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# Simultaneous extraction, separation, and analysis of 5-caffeoylquinic acid and caffeine from coffee co-product by PLE-SPE × HPLC-PDA two-dimensional system

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ABSTRACT

This study proposed an integrated and automated procedure to extract, separate, and quantify bioactive compounds from a coffee co-product by pressurized liquid extraction (PLE) coupled inline with solid phase extraction (SPE) and online with HPLC-PDA (PLE-SPE × HPLC-PDA). The efficiency of the two-dimensional system in performing real-time analysis was verified by comparing HPLC-PDA results acquired by the system (online) and carried out after the extract fraction collection (offline). Different flow rates (1.5 mL/min for 336 min, 2 mL/min for 246.4 min, and 2.5 mL/min for 201.6 min) were evaluated to optimize the extraction, separation, and analysis method by PLE–SPE × HPLC-PDA. Subcritical water at 125 °C and 15 min of static time allowed the highest extraction yields of caffeine and 5-caffeoylquinic acid (5-CQA). Caffeine was retained during the aqueous extraction in the SPE adsorbent and eluted from the column by exchanging the solvent for a hydroethanolic mixture. Thus, caffeine was separated from 5-CQA and other phenolic compounds, producing extracts with different compositions. The solvent flow rate did not have a significant effect (p-value $\geq$ 0.05) on the extraction, separation, and analysis (by online and offline methods) of 5-CQA. However, the online quantification of retained compounds in the SPE (i.e., caffeine) can underestimate concentration compared to offline analysis. Nevertheless, the results suggest that coupling of advanced techniques can be used to efficiently extract, separate, and analyze fractions of phenolic compounds, supplying an integrated method to produce high-added value ingredients for several applications.

## 1. Introduction

Coffee fruit is grown in over sixty countries and is one of the world's most relevant crops (Pham, Reardon-Smith, Mushtaq, & Cockfield, 2019). The post-harvesting processes of coffee cherries involve sequential treatments to remove their skin, pulp, mucilage, parchment, and silverskin. These processes have been performed by dry, wet, semi-dry, and mechanical methods to produce raw coffee beans (Alves, Rodrigues, Antónia Nunes, Vinha, & Oliveira, 2017). The dry process is the most employed in Brazil, Ethiopia, and Haiti. This method consists of drying and dehusking coffee cherries. Thus, producing husk (a mixture of coffee skin, pulp, and parchment) as a co-product. Additionally, defective beans, silverskin, and spent coffee are generated during the coffee processing chain and from the roasting and drink preparation stages.

Coffee co-products have been widely studied as they are produced in large quantities and are rich sources of bioactive compounds, such as caffeine and polyphenolic compounds (Nzekoue et al., 2020). Caffeine, an alkaloid, is one of the main compounds in coffee and is a stimulator of the human body's central nervous system, keeping one awake and energetic (Wallace, 2017). Moreover, caffeine can be used in pain medications and to treat apnea in premature infants, among other potential applications (Van Dam, Hu, & Willett, 2020). On the other hand, coffee also contains high amounts of 5-caffeoylquinic acid (5-CQA), which is the compound most commonly found in extracts obtained from coffee co-products (Lu, Tian, Cui, Liu, & Ma, 2020; Wen, Zhang, Rai, Sun, & Tiwari, 2019). This compound has been linked to beneficial effects on human health, such as reducing the risk of mental illness and regulating glucose and lipid metabolism (Lu et al., 2020). 5-CQA or chlorogenic acid exhibits two isomeric forms also found in coffee extracts: the 3-CQA and 4-CQA, belonging to the group of hydroxycinnamates (Clifford & Wight, 1976).

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The application of phenolic compounds acquired from coffee co-products in the food and pharmaceutical industries has been reviewed by Bondam, Diolinda da Silveira, Pozzada dos Santos, and Hoffmann (2022). They suggest using these compounds to extend the shelf life of food products and as additives or ingredients with antioxidant and antimicrobial activity. On the contrary, in the pharmaceutical industry, the compounds extracted from coffee co-products can be used as food supplements and ingredients in cosmetic products since they present anti-inflammatory, neuroprotective, antimicrobial, and anticancer effects. Cañas et al. (2022), evaluating the gastrointestinal behavior of the coffee pulp phenolic compounds under simulated conditions, observed an increase in the antioxidant capacity throughout the digestive process. Phenolic acids and caffeine were highly bioaccessible during the digestive process, while flavonoids were mainly degraded. According to the authors, non-absorbed phenolic compounds might undergo colonic biotransformation yielding small and potentially more adsorbable phenolic metabolites. Therefore, the phytochemical compounds extracted from coffee co-products have demonstrated potential applications in the food and pharmaceutical industries, demonstrating high bioaccessibility after digestion.

Different technologies and solvents have been employed to extract and isolate these compounds from the coffee co-products. Studies have demonstrated the efficiency of techniques such as Soxhlet, heat-assisted aqueous extraction, heat-assisted magnetic stirring extraction, maceration, high-intensity ultrasound, and pressurized liquid extraction to obtain the compounds from coffee co-products. Typical solvents include water, ethanol, methanol, and mixtures (Aguilera, Rebollo-Hernanz, Cañas, Taladrid, & Martín-Cabrejas, 2019; Nguyen et al., 2019; Nzekoue et al., 2020; Puga, Alves, Costa, Vinha, & Oliveira, 2017; Rebollo-Hernanz et al., 2021; Shang, Xu, Lee, & Um, 2017). High-intensity ultrasound extraction (HIUS) and pressurized liquid extraction (PLE) have stood out for allowing higher yields using hydroalcoholic solvents ( Alves et al., 2017; Shang et al., 2017). However, most extraction studies have expressed the results in total phenolic content (TPC), while few studies assessed all extraction conditions by more robust methods using chromatography. HIUS allows shorter processes than PLE, but PLE enables the use of subcritical water (at temperatures and pressures below the critical points of 374 °C and 22.1 MPa, respectively). These conditions favor the extraction of less polar compounds (Zarzycki & Gilbert, 2020). Additionally, PLE technology enables dynamic processes, while HIUS has been studied in batch processes. Furthermore, PLE also allows combination with other techniques, including HIUS (Dias, de Aguiar, & Rostagno, 2021).

However, extracts produced with these techniques contain several compounds and present low purity, limiting their direct application. After extraction, compounds can be separated and purified to obtain an ingredient for food and pharmacological applications. Solid phase extraction (SPE) has been studied after extraction to separate and concentrate phenolic compounds due to its selectivity in the concentration and elution steps (Rodríguez, Llompart, & Cela, 2000). Thus, the target compound can be isolated in a fraction using an adsorbent compatible with the eluent and the analytical system.

One way to accelerate the obtaining of purified extracts is to perform the inline separation step after extraction. There are reports of the extraction and separation of compounds from mate herb and apple pomace by PLE coupled inline with SPE (Da Silva et al., 2020; Souza et al., 2021). The coupling of PLE with SPE allows the separation of compounds during the extraction process. It also increases the recovery of more hydrophobic polyphenols, such as flavonoids and dihydrochalcones, and eliminates the need for filtration or centrifugation of the extracts. Unfortunately, there are no reports of PLE-SPE for coffee and co-products.

On the other hand, the analysis of the extracts is conventionally carried out after extraction/separation/purification processes (i.e., offline). High-performance liquid chromatography (HPLC) associated with photo-diode array (PDA) and mass spectrometry (MS) detectors (HPLC-PDA-MS) is the method of choice due to its excellent performance in obtaining information about individual compounds and allowing their identification and quantitation (Sanches et al., 2022). Online coupling of HPLC-PDA with PLE-SPE has been reported for mate leaves, semi-defatted açai, and apple pomace, providing several advantages, such as real-time monitoring of the process and potential for automation and artificial intelligence method development (Da Silva et al., 2023; Maciel-Silva et al., 2022; Viganó et al., 2021). However, these studies did not compare HPLC-PDA online and offline analyses. Da Silva et al., 2023 reported that they performed online analyses during their extraction but did not compare the results acquired by the online method with offline ones. This comparison is essential since a small aliquot of a fraction is analyzed in the online HPLC-PDA analysis and may overestimate or underestimate the results if the online method is not well adjusted. In this sense, studies comparing HPLC-PDA in integrated systems by online and offline methods need to be further studied to obtain quantitative information during the process and not after (i.e., offline), allowing dynamic control of the process while saving time in decision-making.

Therefore, this study aimed to propose an integrated and automated procedure to extract, separate, and analyze 5-CQA and caffeine from a coffee co-product by pressurized liquid extraction (PLE) coupled inline with solid phase extraction (SPE) and online with HPLC-PDA. For this proposal, the HPLC-PDA analysis was performed by comparing online and offline responses at different flow rates (1.5, 2, and 2.5 mL/min) applied for 336, 246.4, and 201.6 min, respectively.

## 2. Material and methods

### 2.1. Coffee co-product preparation

A real coffee co-product (a mixture of approximately 60:40 (w/w) Arabic coffee husk: green Arabic coffee beans) was donated by Sítio do Belo do Biriça, Bragança, SP, Brazil, presenting 15.3 ± 0.3% moisture, was dried at 60 °C for 24 h and ground. After that, the moisture was again measured by gravimetric method (Bradley, 2010), and the Sauter mean diameter was determined using sieves from 16 to 80 mesh (WS Tyler, Wheeling, USA). Thus, the raw material presented 3.8 ± 0.1% and 0.44 ± 0.01 mm of moisture and mean Sauter diameter, respectively. The raw material was stored under refrigeration at 7 °C for further assays. The following operational procedures are presented in the flux diagram in Fig. 1.

