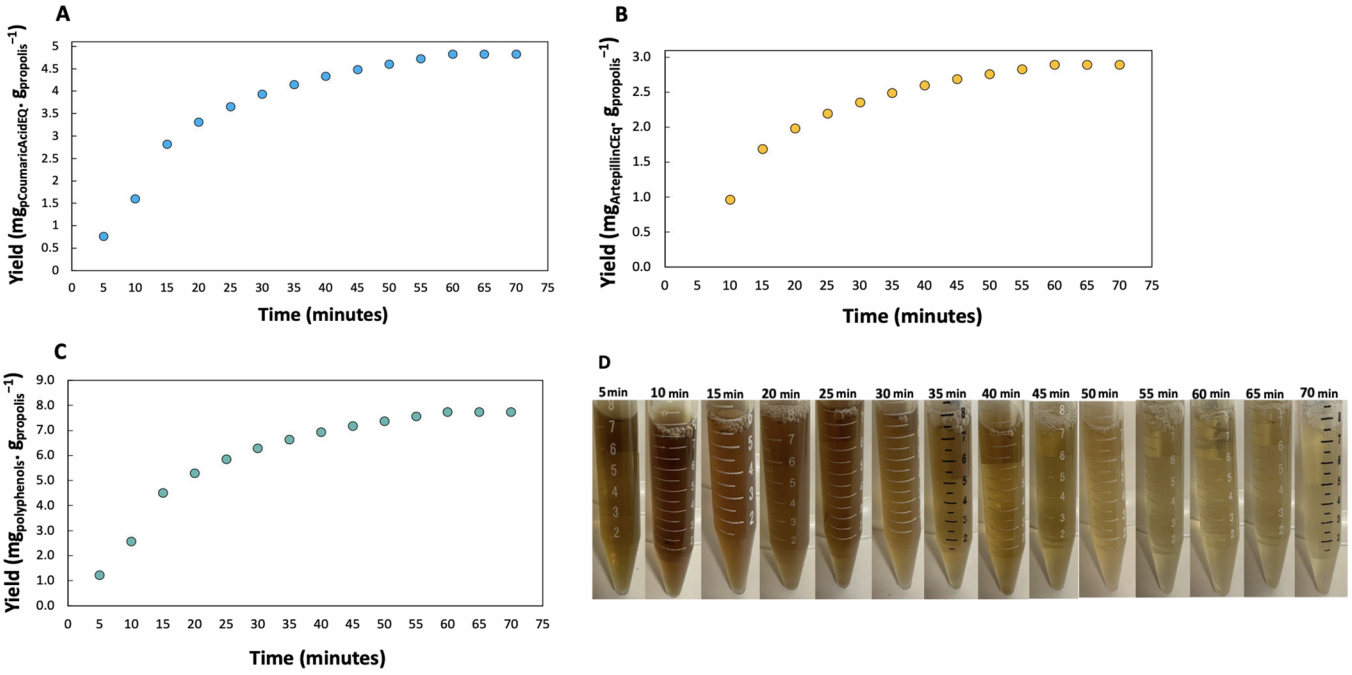

Figure 2. Kinetic curve of propolis polyphenol extraction during 70 min, at 130 °C and 150 mLsolvent·$g_{propolis}^{-1}$ with 2 mLsolvent·min^{-1} and 1500 psi, by aqueous PLE-based extraction. (A) Extraction yields relative to the first part of the chromatogram (peaks 1 to 8), calculated using the p-coumaric acid curve ($mg_{p\text{-coumaricacidEq}}{\cdot}mg_{propolis}^{-1}$). (B) Extraction yield relative to the second part of the chromatogram (peaks 9 to 14), calculated using Artepillin C ($mg_{ArtepilinCEq}{\cdot}mg_{propolis}^{-1}$). (C) Results of the total extraction yield ($mg_{polyphenols}{\cdot}g_{propolis}^{-1}$). (D) Representative photos of the extract collected every 5 min.

the chromatogram. An explanation for this lies in the compounds of lower polarity found in the second part. Extracting these compounds using water requires simultaneous application of high temperature and pressure. Studies reveal that low-pressure extraction, using water (100%) as a solvent for propolis compound extraction, fails to retrieve these compounds, even under elevated temperatures.[1,8] It is noteworthy that while higher temperatures tend to enhance the extraction yield, the optimal temperature identified was 130 °C. At 160 °C, the extraction yield declined, probably due to the degradation of thermosensitive phenolic compounds.[1]

After optimizing PLE temperature at 130 °C, the solid-to-feed ratio (SF) was evaluated: 120, 150, 240, and 460 ($mL_{solvent}{\cdot}g_{propolis}^{-1}$). These variables were selected considering other studies that combined the use of high pressure in propolis samples.[16,17,36,38] Additionally, it is noteworthy that SF values below 100 do not appear necessary for effectively recovering phenolic compounds from propolis.[10] Table 2 presents the outcomes of this extraction, after the analysis by the UHPLC-PDA method offline. Figure S3 of the Supporting Information presents the chromatogram recovery each SF previously described.