### 2.2. PLE optimization

The PLE was carried out in the integrated system PLE inline with SPE and online with HPLC-PDA (PLE-SPE × HPLC), as described in detail in Figs. 2 and S1, according to Viganó et al. (2021). The PLE process was optimized by evaluating the static time (5, 10, and 15 min) and temperature (50, 87.5, and 125 °C) on caffeine and 5-CQA extraction from coffee co-product. These variables were chosen considering previous studies performed by the research group. Moreover, Shang et al. (2017) also observed that temperature and sample loading weight, among other variables, such as flow rate and pressure, significantly affected the extraction of phenolic compounds from spent coffee. The sample mass (0.2 g) was chosen through preliminary tests, noting that when using the online system, the highest concentration fraction obtained during extraction would have to be within the LC standard curve without dilution (the details of this step were presented in Section 2.6). The remaining space of the stainless-steel extraction cell (111.5 × 20 mm, Citua, Campinas, SP, Brazil) was filled with approximately 27 g of glass beads. The cell was coupled into the system and pressurized until 150 MPa with water at a 5 mL/min flow rate. After the pressurization, the heating was initiated at the extraction temperature (50, 87.5, or 125 °C). The temperature profiles of the PLE oven were measured to standardize the heating time to achieve each temperature (50, 87.5, and 125 °C) of extraction. Fig. 3 presents the heating profiles. The initial PLE oven

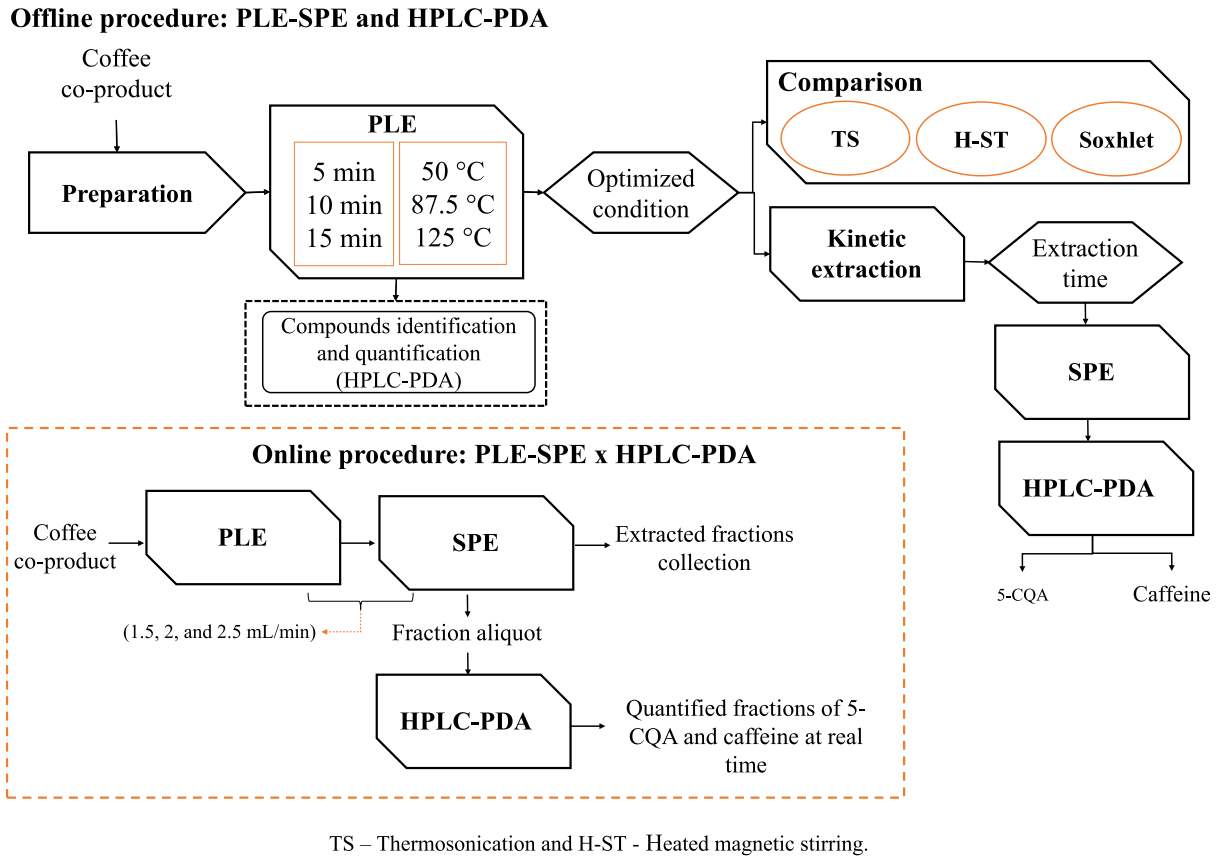



**Fig. 1.** Flow diagram of the processes for obtaining 5-caffeoylquinic acid (5-CQA) and caffeine from coffee co-product.

temperature was room temperature (25 ± 5 °C). The heating time started when the oven was turned on after the pressurization of the system. Thus, the system reached 50, 87.5 °C, and 125 °C after heating for 5, 10, and 15 min, respectively. These heating times were standardized for PLE extractions.

The static time (5, 10, or 15 min) started after the system reached the temperature. The extraction was performed with water at 2 mL/min using a solvent/feed ratio (S/F) of 50 mL/g. The extraction pressure remained at 10 ± 1 MPa. Thus, the extract was collected for 5 min and stored under refrigeration (7 °C) for further analysis. This step was carried out using a central composite design (CCD), including three central points, totaling eleven assays, as presented in supporting information Table S1. The 5-CQA and caffeine content responses determined the best condition.

### 2.3. Caffeine and 5-caffeoylquinic acid identification and quantification

A method using high-performance liquid chromatography on a 2695D separation module (Waters Alliance, Milford, USA) equipped with a 2998 diode array detector (HPLC-PDA) was developed to identify and quantify the compounds present in coffee by-product extracts. The compounds were separated on a Kinetex C18 column (100 × 4.6 mm id, 2.6 µm, Phenomenex, Torrance, USA) at 50 °C using a flow rate of 1 mL/min. The mobile phase consisted of 0.1% acetic acid (v/v) in water (solvent A) and 0.1% acetic acid (v/v) in acetonitrile (solvent B). The following gradient was used: 0 min (95% A); 2 min (90% A); 2.5 min (85% A); 4 min (75% A); 5 min (55% A); 6 min (40%A); and 7 min (95% A) for 9 min. 2 min of delay was added between one injection and another in that the HPLC system returned the initial solvent composition for the analysis. Thus, the total time for each of the analyzes was 11 min. The caffeine and 5-CQA were detected at 270 and 325 nm, respectively. Compounds were identified by comparing their retention time and UV–vis spectra to their reference standard. 5-caffeoylquinic acid (titration ≥ 95%) and caffeine (purity ≥ 99%) standards were purchased from Sigma-Aldrich (Barueri, SP, Brazil).

Moreover, the confirmation of the compounds (5-caffeoylquinic acid and caffeine) was performed employing the same chromatographic method in an ultra-high performance liquid chromatography (UPLC) with a PDA and QDA detector (Waters Co., Milford, MA, USA). The detection of the compounds by mass spectrometry was performed using a positive cone voltage of 13 V and a capillary voltage of 1.0. A selective ion recording (SIR) method searching for the ionized molecular weight of 5-CQA (355 *m/z*) and caffeine (195 *m/z*) was employed. The compounds were quantified using Empower 3 software (Waters, Milford, USA) using a standard curve (5 to 200 ppm). The 5-CQA and caffeine content were determined according to Equation (1). The coffee *coffee co − product mass* was the mass of the raw material dried at 60 °C, presenting a moisture of 3.8 ± 0.1%, according to Section 2.1.

$$5-caffeoylquinic acid \Big/ Caffeine\ content = \frac{mass(\mu g)}{coffee\ co-product\ mass(g)} \quad (1)$$

### 2.4. PLE extraction kinetic curves

The best condition of static time and temperature were used for kinetic extraction. Eighteen fractions were collected using a water flow of 2 mL/min, totaling an extraction time of 150 min and an S/F of 1500 mL/g. The collection was performed at each 2 min of extraction for 20 min, at each 10 min from 20 to 60 min, then after 86, 112, 138, and 150 min of extraction. The quantification of caffeine and 5-CQA was carried out as described in 2.3 section.