Table 2 presents the extraction yield relative to the first part of the chromatogram (peaks 1 to 8, in Figure S3 of the Supporting Information), calculated using the *p*-coumaric acid curve ($mg_{p\text{-coumaricacidEq}}{\cdot}g_{propolis}^{-1}$). These compounds refer to ferrulic, *p*-coumaric, and caffeioquinic acids and derivatives. Also, presents the yields relative to the second part of the chromatogram (peaks 9 to 14 in Figure S3 of the Supporting Information), relative to the Artepillin C curve ($mg_{ArpilllinCEq}{\cdot}g_{propolis}^{-1}$). These compounds refer to cinnamic acids and flavonoids (Artepillin C, quercetin, kaempferide, and baccarin). The total of polyphenols extracted ($mg_{polyphenols}{\cdot}g_{propolis}^{-1}$) are also exhibited in Table 2. It is possible to highlight that the highest yield found was in an SF of 150 $mL_{solvent}{\cdot}g_{propolis}^{-1}$ (4.282 ± 0.192 $mg_{polyphenol}{\cdot}g_{propolis}^{-1}$). It is worth mentioning that at the highest SF of 460 $mL_{solvent}{\cdot}g_{propolis}^{-1}$, there was a significant yield decline (0.324 ± 0.042 $mg_{polyphenol}{\cdot}g_{propolis}^{-1}$).

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This decline occurred mainly due to the nonextraction of the components relative to the second part of the chromatogram, mainly due to the low solubility of the compounds Artepillin C, quercetin, kaempferide, baccarin, cinnamic acid, and derivatives, in water (as they are nonpolar). This factor, added to the low quantity of the propolis sample initially extracted, intensified the difficulty of water in extracting these compounds.[9] These results significantly highlight the absence of a defined linear correlation between the proportion of SF and the total yield of phenolics extracted from propolis in PLE extractions. This reinforces the idea that in highly complex samples, as in the case of propolis, it is crucial to conduct optimization studies to identify the conditions that guarantee a better extraction yield.

Following the optimization of PLE conditions, a kinetic curve was constructed to evaluate the extraction duration and verify the development of a thorough process. This curve was constructed based on the phenolic compound yield analyzed with the offline UHPLC-PDA method previously described. Extraction fractions were collected every 5 min, representing the cumulative total after the kinetics analysis, as depicted in Figure 2. Figure 2A presents the kinetic curve relative to extraction of ferrulic, *p*-coumaric, caffeioquinic acids, and derivatives, calculated using the *p*-coumaric acid curve ($mg_{p\text{-coumaricAcidEq}} \cdot g_{propolis}^{-1}$). Figure 2B presents the kinetic curve relative to extraction of cinnamic acids and flavonoids (artepillin C, quercetin, kaempferide, and baccarin), calculated using Artepillin C curve ($mg_{ArpilllinCEq} \cdot g_{propolis}^{-1}$). Figure 2C presents the kinetic curve of cumulative polyphenols extracted ($mg_{polyphenols} \cdot g_{propolis}^{-1}$), and Figure 2D presents the representative photos of the extract collected every 5 min.

This analysis made it possible to realize that the extraction under the optimized operational conditions using water as the solvent was completed in 70 min, where no more phenolic compounds were detected in the extract. In addition, it is essential to highlight that from between 40 and 50 min of extraction, the kinetic curve reached the diffusion-controlled stage, characterized by being the slow stage of the extraction process, showing an exhaustive approach.[39] However, the maximum amount of polyphenols extracted by this methodology was 7.31 $mg_{polyphenol} \cdot g_{propolis}^{-1}$. Comparing this result with others already reported in the literature, including using PLE systems, it was significantly lower.[16,17,36] One explanation for this was using water as the solvent, as it favors the extraction of polar compounds while making the extraction of less polar compounds,[8] like cinnamic acid derivatives, more challenging. While acknowledging the effectiveness of the PLE method and optimizing the operational variables influencing extraction efficiency, the yield obtained was unsatisfactory compared with those obtained with ethanol. Recently, using this same green propolis biomass, we developed a sustainable ultrasound-assisted extraction process using an amino-acid-based eutectic solvent. Initially, as well as in this study, our main objective was to replace ethanol as an extractant media. Indeed, ethanol can be considered a "Generally Recognized as Safe" Solvent and produced by renewable bias, minimizing the carbon footprint associated with its production and enabling the development of more eco-friendly extraction platforms.[40,41] Nevertheless, ethanol cannot be regarded as an inert substance; on the contrary, its utilization in pharmaceutical preparations is linked to safety concerns. In this sense, an extraction approach using water is still desired. Despite the eutectic solvent-based processes being successfully developed, including adding a more pronounced biological effect for the extract (improved antihypertensive, antimicrobial, and antioxidant activity compared to those obtained with water and ethanol), a maximum recovery was still obtained with the ethanol-based process.[1] In this scenario, given some challenges regarding the viscosity of the eutectic solvents in pressurized systems that is still under evaluation, and considering both cost-effectiveness and compatibility with the system, ethanol emerges as the ideal choice to be studied for building a comprehensive PLE coupled with PDA detection.