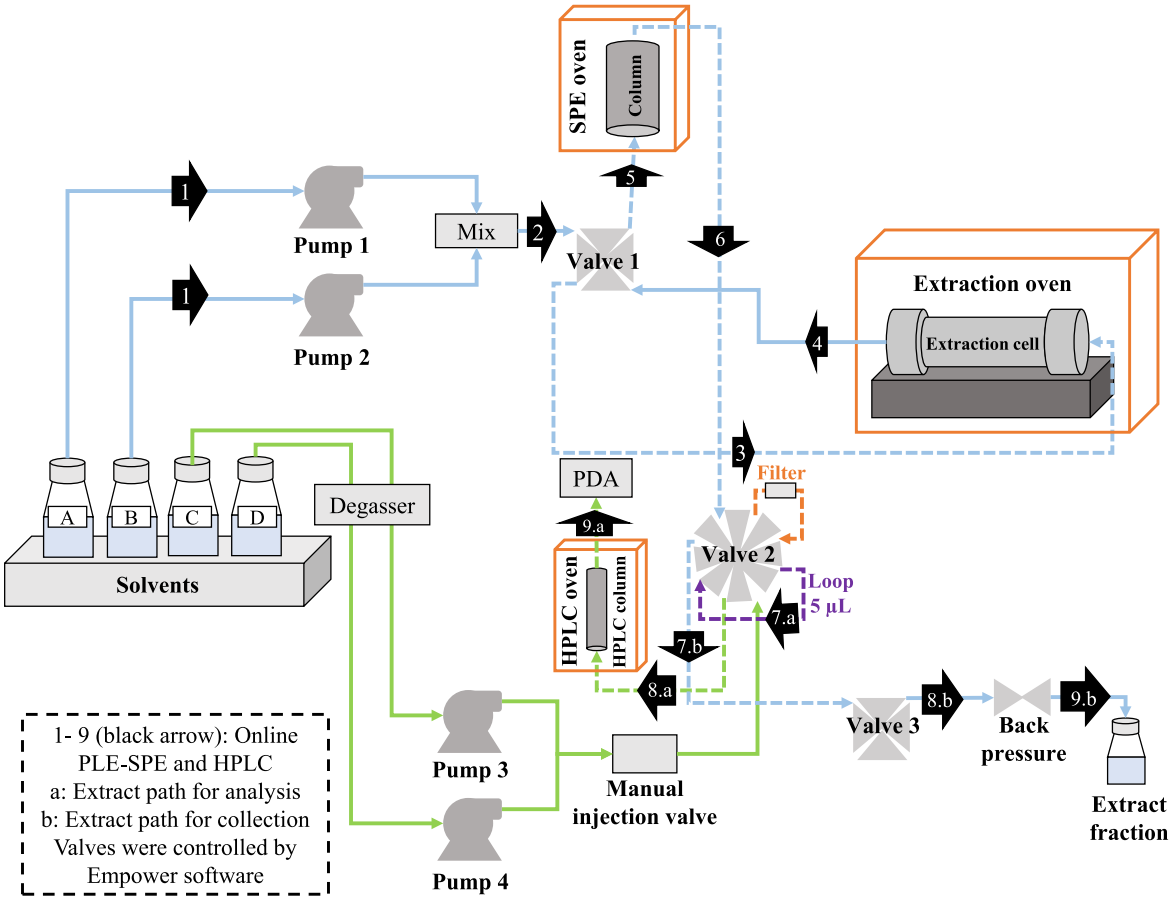



**Fig. 2.** Diagram of the 2D PLE-SPE × HPLC-PDA system configuration. Blue line: Path from extraction to collection; Green line: Path of chromatography solvents and extract to be analyzed.

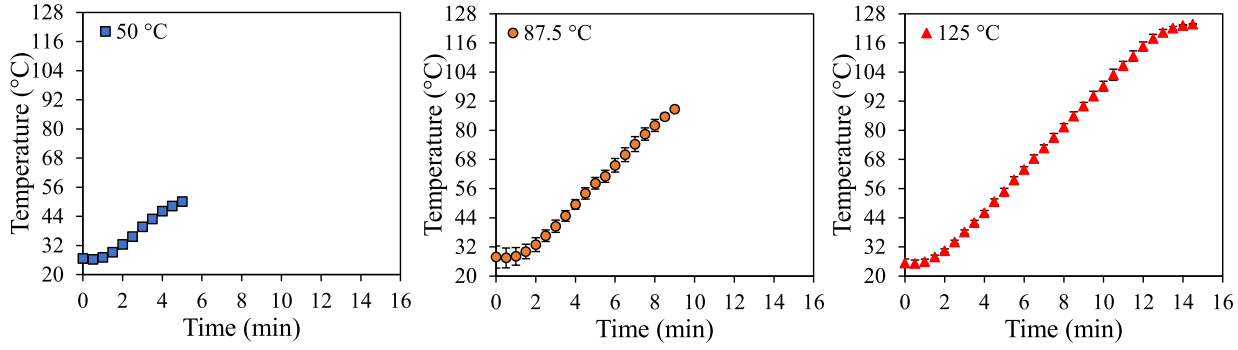



**Fig. 3.** Heating profiles of the PLE oven to achieve 50, 87.5, and 125 °C (n = 2).

### 2.5. Extraction techniques comparison

The extraction yield acquired by PLE extraction kinetic using optimized static time and temperature was compared with the ones obtained by Soxhlet, thermosonication, and heated magnetic stirring techniques at similar conditions. Each extraction was performed using 0.2 g of sample, S/F of 1500 mL/g, and water as solvent. The extraction time was 150 min for each extraction. Soxhlet extraction was performed by placing the raw material in a paper cartridge to be refluxed for 2 h and 30 min in a Soxhlet apparatus at water condensing temperature. The thermosonication was carried out in ultrasound bath type (Elmasonic P, Singen, Germany) at 37 kHz, 320 W, and 80 °C. C-MAG HS IKA® hot plate magnetic stirrer at 1500 rpm and 80 °C was employed to perform the heated magnetic stirring extraction. The extractions were performed in triplicate. The extract acquired by each technique was analyzed according to its caffeine and 5-CQA content (Section 2.3).

### 2.6. Online extraction, separation, and analysis of phenolic compounds

The online extraction, separation, and analysis of compounds from coffee co-product were performed using the integrated system PLE inline with solid phase extraction (SPE) and online with HPLC-PDA, as illustrated in Figs. 2 and S1 (Viganó et al., 2021). The SPE cell is in line with the PLE, as shown in Figs. 2 and S1 (real system). Thus, the solvent

passes through the PLE and the SPE cells using a valve connecting the two cells. First, the SPE was prepared by filling the column with 2 g of PoraPakTM Rxn RP Bulk adsorbent (Waters, Milford, USA). The remaining space of the SPE cell (50 × 4.6 mm) was filled with glass beads. Thus, the column activation and conditioning were performed by pumping 30 mL of ethanol and 30 mL of water at 3 mL $min^{-1}$. After adsorbent preparation, the extraction cell was prepared and coupled to the system, using 0.2 g of sample and glass beads, as mentioned in section 2.2. Thus, the extraction was initially performed using water, employing static time and temperature (125 °C and 15 min) that allowed the highest yields pre-established for PLE.

The S/F of 1500 mL/g evaluated in this step was chosen considering the total extraction of 5-CQA and caffeine observed in the results acquired through the kinetic extraction curve obtained by PLE at 125 °C and 15 min of static time (Fig. 6A). After extraction using water up to S/F of 1500 mL/g, the system started the second extraction stage by pumping ethanol:water (50:50 v/v) through the PLE-SPE cells to elute the compounds retained in the SPE. In this second stage, adding the solvent volume used in the first stage, the extraction reached an S/F of 2500 mL/g. This solvent volume was chosen through preliminary tests, aiming for total caffeine elution. During the extraction process, the system performed online automatic injections in the HPLC-PDA by transmitting a signal to valve 3 that allowed the passage of a small extract aliquot to a 5 μL loop (Fig. 2). Thus, the extract passed through the filter, the loop, and was then injected into the chromatographic column. The first injection was performed at 5.5 min of extraction. After the first injection, the equipment performed another injection every 11.2 min, considering the time of the HPLC-PDA quantification method (11 min) and the delay to save the result (0.2 min). The operation of the PLE-SPE × HPLC online system is carried out automatically. Before the samples' extraction, separation, and chromatographic analysis, the system was programmed concerning the solvent flows, extraction time, and chromatographic injection time using the Empower 3 software.

Different solvent flow rates (1.5, 2, and 2.5 mL/min) were studied at approximately the total S/F of 2500 mL/g to extract and separate the compounds. These different flow rates were evaluated to observe the system's performance in quantifying compounds by varying the extraction speed, considering that at higher flow rates, the extraction of compounds occurs faster, and fewer chromatographic analyses can be performed. The extracts fractions were also collected every 11.2 min to analyze them using the same HPLC-PDA method offline. Moreover, each fraction's volume was also measured for further quantification. The conditions employed for each flow rate and extraction time are presented in Table 1.

The calibration curve using 5-CQA and caffeine standards to quantify online was conducted by pumping a solution containing these standards in different concentrations (0.625, 1.25, 12.5, 50, 100, and 200 ppm) by the PLE-SPE × HPLC system. However, instead of the PLE and SPE columns, by-passes were coupled to allow the passage of standard solutions. Thus, using the same programming performed for extraction, the solution containing the standard was injected every 11.2 min.

After the flow rate study, another study employing the flow rate of 1 mL/min and a different proportion of the hydroethanolic mixture to elute caffeine from SPE was also carried out. Thus, the first step of the extraction was performed using water as solvent at the flow rate of 2.5 mL/min, and in the second step, ethanol:water (40:60 v/v) was used at 1 mL/min to elute the caffeine. The online and offline fractions of the second extraction step were evaluated. This step also verified the system's performance using different sample masses (0.2, 0.4, and 0.6 g) at the same amount of solvent and extraction time of the flow rate of 2.5/1 mL/min, according to Table 1.

### 2.7. Statistical analysis

The mean difference was verified by analysis of variance (ANOVA) using the Minitab 18® software with a 95% confidence level (p-value $\leq$ 0.05). Tukey's test of means was performed at a 95% confidence level (p-value $\leq$ 0.05). The DCC experimental design and the analysis to obtain the response surfaces were performed in Statistic 7.0 and Design Expert v. 13 software.

## 3. Results and discussion

### 3.1. Identification of compounds in the sample

The analysis of the extracts obtained from the coffee co-product revealed the predominance of 5-CQA and caffeine. A representative chromatogram of the extracts obtained by PLE and the comparison of the UV–Vis spectra of these compounds with authentic standards are presented in Fig. 4. Six well-defined peaks were observed in the chromatograms. The peaks 4 and 5 were identified as 5-CQA and caffeine, according to their mass spectrometry and the relation between the retention time and UV spectrum of standards. Fig. S2 of supplementary material presents the results confirming the identification of peaks 4 and 5 as 5-CQA and caffeine by mass spectrometry. The retention times of the compounds (5-CQA and caffeine) analyzed by UPLC-PDA and QDA (Fig. S2) were different from those previously observed by HPLC-PDA (Fig. 4). This difference may be associated with the injection of samples since the HPLC presents a manual injector while the UPLC has an automatic injector. However, the standards confirm the identification of the compounds analyzed by the different equipment. Moreover, Fig. S2-D demonstrated that more than one peak was identified with the ionized molecular mass of 5-CQA (355 $m/z$), indicating a 5-CQA isomer. However, the peak identified as 5-CQA had the same retention time and molecular mass as its standard.