Considering the previously obtained results, the solvents, water 100%, and ethanol 100% were compared in terms of propolis polyphenol extraction yield. The optimized conditions identified in water PLE of 130 °C and SF 150 $mL_{solvent} \cdot g_{propolis}^{-1}$ and the fixed conditions of 2 $mL_{solvent} \cdot mL^{-1}$ and 1500 psi were used. To compare the solvent extraction efficiency, a kinetic curve made by $mg_{polyphenol} \cdot g_{propolis}^{-1}$ (analyzed offline by UHPLC-PDA) of extracts collected every 5 min during 70 min of extraction was made (Figure 3A). Figure 3B presents the chromatograms recovered by the UHPLC-PDA, 290 nm, of each solvent (water and ethanol, 100%).

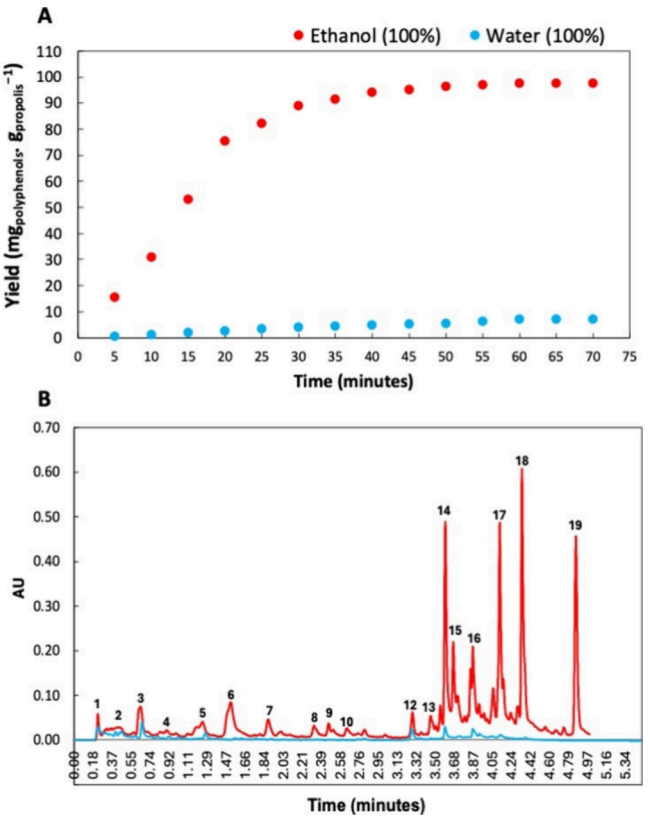

Figure 3. (A) Kinetic curve of propolis polyphenol extraction for 70 min, at 130 °C, 150 $mL_{solvent} \cdot g_{propolis}^{-1}$, 2 $mL_{solvent} \cdot min^{-1}$ and 1500 psi. With different solvents, ethanol (100%) and water 100%. (B) Chromatography recovered at 290 nm using the UHPLC-PDA method. The numbers represents a peak recovery: (1) ferulic acid; (2) feruloylquinic acid; (3)*p*-coumaric acid; (4) caffeoylquinic acid, (5) dicaffeoylquinic acid (a); (6) dicaffeoylquinic acid (b); (7) dicaffeoylquinic acid (c); (8) tricaffeoylquinic acid (a); (9) tricaffeoyquinic acid (b); (10) 3,4-dihydroxy-5-prenyl-cinnamic acid; (11) dihydrokaempferide; (12) quercetin; (13) dihydrokaempferide derivative; (14) n.i.; (15) chrysin; (16) kaempferide; (17) artepillin C; (18) baccarin; (19) n.i.

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Through the results presented in Figure 3, it is possible to clearly notice that the ethanol extraction provided a greater yield of propolis polyphenol extraction compared to water. With recovery after 70 min, 97.56 and 7.31 $mg_{polyphenol} \cdot g_{propolis}^{-1}$ (Figure 3A) were obtained, using ethanol 100% and water 100% as solvents, respectively. Furthermore, through the chromatograms presented in Figure 3B, it is possible to note that ethanol was able to recover a greater diversity of phenolic compounds compared to water. Table S3 of the Supporting Information presents the extracted compounds by ethanol, as well as its UV/vis absorption. When compared with Table S2, which presents the compounds extracted using water as solvent, it is possible to notice that ethanol extracted a greater diversity of quinic and cinnamic besides flavonoids (quercetin, dihydrokaempferide, and derivatives) compared to water. Due to these findings, the ethanol (100%) was chosen for subsequent extractions in the PLE−PDA system. It is possible to observe in Figure 3A that from 30 min forward, a diffusion-controlled phase begins, signifying the slow phase of the extraction process.[39]

Ethanol-Pressurized Liquid Extraction Coupled Inline with Photodiode Array. PDA was employed for process monitoring with the aim of real-time control to guarantee exhaustive extraction. The prior knowledge of the propolis extraction kinetic curve is pivotal in evaluating the inline technology and ensuring accurate monitoring by the PDA. Figure 4A presents the chromatogram generated by the PDA recovery at 290 nm, and Figure 4B presents the representative kinetic curve of this extraction recovery every 5 min.

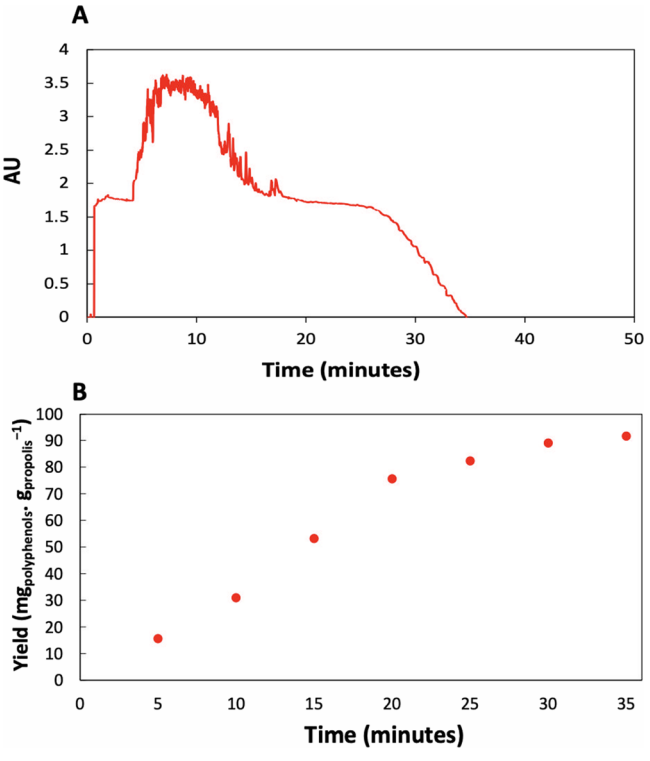

Figure 4. Pressurized liquid extraction coupled inline with a photodiode array. Extract recovery at PLE at 130 °C, 2 $mL_{solvent} \cdot min^{-1}$, 150 $mL_{solvent} \cdot g_{propolis}^{-1}$, and 1500 psi, using ethanol (100%) as the solvent; the extraction was monitored with PDA real-time analysis. (A) PDA recovery at 290 nm and (B) kinetic curve of propolis polyphenol extraction during 35 min recovery every 5 min.

It is possible to see that no signal was detected by the PDA after 35 min, suggesting the extraction interruption at this point. A comparison of the extraction yield after 35 min, as illustrated in Figure 4B, with the extraction yield obtained under identical conditions at the end of 70 min (depicted in Figure 3) reveals results of 94.126 and 97.563 $mg_{polyphenol} \cdot g_{propolis}^{-1}$, respectively. Therefore, 35 min of extraction was considered the optimal time for extraction in these operational conditions.

This outcome underscores that real-time monitoring by PDA facilitated the determination of optimal extraction time, thereby averting unnecessary energy consumption and higher-volume use of solvents. This discovery holds significance, as reducing extraction time while maintaining a high yield is a primary focus for companies involved in producing propolis-based products. Maximizing the utilization of raw materials, particularly in producing extracts rich in phenolic compounds, is crucial for ensuring a product's success, as Contieri et al.[1] emphasized.

Propolis Polyphenol Quantification Inline (PLE−PDA). To comprehensively evaluate the nuances of real-time monitoring, we studied how to transform the area values read from the PDA into phenolic compound quantification on the extracts after a PLE. To develop an inline quantification method, calibration lines were constructed with different wavelengths, 290, 320, and 360 nm (Figure S5 of the Supporting Information). The selection of these wavelengths was deliberate, aligning with the peak absorption points for key green propolis biomarkers—namely, *p*-coumaric acid at 290 nm and Artepillin C at 320 nm. Notably, these biomarkers, representing distinct polarities, exemplify the diverse compound properties inherent in propolis extracts. *p*-Coumaric acid showcases predominance in higher polarity, while Artepillin C embodies a less polar nature.[42,43] The wavelength of 360 nm was chosen to be the maximum absorbance of other phenolic compounds also extracted by ethanol and to differentiate the maximum absorbance of the propolis biomarkers.

For this evaluation, calibration curves were made. The *x*-axis corresponds to the total area obtained through PDA in real time, after exhaustive extraction by PLE, using different quantities of crude propolis (0.25, 0.20, 0.125, 0.065, and 0.0325 g). The *Y*-axis corresponds to the total quantification of phenolic compounds extracted in this PLE, analyzed offline by UHPLC-PDA. Figure S4 of the Supporting Information presents all the graphs generated by the PDA real-time analysis on the three wavelengths (290, 320, and 360 nm) during the different amounts of propolis extractions. Through the figures, it is possible to notice that there is a directly proportional relationship between the amount of sample extracted and the amplitude of the signal generated by the PDA. Also, from Figure S4 of the Supporting Information, it is noticeable that the extraction reached a signal level of "0" more quickly when a smaller amount of sample was used. This result demonstrates that real-time monitoring by PDA assists in determining the extraction time, thereby avoiding unnecessary energy consumption. After this real-time analysis, the total areas of all graphs were recovered to construct the calibration curves. Furthermore, the total content of phenolic compounds extracted in each assay was quantified by UHPLC-PDA to construct calibration curves (Figure S5A−C of the Supporting Information). Table 3 presents the recovered values.