Some studies report the co-elution of compounds extracted from coffee raw materials since this material presents structural isomers. For instance, 5-CQA, also known as chlorogenic acid, exhibits two isomeric forms: the 3-CQA and 4-CQA. Furthermore, coffee extracts contain dicaffeoylquinic and feruloylquinic acids (diCQAs and FQAs), each presenting three distinct isomers: the 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA for diCQAs, and the 3-FQA, 4-FQA, and 5-FQA for FQAs (Clifford & Wight, 1976). However, the study of Jeon et al. (2017) verified just two chromatographic co-elutions (4-FQA and 5-FQA) and (3-FCA and 4-CQA) of compounds extracted from coffee. They and other reported studies did not observe the co-elution of 5-CQA with its isomers. They also noted that several studies had demonstrated the detection of the 3-CQA in a lower retention time than the 5-CQA. In this sense, the

**Table 1**
Conditions employed to study the online extraction, separation, and analysis of 5-CQA and caffeine from coffee co-product.

| Flow rate (mL/min) | Aqueous extraction time (min) | Ethanol-water extraction time (min) | Total extraction time (min) | Total volume of water | Total volume of ethanol–water | Number of HPLC-PDA injections |
|---|---|---|---|---|---|---|
| 1.5 | 200 | From 201 to 336 | 336 | 300 | 504 | 29 |
| 2 | 150 | From 151 to 246.4 | 246.4 | 300 | 492.8 | 22 |
| 2.5 | 120 | From 121 to 201.6 | 201.6 | 300 | 504 | 18 |
| 2.5/1* | 120 | From 121 to 243.2 | 243.2 | 300 | 243.2 | 11 |

*The experiment was performed at two different flow rates: 2.5 mL/min to extract the 5-CQA fraction; and 1 mL/min to analyze the caffeine extraction in eleven fractions.

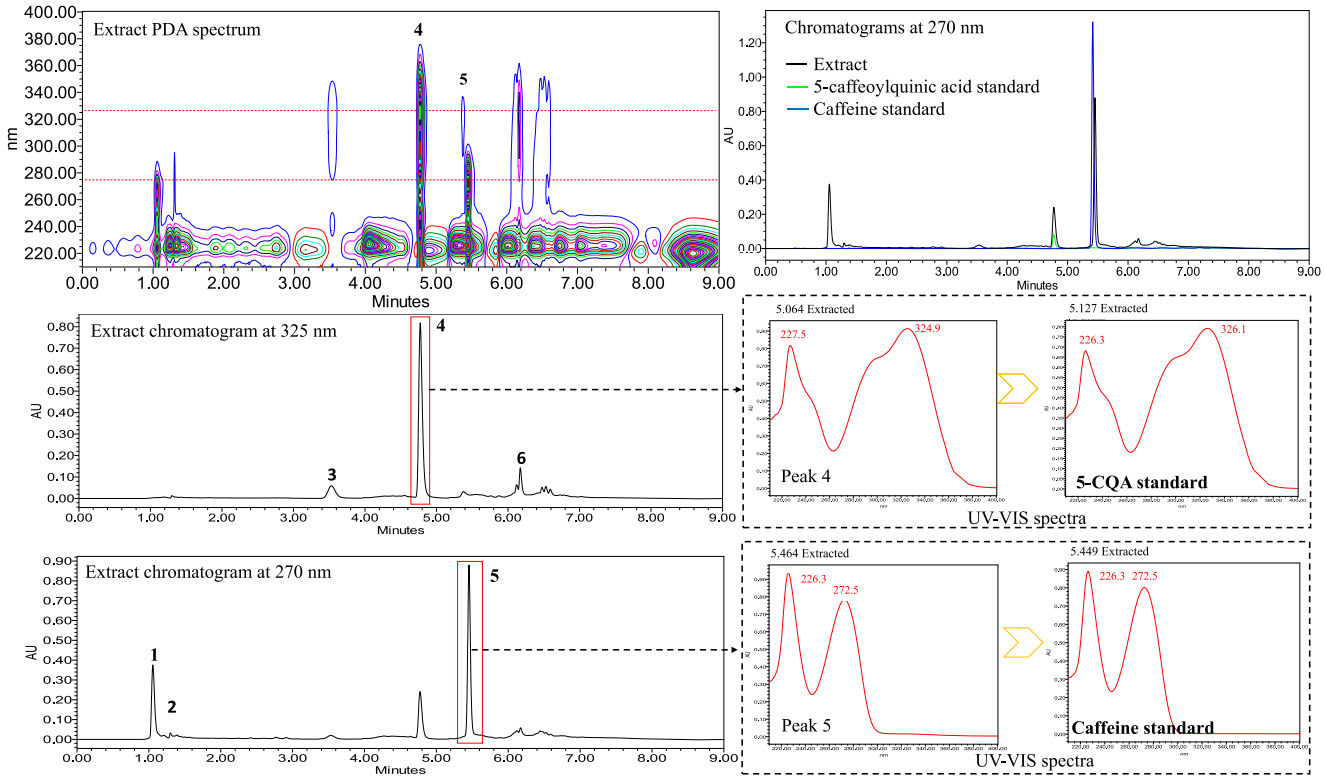



**Fig. 4.** 5-caffeoylquinic acid (5-CQA) and caffeine identification in PLE extract acquired at 125 °C with water at 2 mL/min from coffee co-product.

peak before that of 5-CQA observed in our chromatograms may refer to 3-CQA (Fig. S2-D). However, we do not have the pattern of this compound in our laboratory to confirm its identification. Direct comparisons between chromatographic methodologies employing distinct experimental conditions do not serve as conclusive evidence that the method developed in this study does not allow the co-elution of 5-CQA with its isomers. However, they indicate a consistent trend wherein liquid chromatography techniques have successful separated 5-CQA from its isomers across various methodologies. Despite the possibility of co-elution between 5-CQA and isomers, achieving these research objectives within the context of comparative analysis is possible. Moreover, in order to advance the development of online methods utilizing the two-dimensional system, it became imperative to establish an analysis method of superior speed compared to those previously developed and reported in the scientific literature. Furthermore, the purity plot presented in Fig. S3 and purity data acquired by PDA in Table S2 displays the purity angle (green line) below the purity threshold (blue line) across the entire peak. Thus, demonstrating that the peaks are spectrally homogenous based on the PDA data. Nonetheless, isomers share strikingly similar UV–Vis and MS characteristics, posing a significant challenge in distinguishing whether these compounds co-elute and requiring a specific analytical study.

Most studies that evaluated the extraction of phenolic compounds from coffee co-products expressed the yield results in total phenolic content (TPC) (Kieu Tran, Kirkman, Nguyen, & Van Vuong, 2020; Myo & Khat-udomkiri, 2022; Ribeiro, Luzia, & Jorge, 2019; Silva et al., 2021). However, Silva et al. (2021) and Kieu Tran et al. (2020) quantified the major phenolic compounds found in the coffee husk and pulp extracts, such as gallic acid, 5-CQA, caffeic acid, and caffeine. As observed in the chromatogram acquired in this study, Kieu Tran et al. (2020) also show the peaks representing 5-CQA and caffeine at close retention times. However, the extracts obtained in this study did not present gallic and caffeic acid, as found by Silva et al. (2021). These standards' spectra and retention times differed from the peaks observed in our extracts. This difference is probably associated with the coffee material characteristics since the solvents employed for the extraction were the same (water and ethanol). The raw material of Silva et al. (2021) was coffee husk from arabica and typica coffee from Taquaritinga do Norte, PE, Brazil, while the coffee co-product used in this study was a mixture of arabica husk and defective beans from the state of São Paulo. In this sense, raw materials grown in different regions and seasons from other species may have different compositions of phenolic compounds (Kaisangsri et al., 2020).

The online and offline standard curves acquired for 5-CQA were presented in Eqs. (2) and (3), respectively. Eqs. (4) and (5) showed the online and offline curves determined for caffeine standard.

$$Online\ 5-caffeoylquinic\ acid(ppm) = \frac{Area}{73048} - 279987 \quad (2)$$

$$Offline\ 5-caffeoylquinic\ acid(ppm) = \frac{Area}{43432} - 59575 \quad (3)$$

$$Online\ caffeine(ppm) = \frac{Area}{82846} - 346873 \quad (4)$$

$$Offline\ caffeine(ppm) = \frac{Area}{42237} - 198237 \quad (5)$$

The online quantification of the same standard solutions allowed higher areas than the offline one, as observed in Eqs. (2)–(5). This difference was associated with the dead volume between the valve ports, which probably is higher in the online path than for the offline one in valve 3 (Fig. 2).