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Table 3. Recovery Values Are Used to Construct the Calibration Curve at Different Wavelengths[a]

wavelength	initial amount of crude propolis (g)	total area in line (AU)	total polyphenols recovered ($mg_{polyphenol}{\cdot}g_{propolis}^{-1}$)
290	0.250	13,381,606,290	181.15
	0.200	4,107,693,539	128.15
	0.125	903,278,282	106.44
	0.065	293,335,586	41.63
	0.0325	35,471,160	18.10
320	0.250	13,038,851,142	244.66
	0.200	4,866,854,751	174.57
	0.125	908,181,528	122.624
	0.065	367,845,970	60.74
	0.0325	92,326,580	26.41
360	0.250	9,592,431,363	109.82
	0.200	4,754,855,970	77.85
	0.125	1,440,585,545	62.61
	0.065	426,994,514	26.51
	0.0325	3,547,116	11.52

[a]The table presents the total area inline, corresponding to the total area obtained through PDA signal in real time, after exhaustive extraction by PLE, using different quantities of crude propolis (0.25, 0.20, 0.125, 0.065, and 0.0325 g) and presents total quantification of phenolic compounds extracted by this PLE, analyzed offline by UHPLC-PDA.

With these results, it is possible to see that the area values, as well as the propolis phenolic compound quantification, varies when the extract is analyzed at different wavelengths, regardless of the amount of initial sample. This probably occurs due to the complexity of green propolis and the existence of several phenolic compounds. The significant signal variation between different wavelengths indicates that certain compounds are extracted preferentially than others. Through the results, it was noticed that a greater area and quantity of polyphenols were found at 320 nm. This result indicates that the largest number of phenolic compounds extracted presents maximum absorption in UV_{vis} and PDA close to 320 nm. Examples are Artepillin C (one of the biomarkers of green propolis), quinic acids, and ferulic acid. This observation provides important insights into the types of compounds extracted by PLE.

After constructing the straight line and obtaining an adequate R^2, we validated the straight lines by applying the PLE−PDA extraction method to the same sample of green propolis extracted with a different solvent (ethanol:water 50% v/v). An exhaustive extraction was carried out using the initially optimized conditions at 130 °C, 2 $mL_{solvent}{\cdot}min^{-1}$, 0.125 g of propolis, and 1500 psi. After removing the total area from the graphics, it was recovered and inserted into the discovery curve to obtain the inline result. After this, the same sample was read offline using the UHPLC-PDA method and the coefficient of variation was calculated. Table 4 presents the results.

It was possible to observe that the greatest variation in results was obtained at 290 nm. This variation can be explained not only by the differences between the analysis methods but also by the complexity of the raw material. The preliminary results suggest that it is feasible to perform quantification entirely inline. Although further studies are necessary to ensure proper validation, these findings underscore the potential of the inline PLE−PDA method as a valuable tool for real-time monitoring during production. Additionally, it can significantly

Table 4. Validation with Straight Lines by Applying the PLE−PDA Extraction[a]

wavelength (nm)	inline quantification ($mg_{polyphenol}{\cdot}g_{propolis}^{-1}$)	offline quantification ($mg_{polyphenol}{\cdot}g_{propolis}^{-1}$)	error (%)*
290	133.98	105.95	26
320	148.65	131.58	12
350	54.92	43.56	20

[a]The extraction was conducted using the same sample of green propolis using ethanol:water (50% v/v) as solvent. An exhaustive extraction was carried out using the initially optimized conditions at 130 °C, 2 $mL_{solvent}{\cdot}min^{-1}$, 0.125 g of propolis, and 1500 psi. The table presents the inline quantification corresponding to the phenolic compound value generated by the calibration curve and the offline quantification corresponding to the phenolic compounds analyzed offline by UHPLC-PDA.

aid in determining the optimal conditions for the extraction process, thereby enhancing the efficiency and consistency in manufacturing. Implementing this method could lead to more accurate control and better-quality end products, aligning with industry standards and regulatory requirements.

Greenness Assessment Criteria by Analytical GREEnness. When developing a new method, it is essential to evaluate its environmental impact, as this encourages adjustments that can still be made to reduce its inherent implications. Thus, the AGREE methodology evaluated the greenness of the proposed method. The results of the new method, PLE−PDA, were compared with different analysis methodologies published in the literature, including ultra high-performance liquid chromatography (UHPLC) offline and high-performance liquid chromatography (HPLC), both performed under online and offline detection operating modes. These methods also utilized propolis as the raw material, with two of them incorporating green propolis, which is the focus of our analysis here—Table S4 of the Supporting Information summarizes the variables used on those methods.[8,28,29] The GREEnness analytical calculator was used to calculate the environmental score of each methodology. This method evaluated the impact considering each of the 12 principles of Green Analytical Chemistry (GAC).[30] The metric is divided into 12 criteria scored from 0 to 1, with those closer to 1 being greener. The criteria presented in this metric consider process characteristics such as material requirements (quality and quantity), waste generation, energy consumption, analyst safety, and the general approach of the analytical procedure, such as the number of pretreatment steps and location of the analytical device concerning the object of investigation.[30] The pictograms resulting from the metric are shown in Figure 5, and the scores for each draw are presented in Table S5 of the Supporting Information

It was observed that the proposed method received the highest score (AGREE 0.9) and, consequently, was considered the most environmentally friendly analytical approach among those evaluated. One of the reasons that the method achieves a higher score is the type of coupling used. According to the metric, for an analytical method to be considered greener, it should be applied directly to avoid sample treatment (criterion 1), measurements should be performed in situ (criterion 2), there should be integration of processes and operations for energy and solvent savings (criterion 4), and it should be automated (criterion 5).[30] Thus, the inline coupling can fulfill all these criteria and, consequently, is awarded the maximum score. Additionally, it can be noted that the online coupling

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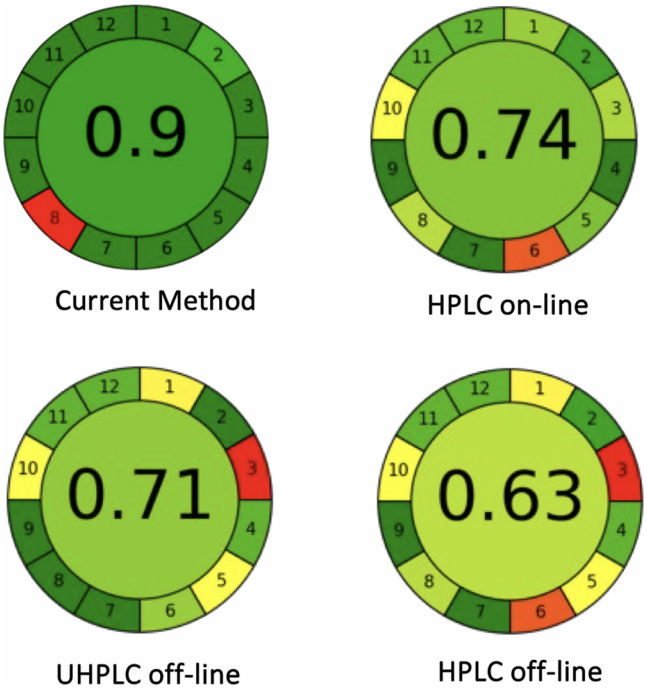

Figure 5. GREEnness scores of different analysis methods (current method; HPLC online method;[28] UHPLC offline method;[8] and HPLC offline[29]).

meets some of these criteria, while the offline analyses do not, resulting in lower scores for all of these criteria.

Another reason the current PLE−PDA process is considered the most environmentally friendly is that other evaluated methods employed highly volatile and toxic solvents as mobile phases, which can have detrimental effects on both the environment and the health of those who handle them. In the proposed method here, the mobile phase is derived from the extraction (the same extraction solvent container is used for analytical purposes), which is consequently composed of a propolis extract generated using 100% ethanol. Thus, the use of ethanol instead of acetonitrile (ACN) or methanol (MeOH), employed in other methods, led to significant improvements in sustainability and safety, as evidenced by high scores in criteria 10, which emphasizes the importance of using reagents obtained from renewable sources; criterion 11, which promotes the elimination or substitution of toxic reagents (once ethanol has a lower toxicity profile than ACN and MeOH); and criterion 12, which highlights the importance of prioritizing operator safety, in which ethanol could also be a suitable candidate.[44,45]

However, it is important to note that the method proposed here was inferior to all others in criterion 8, which advocates for multianalyte or multiparameter methods being preferred over methods that analyze one analyte at a time. This outcome was expected and occurred precisely because PDA was used to monitor the extraction process in real time rather than for the characterization of compounds, as in chromatographic methods. Despite this limitation, as assessed earlier, the different wavelengths generated by PDA indicate the class of compounds being extracted. In addition, when nonseparation between compounds is required, safeguarding the chromatographic column life span is highly welcome. Moreover, recognizing that the primary benefits of utilizing propolis bioactive compounds stem from the synergism among the components in the extract, we do not recommend separating these compounds into different fractions. In this context, chromatographic separations are deemed unnecessary. Expanding on the issue of chromatographic methods, an important finding presented in Figure 5 was that the UHPLC offline chromatographic method scored higher than the HPLC offline method. This can be explained by the fact that the UHPLC method is faster and consumes less energy and solvent, earning higher scores in these criteria (criteria 6 and 8).

Considering the presented data, it was evident that the method proposed here (PLE−PDA) demonstrated a low environmental impact, making it a viable alternative for process monitoring. Furthermore, the findings also reinforce the idea that the inline coupling of techniques, combined with a rapid detection method like UHPLC and the use of nontoxic mobile phases, represents the best option for a greener analysis. For propolis, these modern analyses remain unexplored and must be encouraged by the scientific community.

GREEnness Assessment by the Path2Green Metric. The sustainability of the proposed extraction process was evaluated using the *Path2Green* metric, which is based on 12 principles of green extraction processes. This metric provides a comprehensive analysis of the environmental impact of the current PLE−PDA platform. The result, calculated using the *Path2Green* mobile app, is illustrated in Figure 6, and the final

Figure 6. Pictogram illustrating the final score of the *Path2Green* metric, which is based on the 12 principles of green extraction. The principles are depicted around the pictogram in varying shades of green, yellow, and red: green represents high adherence to the principle, yellow indicates a neutral score, and red signifies poor adherence.

scores for each principle are detailed in Table S6 of the Supporting Information. Each principle is scored on a scale from −1 to 1, with scores closer to 1 indicating better adherence to the green principles. The criteria in this metric encompass attributes from both pre- and postextraction procedures, providing a comprehensive assessment of the process's sustainability.