### 3.2. Selection of PLE conditions and its kinetic for extracting 5-CQA and caffeine

Initially, the effects of static time (5, 10, and 15 min) and temperature (50, 87.5, and 125 °C) on 5-CQA and caffeine extraction by PLE

were evaluated. Through CCD, statistical models were generated to describe the extraction of caffeine and 5-CQA from coffee solid residues. Analysis of variance (ANOVA) of the experimental data was performed, and the significant effects of the process variables were used in the models. Eqs. (6) and (7) show the 5-CQA and caffeine concerning the extraction temperature (T) and the static time (St), respectively, in coded form. The models showed coefficients of determination ($R^2$) for the extraction of 5-CQA and caffeine of 0.88 and 0.86, respectively. Analysis of variance showed that both models were significant ($p < 0.05$).

$$5-caffeoylquinic\ acid\ extraction\left(\frac{\mu g}{g}\right) = 276 + 685(T)^2 + 454(St) \quad (6)$$

$$Caffeine\ extraction\left(\frac{\mu g}{g}\right) = 759 + 334(T) + 466(St) \quad (7)$$

The Fischer-F test ($F_{calculated}/F_{tabulated}$) applied to the models yielded $F_{calculated}$ values 10.5 for 5-CQA extraction and 12.6 for caffeine extraction. In contrast, the $F_{tabuleted}$ value was 5.79. This result suggests that both models were suitable for predicting the extraction of 5-CQA and caffeine from coffee solid residue, as a predictive model typically exhibits an $F_{calculated}$ value higher than the $F_{tabulated}$ value. Moreover, the optimal conditions were experimentally evaluated to verify the accuracy of the mathematical model. The predicted values of 1415 and 1559 μg/g were determined by Eqs. (6) and (7) using the coded best conditions (+1) for 5-CQA and caffeine, respectively. The experimental values acquired at the same conditions (15 min of static time and 125 °C) were 1712 ± 45 μg/g and 1917 ± 187 μg/g of 5-CQA and caffeine, respectively. Therefore, the model demonstrated predictive capability as the values obtained through experimental validation closely matched the predicted values.

Thus, the surface responses were generated and shown in Fig. 5. Table S1 of the Supplementary Material also presents the CCD design and numerical results obtained. An increase in 5-CQA and caffeine extraction yield was significantly affected by increasing the temperature from 50 to 125 °C (p-value < 0.05). However, the rise of temperature from 50 to 87.5 °C decreased the 5-CQA content in the extract at any static time, while the increase from 50 or 87.5 °C to 125 °C increased the content. The reduction of the 5-CQA content and then its increase according to the rise in temperature is probably associated with the waters' characteristics (extractor solvent) at each temperature. The extraction temperature increase from 50 to 87.5 °C may have caused the thermal degradation of the 5-CQA (Dawidowicz & Typek, 2010). Liu, Yang, Zhang, and Majetich (2010) also observed an increase in the

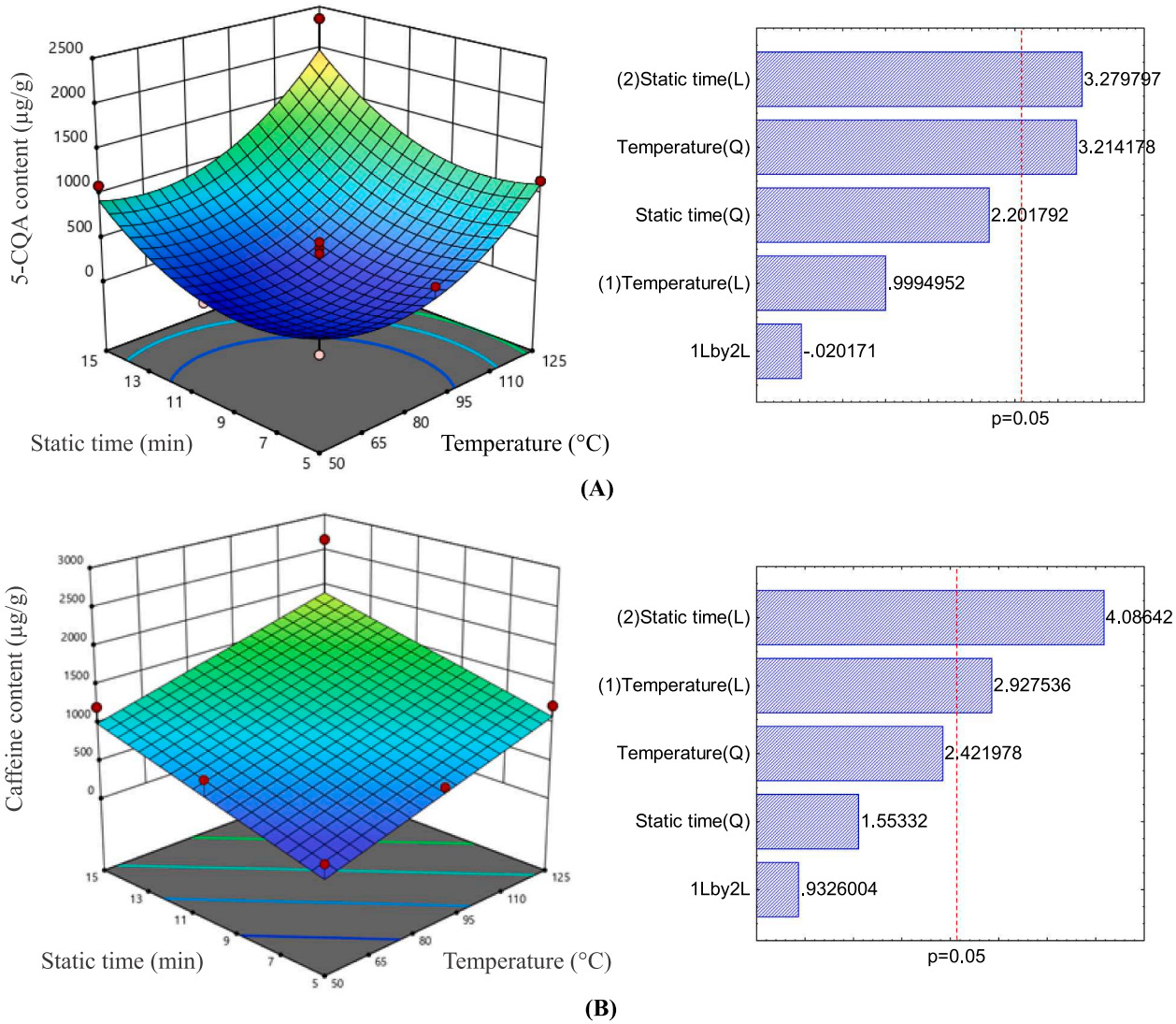



**Fig. 5.** Response surfaces and Pareto plots of static time and temperature effects on (A) 5-caffeoylquinic acid (5-CQA) and (B) caffeine content.

extraction of 5-CQA by the ultrasound-assisted extraction temperature rise from 20 to 50 °C and a reduction in the extraction by increasing the temperature to 60 °C. They attributed the lower 5-CQA content in the extract acquired at 60 °C to its thermal degradation. On the other hand, at 125 °C, the water is in a subcritical state. Thus, it may have promoted some thermal degradation of 5-CQA but also favored the extraction, allowing a higher yield of this compound. The dielectric constant of water is decreased at subcritical conditions. This lower water dielectric constant allows higher thermal vibration between the molecules, weakening hydrogen bonds, reducing its surface tension, and facilitating its penetration into the matrix (Costa, Strieder, Saldaña, Rostagno, & Forster-Carneiro, 2023). Thus, the water at subcritical conditions (125 °C), despite of the fact that it may promote some thermal degradation of 5-CQA, also has allowed the higher extraction of 5-CQA and caffeine due to its lower dielectric constant at this state.

Shang et al. (2017) also verified that PLE temperature was the parameter that most influenced the extraction of caffeine and phenolic compounds from spent coffee. They obtained higher extraction yields by increasing the PLE temperature from 80 to 195 °C using an ethanol/water mixture (70/30, v/v), 10 min of static time, 10 MPa, and 40% flush volume of the extraction cell. According to them, high temperatures favored the contact of the solvent with the caffeine and phenolic compounds by increasing the diffusion rate, mass transfer, and solubility and decreasing the surface tension and viscosity of the solvent. Although Shang et al. (2017) reported that they obtained better extraction results using 70% ethanol, they did not compare their results using pure water as a solvent. Moreover, water was chosen as the extractor solvent in this first step of the study, considering using a hydroethanolic mixture in the second step to separate caffeine.

The duration of static time had a notable impact on the extraction of 5-CQA and caffeine (Fig. 5). Longer static times resulted in an extended contact period between the solvent and solute, facilitating enhanced mass transfer. Moreover, the prolonged heating duration required to reach a temperature of 125 °C (Fig. 3) probably contributed to the improved extraction of caffeine and 5-CQA by extending the interaction time between the solvent and the sample. Therefore, the PLE conditions (15 min of static time and 125 °C) were selected for the next steps of this study since they allowed the highest extraction yields.

After selecting PLE static time and temperature, an extraction kinetic was performed at this condition using water at 2 mL/min. Fig. 6 presents the 5-CQA and caffeine extraction kinetic curves and their chromatograms. Typical kinetic curves can be observed in Fig. 6A, including the three extraction phases: constant extraction rate (CER), falling extraction rate (FER), and diffusional controlled (DC) (Palma, Barbeiro, Piñero, Liazid, Barroso, Rostagno, Prado, & Meireles, 2013; Prado & Rostagno, 2022). In the CER step, the compounds are extracted by convection at a constant rate since the solvent easily accesses the solute. This phase was observed until about 20 min of 5-CQA and caffeine extraction. In the FER phase, the extraction rate decreases fast due to lower mass transfer area and the start of diffusion. In this sense, in the FER step, both convection and diffusion extraction mechanisms act. Thus, the FER that represents the extraction time when the compounds started to be extracted by diffusion due to their interactions with raw materials was observed until 45 min for 5-CQA and caffeine. A low extraction stage characterizes the DC period since the extraction rate depends only on mass transfer by diffusion. Then, the DC is verified in the final curve representing the extraction by diffusion. The behavior of 5-CQA and caffeine extraction was similar.