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The method achieved a *Path2Green* score of 0.350, indicating an excellent pathway toward sustainability and representing a carbon footprint of around 343 $g_{CO2}{\cdot}g_{biomass}^{-1}$. This high score can be attributed to the high scores obtained for several principles, specifically principles 1, 2, 4, 5, 6, 8, and 10. For principle 2 (transport: preserving biomass integrity while minimizing transportation's environmental impact), the propolis was sourced from the same city where the extraction process was conducted. This local sourcing strategy effectively minimizes the environmental impact associated with transportation, aligning with recommendations to enhance resource self-sufficiency and reduce dependence on external resources. By minimizing transportation distances, the process not only reduces carbon emissions but also supports local economies and ensures the freshness and integrity of the biomass used in the extraction. The green propolis was cultivated by small local producers, which aligns with principle 1 (biomass: select biomass that is naturally sourced or requires minimal resource usage for production). Although this did not yield a maximum score due to the scale of cultivation, it emphasizes eco-conscious practices and environmentally friendly approaches. Principle 3 (pretreatment: optimization for pretreatment avoidance and cost-effective techniques) received a score of −0.20 due to the standardization of the propolis biomass before extraction. Specifically, applying physical pretreatment to standardize particle size ensures good results and reproducibility. It is important to note that physical pretreatments are more environmentally friendly compared to biological and chemical methods. Additionally, they are more cost-effective because they typically require less energy and fewer resources, thus aligning with sustainable extraction practices. Additionally, principle 4 (solvents: minimize solvent usage, prioritizing those of biological origin, biodegradable, and nontoxic) received high scores due to the absence of sample preparation steps and the use of ethanol as the solvent. Ethanol, being biodegradable and relatively nontoxic, aligns well with the principle's focus on minimizing environmental impact and promoting safer, more sustainable extraction practices. Principles 6 (purification: final application dictates the extent of purification) and 8 (post-treatment: functionalization of natural products postextraction to maximize their benefits) received high scores because the extract is ready to use and contains no toxic solvents or degradation byproducts. This approach ensures that the extract meets the requirements for its intended application without additional purification or functionalization steps, thereby enhancing both its effectiveness and safety. This aligns with principle 10 (application: ensure safety for applications in several domains), as the safe extract can be utilized in a variety of products, including cosmetics, pharmaceuticals, skincare, cleaning, and hygiene items. This versatility not only ensures that the extract meets safety standards across different applications but also provides potential economic returns and investment opportunities due to its broad applicability and market potential. Principle 5 (scaling: ensure reproducibility and a continuous extraction flow) also received a positive score (+0.50) due to the use of inline coupling. This scalable technique involves minimal steps and operates within a semi-continuous flow framework with automated systems, ensuring reproducibility and efficient scaling of the extraction process. This minimizes process interruptions, enhances predictability and productivity, and achieves high production in a short time frame. High-pressure extraction guarantees a high extraction yield, earning a high score (+0.50) for principle 7 (yield: maximize the utilization and valorization of the biomass). However, the high energy demand characteristic of modern extraction techniques led to a penalty for principle 9 (energy: prioritize using clean energy sources and high-efficiency extraction techniques), resulting in a score of −0.50. Despite using renewable energy sources, the significant energy required to operate the pressurized system and integrate it with analytical tools has an impact. While this is acceptable, there is room for improvement in enhancing energy efficiency and reducing overall consumption to better align with sustainability goals. Challenges were noted for principles 11 (repurposing: trace strategies to perform closed-loop extraction systems, preferably using nonvirgin materials) and 12 (waste management: refine waste reduction and ensure effective waste management), where maximum scores were not achieved due to waste generation. This challenge is not unique to the proposed method, as waste generation is often inevitable in extraction processes despite efforts to minimize it. Overall, the Path2Green metric provided valuable insights into the sustainability of the method, highlighting its strengths and areas for improvement in achieving greener extraction processes.

Method Practicality Assessment by the Blue Applicability Grade Index (BAGI). To complement the sustainability analysis, the practicality and applicability of the PLE−PDA process were evaluated using the Blue Applicability Grade Index (BAGI).[31] This parameter evaluation is essential in a laboratory routine. When carrying out the BAGI analysis, the real applicability of the developed method can be effectively discussed. The pictogram of the BAGI result (calculated via the web application) is presented in Figure 7,

Figure 7. Pictogram illustrating the final score of the BAGI metric based on 10 principles. Four discrete scores of equal weights are used in the assessment: 10, 7.5, 5.0, and 2.5 points correspond to dark blue (no. 0c305b), blue (no. 3a89c1), light blue (no. adcffd), and white (#FFFFFF), respectively. The final score is the sum of the 10-principle results.

with detailed results for each principle provided in Table S7 of the Supporting Information. In this metric, 10 principles can be scored in four scores of equal weights, 10, 7.5, 5.0, and 2.5, which correspond to colors dark blue (#0c305b), blue (#3a89c1), light blue (#adcffd), and white (#FFFFFF), respectively. The final score is the sum of the 10 principles results; therefore, the closer to 100, the better overall performance.