Fig. 6B and C present the chromatograms acquired by each kinetic fraction. The 5-CQA was identified and quantified at 325 nm and caffeine at 270 nm. 5-CQA is more polar than caffeine, appearing on the chromatogram at a shorter retention time of 5.06 min than caffeine (5.46 min). Furthermore, other peaks were observed in the chromatograms obtained at 270 and 325 nm, indicating other compounds extracted from coffee co-product. However, this study aimed to quantitatively monitor the extraction kinetic of 5-CQA and caffeine at the temperature and static time that previously provided the highest yield. Thus, at the flow rate of 2 mL/min, we selected a time of 150 min to perform the extraction using subcritical water, aiming for the total extraction and quantification of compounds under the chosen conditions.

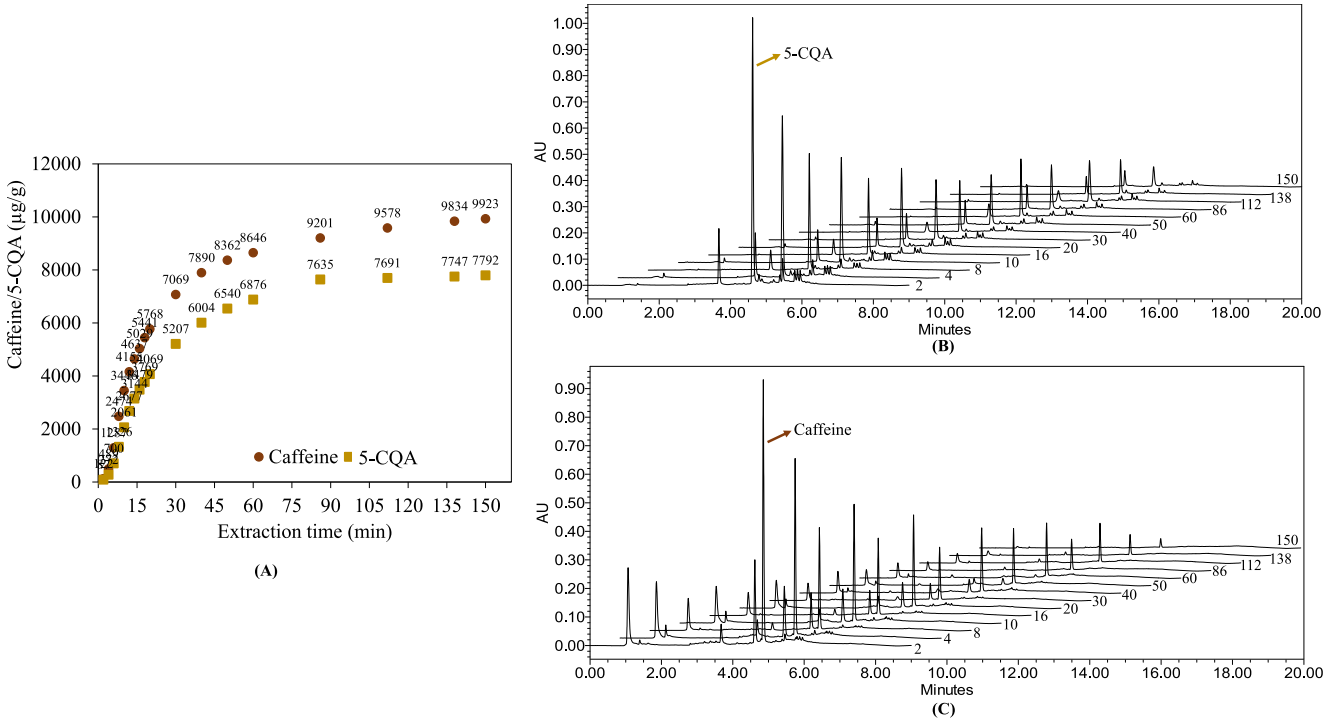



**Fig. 6.** PLE kinetic results acquired with water at 2 mL/min: (A) kinetic curves of 5-caffeoylquinic acid (5-CQA) and caffeine extracted from coffee co-product and chromatograms of fractions acquired (n = 2) (B) at 325 nm and (C) at 270 nm. The numbers next to the chromatograms refer to the extraction time (min).

### 3.3. PLE comparison with other extraction techniques

Table 2 presents the 5-CQA and caffeine content of extracts acquired by PLE, Soxhlet, thermosonication, and heated magnetic stirring. These techniques were compared because, as PLE, they employ more than one form of energy. Soxhlet uses percolation and heat, thermosonication applies acoustic energy and heat, and heated magnetic stirring uses magnetic stirring and heat.

Thermosonication and heated magnetic stirring extracted higher yields of 5-CQA than PLE and Soxhlet techniques. This result is probably due to the extraction temperature and amount of solvent in permanent contact with the sample. Thermosonication and magnetic stirring methods were performed at lower temperatures (80 °C) and in batches, remaining the total volume of solvent in touch with the raw material during all extraction times. On the contrary, PLE and Soxhlet were carried out at higher temperatures (≥100 °C) and are dynamic techniques in which fresh aliquots of solvent pass through the sample. The study performed by Upadhyay, Ramalakshmi, Rao, and L. (2012) demonstrated that increasing the extraction temperature from 70 to 90 °C decreased the 5-CQA content in extracts acquired from green coffee beans by microwave-assisted extraction. The same behavior was observed in this study by increasing the extraction temperature from 50 to 87.5 °C. Therefore, the higher temperatures achieved by PLE and Sohxlet extraction probably promoted the thermal degradation of 5-CQA.

Furthermore, 5-CQA is a polar compound, and the coffee co-products are probably a food matrix that requires higher volumes of water to enhance the penetration of the solvent in the plant cell matrix. Frosi, Montagna, Colombo, Milanese, and Papetti (2021) observed that water promoted higher 5-CQAs extraction from coffee husk than ethanolic solutions or 2% water acidified with citric acid. Moreover, the acoustic cavitation and the intense agitation may also have enhanced the extraction of 5-CQA by thermosonication and heated magnetic stirring, respectively. Acoustic cavitation disrupts the plant materials through physical forces developed during the process, improving the mass transfer and releasing extractable compounds in the solvent (Kumar, Srivastav, & Sharanagat, 2021). On the contrary, the intense stirring also may have increased the diffusion transfer, favoring the transfer of the compound from the solid coffee material to the solvent.

On the contrary, PLE using subcritical water allowed higher caffeine extraction from coffee co-product than the other techniques. The solubility of a compound in subcritical water is influenced by the chemical structure of the solute (degree of conjugation of the aromatic rings and the presence of different side groups around the hydrophobic organic compounds) and the complex interactions between the water and the solute. According to Ko, Nam, and Chung (2020), the greater the aromatic hydrocarbon rings, such as triterpene saponins, the higher the solubility at higher temperatures (220 °C) of subcritical water. Thus, the water at subcritical conditions probably favored the extraction of caffeine due to its structure by increasing mass transfer and the solubility of this compound. Furthermore, the higher temperature employed in PLE extraction probably enhanced the caffeine extraction by decreasing the water viscosity and surface tension and increasing the surface contact between solvent and raw material (Herrero, Sánchez-Camargo, Cifuentes, & Ibáñez, 2015). According to Mustafa and Turner (2011), these conditions allow the formation of cavities by the solvent efficiently, thus permitting non-thermolabile compounds to dissolve faster in the solvent.

**Table 2**
5-CQA and caffeine extraction content of the extracts acquired by PLE, Soxhlet, thermosonication, and stirring.

| Extraction technique | 5-CQA content (μg/g) | Caffeine content (μg/g) |
|---|---|---|
| PLE | 7064 ± 1024[b] | 9951 ± 330[a] |
| Soxhlet | 8788 ± 447[b] | 6207 ± 136[c] |
| Thermosonication | 11546 ± 87[a] | 8666 ± 87[b] |
| Heated magnetic stirring | 12609 ± 1076[a] | 8982 ± 555[b] |

Mean values ± standard deviation (n = 3). Values followed by different letters in the same column show differences by Tukey's test at 95% significance (p-value ≤ 0.05).

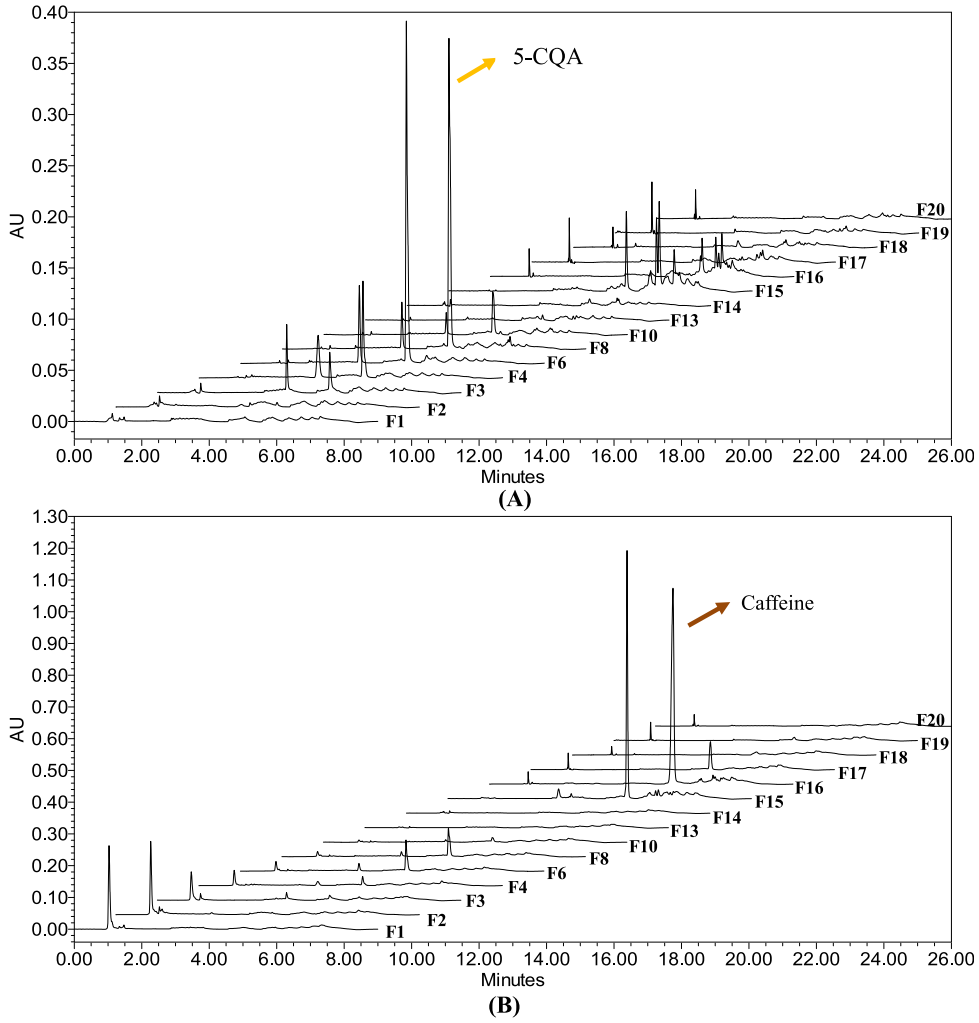
### 3.4. Inline PLE-SPE and online HPLC-PDA quantification of 5-CQA and caffeine