As illustrated in the center of the pictogram (Figure 7), the PLE−PDA process achieved a score of 85. It is considered an excellent result as are those recommended to the methods that achieve at least 60 points.[31] On the inside of the pictogram, it is possible to observe the colors corresponding to principles 1 to 5. Among these, principles 1 to 3 correspond to the analytical determination steps, while principles 4 and 5 relate

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to the sample preparation steps. Regarding analytical determination, all three principles (1: type of analysis; 2: number of analytes that are simultaneously determined, and 3: analytical technique-instrumentation) did not reach the maximum score.

Principle 1 was scored in 7.5 because the PLE−PDA method is quantitative and not both quantitative and confirmatory, which would give the maximum score of 10 for this principle. Principle 2 scored 2.5 since the analytical method developed here does not allow for the identification of isolated compounds, being instead classified as a single-element analysis (based on a monitoring curve). The maximum score for principle 2 is achieved when a multielement analysis is performed, involving more than 15 compounds simultaneously. The penalties observed in these principles were expected since the PDA was employed to monitor the extraction process in real time rather than to characterize compounds. However, as also highlighted in the AGREE method evaluation section, particularly in criterion 8, the various wavelengths generated by the PDA provide valuable insights into the classes of compounds being extracted. Furthermore, the absence of compound separation contributes to extending the chromatographic column's lifespan, which is a critical attribute for any analytical method. Regarding principle 3, the score of 7.5 was due to the type of instrumentation used. The maximum score refers to portable instrumentation (e.g., smartphone-based detectors, portable GC).

Despite these disadvantages, the principles covering the sample preparation steps (4: number of samples that can be treated simultaneously and 5: the sample preparation scale) obtained maximum scores (10). These scores are due to the absence of a sample preparation step, as PDA detection is performed in line after PLE. These positive results are particularly significant in a laboratory routine, as the sample preparation step is often the most tedious and labor-intensive part of the analytical process.[31] Furthermore, the absence of this step eliminates energy consumption and waste generation, significantly reducing the environmental impact of the analytical method.[18]

On the outer section of the pictogram (Figure 7), it is possible to observe the colors associated with principles 6 to 10. This principle simultaneously addresses aspects of analytical determination and sample preparation. For these principles, the only one that did not achieve the maximum score was principle 6: number of samples that can be analyzed per hour. This principle received a score of 7.5, which corresponds to analytical methods capable of analyzing 5−10 samples per hour. A maximum score would only be achieved if the method could analyze more than 10 samples per hour. It is important to note that the analysis time of the PDA varies depending on the wavelength used, as shown in Figures S4 and S6. Therefore, the number of samples to be analyzed per hour is a variable parameter, even within the same sample.

For the other principles (7: type of reagents and materials used in the analytical method; 8: requirement for preconcentration; 9: automation degreep; and 10: amount of sample), the maximum scores are attributed due to the use of a common commercially available reagent (propolis ethanolic extract) in the PDA analytical procedure; no preconcentration was required to meet the necessary sensitivity or regulatory criteria, and fully automated methods (the PLE−PDA *inline* system does not require manual injection) and low sample quantities were required for bioanalytical samples, specifically <10 g of raw propolis.

With the positive result of this analysis, it became evident that the method developed here is not only applicable in laboratory environments but also offers significant advantages in terms of sustainability, practicality, and scalability. The absence of complex sample preparation steps, the ability to perform real-time analysis, and the reduction in reagent and energy consumption make this method a viable alternative for routine laboratory implementation. Furthermore, its ability to be easily scaled for different sample volumes and the minimization of generated waste enhance its potential for large-scale applications.

CONCLUSIONS

The pressurized liquid extraction (PLE) coupled with a photodiode array detection (PDA) system has demonstrated significant advantages in extraction and real-time monitoring, particularly with complex samples such as green propolis. This system allows for the precise determination of extraction kinetics and real-time tracking of the full valorization process of green propolis. It offers exceptional control over pressure, solvent flow, and temperature, making it a more environmentally friendly and efficient method compared with offline approaches, which often involve complex procedures, higher solvent use, and less precise control. The PLE−PDA approach mitigates potential errors and enhances the overall process efficiency and sustainability. Our results also indicate high reproducibility between analyses, and the PDA detector's capability to read at different wavelengths provides valuable real-time insights into the components being recovered. Furthermore, the inline method is a more sustainable alternative to offline protocols, which typically require more time, solvent, and energy. The innovative solutions presented here pave the way for developing advanced extraction and analysis methods. We encourage further research to validate this method for a wider range of propolis samples and other natural products.

ASSOCIATED CONTENT

Data Availability Statement

The data supporting this article have been included as part of the Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.4c08602.

3D representation apparatus PLE−PDA; chromatogram recovery of different conditions; PDA real-time analysis and calibration curves and at different wavelengths integrating the peaks areas; calibration curve equation; compounds extracted by water and ethanol at 290 nm by PLE; and different analysis methods reported in the literature for propolis and assessment by AGREE, *Path2Green*, and Blue Applicability metrics (PDF)

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