The SPE adsorbent retained caffeine during the aqueous extraction of the compounds from coffee co-products. Fig. 7 presents the chromatographic results acquired for coffee co-product extract fractions separated by inline SPE after PLE at 2 mL/min. No compound was identified in the first fractions (F1 and F2), probably because they were retained in the SPE adsorbent. However, 5-CQA, some compounds that absorb at a wavelength of 270 nm, and others that absorb at 325 nm were recovered from F3 to F8. Fractions from 6 to 8 were the ones that most extracted 5-CQA, but also extracted other compounds that adsorb at the wavelength of 325 nm (Fig. 7A). The extractor solvent was changed between the analyzes of fractions 13 and 14 after 150 min of extraction using water. Thus from 150 min, the system started pumping the hydroethanolic mixture. After exchanging the water for the hydroethanolic mixture, two higher peaks were identified in F15 and F16 as caffeine (Fig. 7B). Thus, caffeine was quickly eluted from the adsorbent after changing the solvent polarity. Therefore, the SPE separated caffeine from the other compounds in the extract by comparing the chromatograms obtained after PLE (Fig. 6) with those obtained after PLE inline with SPE (Fig. 7).

Fig. 8 presented solvent flow rates (1.5, 2, and 2.5 mL/min) impact on the extraction, separation, and analysis of 5-CQA and caffeine. First, the separation of caffeine and 5-CQA using any flow rate was observed. At 2 mL/min, fractions containing 5-CQA were extracted by water and quantified up to approximately 150 min of extraction. On the contrary, caffeine was eluted from SPE by the hydroethanolic mixture and quantified from 167 min of extraction at 2 mL/min. However, higher flow rates promoted the faster separation of the compounds. The inline PLE and SPE at 2.5 mL/min allowed the extraction of 5-CQA until about 100 min, while the caffeine was eluted from approximately 125 min to 160 min. On the contrary, the flow rate of 1.5 mL/min promoted the extraction of 5-CQA until about 150 min and caffeine from approximately 225 min to 300 min. Thus, higher flow rates allowed faster extraction and separation of the 5-CQA and caffeine acquired from coffee co-products.

Moreover, the flow rate did not impact the total 5-CQA recovery. The 5-CQA content determined by online and offline quantification methods was statistically the same (p-value = 0.048). Furthermore, PLE-SPE employing water and the hydroethanolic mixture at any flow rate recovered 100% of the 5-CQA extracted by PLE using water. Therefore, the PLE-SPE online system efficiently extracted, separated, and quantified 5-CQA in the studied flow rates. However, the 1.5 mL/min flow promoted higher differences between the yield results acquired for each extraction fraction by online and offline analysis. This displacement of the curves may be associated with online quantification. In lower flow rates, more fractions were analyzed (Table 1), thus also highlighting errors related to the online injection of a small volume (5 μL) of the entire fraction (16.8 mL) at 1.5 mL/min.

On the contrary, the flow rate statistically affected the quantification of caffeine (p-value = 0.019). The higher flow rate (2.5 mL/min) allowed the lowest caffeine quantification. This behavior happened because caffeine is retained in the SPE. Therefore, when the solvent polarity was changed by adding ethanol to water, caffeine was quickly eluted from the SPE in a few fractions (Figs. 7 and 8). The flow increases from 1.5 to 2.5 mL/min speedily eluted caffeine from SPE, significantly complicating its online quantification. Thus, lower flow rates were better for quantifying caffeine extracted from coffee co-product. However, PLE-SPE employing water and the hydroethanolic mixture at any flow rate recovered 100% of the caffeine extracted by PLE using water.



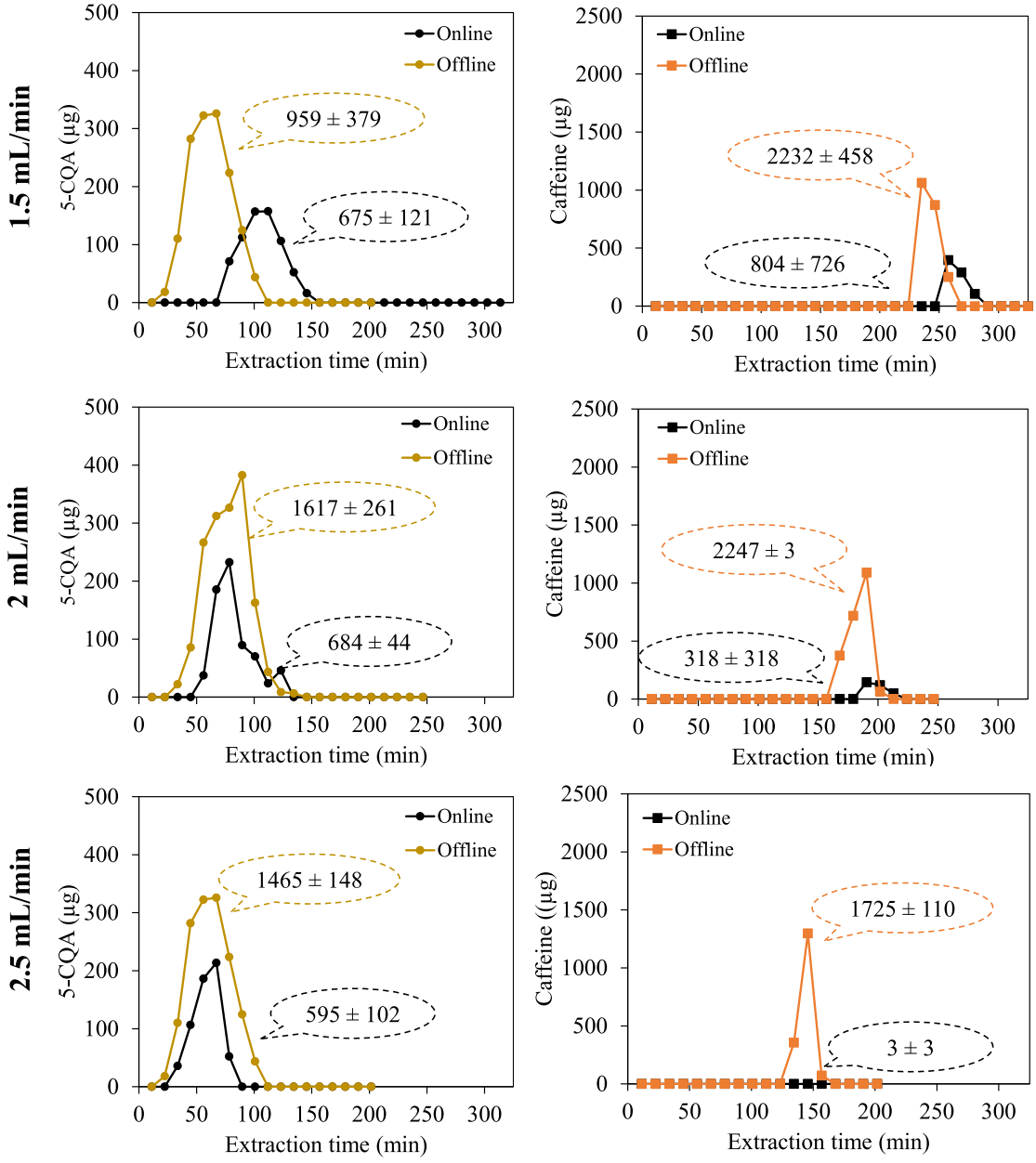

Fractions (F) were acquired at: F1: 11.15 min, F2: 22.65 min, F3: 33.8 min, F4: 44.95 min, F6: 67.25 min, F8: 89.55 min, F10: 111.85 min, F13:145.3 min, F14: 156.45 min, F15: 167.6 min, F16: 178.65 min, F18: 210.5 min, F19: 212.2 min, and F20: 223.5 min of extraction.

**Fig. 7.** The impact of SPE on the chromatographic results acquired for coffee co-product extract fractions obtained by PLE in line with SPE at (A) 325 nm and (B) 270 nm.

Therefore, the system PLE-SPE was efficient for extracting and separating caffeine. On the contrary, the online system was inefficient for caffeine quantification. The online system quantified lower caffeine content in the extracts than the offline method. This difference is probably associated with online quantification. The system performs the online quantification by injecting a small volume (about 5 μL) of the whole fraction into the chromatographic column. This small volume may or may not represent its entire fraction. On the contrary, in the offline method, we inject the sample in the chromatographic column after collecting and mixing all the fraction volumes. Moreover, high deviations were observed in the results obtained for caffeine by the offline method using flow rates of 2 and 1.5 mL/min. This behavior occurs because caffeine was eluted in a short processing time. Therefore, any minor difference between the processes (head loss, pressure difference) can promote a difference in the caffeine content in a specific fraction. Despite this, the flow rate did not affect the total caffeine content quantified by the offline method (p-value = 0.4).

Furthermore, the 1.5 mL/min flow promoted higher differences between the yield results acquired for each extraction fraction by online and offline analysis. This displacement of the curves may be associated again with online quantification. In lower flow rates, more fractions were analyzed (Table 1), thus also highlighting errors related to the online injection of a small volume of the entire fraction. Therefore, this difference or error was not noticed using higher flow rates (2 and 2.5 mL/min).

This advance of the online result, concerning the offline one, was also observed using a lower flow rate to quantify caffeine. Fig. 9 presents the impact of the 1 mL/min flow rate and the 40:60 (v/v) ethanol/water proportion on the extraction and quantification of caffeine from different sample masses (0.2, 0.4, and 0.6 g). The caffeine was quantified in more fractions by decreasing the flow rate to 1 mL/min and adding more water to the hydroethanolic mixture. However, the caffeine content extracted from 0.2 g of the sample quantified by the offline method was significantly higher than that by the online mode (p-value = 0.022). Furthermore, the online system PLE-SPE × HPLC-PDA presented similar behavior, extracting, separating, and analyzing the caffeine extracted from different sample masses (0.2, 0.4, and 0.6). In this sense, the online method quantified approximately 2 × less caffeine content than the offline quantification method. Thus, PLE-SPE showed efficiency in extracting and separating caffeine, but the online quantification method

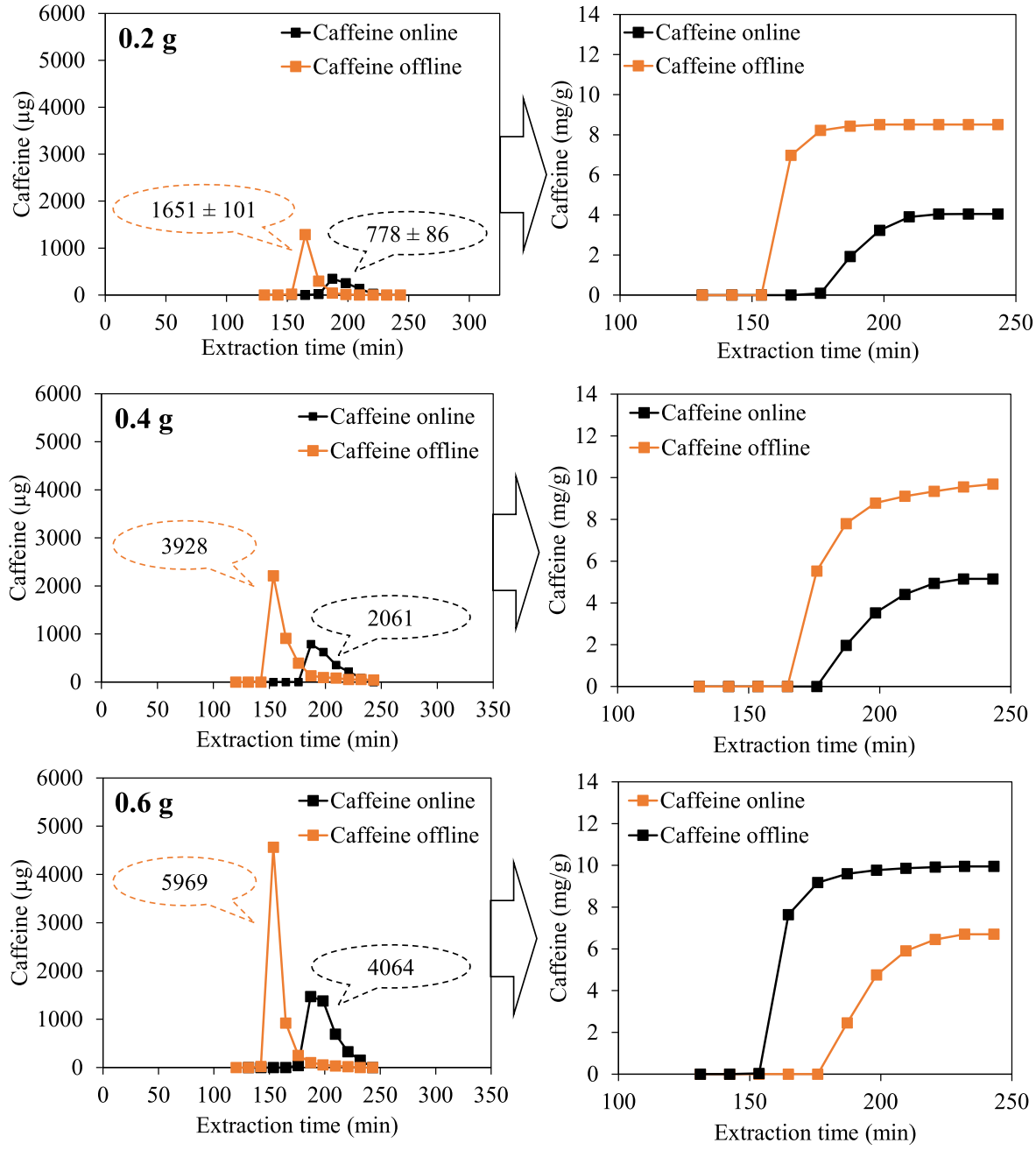



The value shown in the dashed line balloon represents the total value extracted of 5-CQA and caffeine (µg).

**Fig. 8.** Flow rate (1.5, 2, and 2.5 mL/min) impact on the extraction, separation, and online quantification of 5-caffeoylquinic acid (5-CQA) and caffeine (n = 2).

needs improvement. Future studies should try to reduce the chromatographic methods' time to analyze more extract fractions. Furthermore, another possibility is to program the system to inject the samples into the chromatographic column at different times from those evaluated in this study, looking for an aliquot time that better represents the fraction. However, the study needs to consider different flow rates since, as observed in this study, it affected the results acquired for caffeine that was eluted from SPE in a few fractions. Therefore, the system presented some limitations in quantifying compounds extracted/eluted in a short processing time.

Despite the system's limitations for quantifying caffeine elution from SPE, it offers several notable advantages compared to previously proposed methods for obtaining, fractionating, and analyzing bioactive compounds from solid coffee residues. The predominant emerging technology for extracting phenolic compounds from coffee residues has primarily been high-intensity ultrasound, although other approaches involving high-pressure, microwaves, and pulsed electric fields have been explored (Strieder et al., 2023). Most of these researchers have reported their extraction results regarding total phenolic content (TPC) to compare different extraction conditions easily. However, it's crucial to note that chromatographic methods offer a significantly higher level of precision as they directly identify and quantify individual compounds. Additionally, studies employing chromatographic analysis of extracted compounds use several steps, such as solid material filtration, dilution, and additional filtration for subsequent analysis. These steps not only consume time and resources but also introduce potential systematic errors in the quantification process. On the other hand, the proposed process enables the monitoring/quantification of the extracted compounds in real-time. This function is unique and valuable considering the diversity of plant matrices since plants grown in different




The value shown in the dashed line balloon represents the total value extracted of caffeine (µg). The results acquired for 0.2 g present an n=2, while those obtained for 0.4 and 0.6 g present an n=1.

**Fig. 9.** The impact of the flow rate of 1 mL/min and the 40:60 (v/v) ethanol/water proportion on the extraction and quantification of caffeine acquired from 0.2, 0.4, and 0.6 g of sample.

regions and seasons present potential variations in the quantity and composition of bioactive compounds. Additionally, it eliminates sample preparation steps.

Moreover, it is noteworthy that studies proposing the efficient separation of caffeine from 5-caffeoylquinic acid in coffee extracts by SPE were not found. This step could hold significant industrial implications, depending on the intended application of the extract, given the distinct activities of these two compounds. Furthermore, a novel approach, including inline extraction and separation steps, followed by online quantitative chromatographic analysis, was introduced. Other studies from our research group, as referenced throughout the manuscript, proposed extraction and separation of phenolic compounds from different plant matrices by inline PLE-SPE and inline/online analysis of compounds extracted by PLE (PLE × HPLC-PDA) (Da Silva et al., 2020; Souza et al., 2021; Viganó et al., 2021; Da Silva et al., 2023; Maciel-Silva et al., 2022). However, these studies conducted online analyses qualitatively, only demonstrating the extraction profile without providing an online quantification procedure as proposed in this study.

The limitations of this procedure are related to the cost of the equipment and its programming since the entire procedure is performed

automatically after setting in the equipment. Furthermore, as demonstrated in this study, using an integrated process requires a short chromatographic run method so that the collected points represent the sample as a whole.

## 4. Conclusion

This study has demonstrated that the two-dimensional system (PLE-SPE × HPLC-PDA) allows the online extraction, separation, and analysis of the compounds extracted from coffee co-product. PLE was the most efficient extraction technique to recover caffeine from coffee co-product due to the subcritical conditions employed. Moreover, the inline SPE with PLE isolated caffeine from 5-CQA and other compounds in a few fractions. The online quantification also efficiently quantified 5-CQA in any flow rate employed. However, the system presented some limitations in quantifying compounds extracted/eluted in a short processing time. Therefore, this study demonstrated the potential of using co-product from coffee to obtain purer fractions of phenolic compounds, supplying high-valued added ingredients for food and pharmacy industries.

## CRediT authorship contribution statement

**Monique Martins Strieder:** Conceptualization, Writing – original draft, Methodology, Investigation. **Vitor Lacerda Sanches:** Writing – review & editing, Methodology, Investigation. **Maurício Ariel Rostagno:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113690.

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