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Application of an integrative system (2D PLE×HPLC-PDA) for bioactive compound extraction and online quantification: Advantages, validation, and considerations

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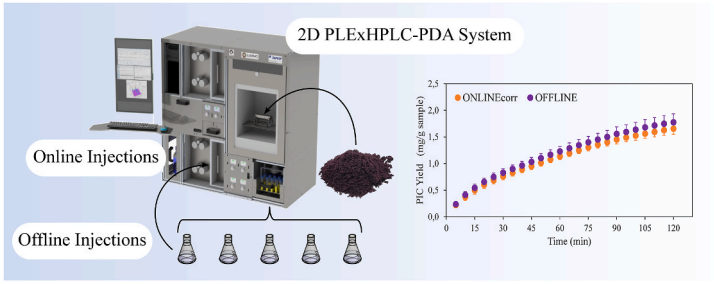
HIGHLIGHTS

- An automated system to analyze bioactive compounds from solid samples is presented.
- 2D PLE × HPLC-PDA system integrates sample preparation online with chromatography.
- The system validation compares online and offline extract quantification.
- The new online method reports significantly equal yields with offline quantification.
- The online method represents a feasible tool for analyzing bioactive compounds.

GRAPHICAL ABSTRACT



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ABSTRACT

Analyzing compounds such as polyphenols in solid samples frequently uses a solid-liquid extraction step. The solid-liquid extraction and analysis integration in a single equipment is not commercially available since several challenges are inherent to this hybridization. In the context of developing more sustainable analytical procedures, innovative techniques are demanded. Given that, this work proposes a new integrative system (2D PLE × HPLC-PDA) and presents its validation for bioactive compound extraction and online quantification, discussing the main advantages and cares that need to be taken. Two food byproducts – passion fruit bagasse and coffee husks – were chosen as solid model samples. The system was configured to perform pressurized liquid extraction (PLE) with periodical automated extract injection in the HPLC, consequently obtaining the online quantification of target compounds from the solid samples. In parallel with the online injections, extract fractions were collected and submitted to offline analysis in which the extraction yield of piceatannol and chlorogenic acid and caffeine were evaluated, respectively, for passion fruit bagasse and coffee husks. The extraction yields obtained by online and offline injections were compared and were significantly equal ($p > 0.05$). Thus, the 2D PLE × HPLC-PDA system represents a feasible tool to integrate solid sample preparation and chemical analysis of biocompounds in a single and online step.

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1. Introduction

The investigation of natural bioactive compounds has grown over the last two decades. Its scope ranges from identifying and quantifying target compounds in raw materials to their applicability in commercial products [1]. Chemical characterization, mainly aiming at quantifying target substances, is crucial to select raw materials, optimizing processes, and evaluating bioavailability and bioaccessibility. However, preparative techniques like solid-liquid extraction techniques are generally demanded prior to the analytical steps [2,3].

Significant advances have been observed in solid-liquid extraction techniques, either in proposing new methods or developing new solvents [4,5]. Much of the progress achieved was driven by the need for green chemistry and sustainable development goals [6]. New extraction methods such as pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) have emerged as alternatives to conventional solid-liquid extraction methods [7] and are currently found consolidated regarding the phenomenological description, applicability, and advantages [8]. Similarly, new solvents such as eutectic solvents and ionic liquids have been proposed as substitutes for volatile organic solvents [9,10], but despite significant advances, they still constitute an area with clear potential for investigation. In addition, analytical techniques for characterizing liquid mixtures have also evolved in the past decades, which culminated in the availability of automatic commercial apparatus that, together with the use of columns packed with fused-core particles, enabled an efficient degree of separation of compounds, short running times and lower solvent consumption [11].

Although significant progress has been achieved, the integration and automation of solid sample preparation and extract chemical analysis are still challenges to overcome. The literature has already documented the online combination of SFE with liquid and gas chromatography, as reviewed by Sánchez-Camargo et al. [12]. While significant progress has been made in coupling SFE with extract characterization techniques, it is recognized that PLE is often employed for analytes where SFE yields are comparatively lower, particularly for polar compounds. Consequently, PLE fills a gap that is not addressed by SFE, serving as a valuable technique. The online hybridization of PLE with extract characterization techniques remains sparsely documented in the literature, and the availability of commercial devices specifically designed for this purpose is still limited [2,11]. Consequently, extraction and chromatography of polar compounds have been mostly performed offline, i.e., sequentially without any integration level, which requires a long time to complete the analysis, increases labor and energy costs [13], and makes the analysis more error-prone. Additionally, it is known that a single solvent cannot extract different groups of substances from the same solid sample, making the comprehensive analysis of biological materials difficult. Hence, systems equipped with valves that enable automatic selection of different solvents improve the analytical process, enabling multiple extraction steps using different solvents to be applied to the same solid sample [2].

An alternative to overcome these challenges was proposed by Viganó et al. [11], which has a patent application [14]. The authors introduced a system to perform PLE followed by SPE in-line and HPLC online. A complete description of the system and its operation details was provided by illustrating a case of the comprehensive analysis of phenolic compounds from *Ilex paraguariensis*. However, system validation was still required to compare online with offline quantification. Therefore, the present work proposes an online method applied to the previously described system that goes up a step in the future of extraction and analysis automation systems. A validation proposal for the 2D PLE × HPLC-PDA system for the extraction of bioactive compounds and extract quantification is presented. Considering the highly available coffee and passion fruit byproducts, these solid matrices were used as sources of bioactive compounds. A detailed validation methodology that could be applied to different kinds of compounds and samples was proposed. Thus, this work presents a detailed protocol for PLE × HPLC online systems, discussing the main advantages and care needed.

2. Material and methods

2.1. Chemicals and sample

Ultra-pure water was provided by a Purelab Flex 3 purifying system (Elga Veolia, High Wycombe, United Kingdom). Absolute ethanol was purchased from Anidrol (Diadema, SP, Brazil). HPLC-grade acetonitrile was from ACS Científica (Sumaré, SP, Brazil) and methanol from Supelco (Darmstadt, Germany). Piceatannol 98%, chlorogenic acid, and caffeine were purchased from Sigma-Aldrich (Barueri, SP, Brazil), and

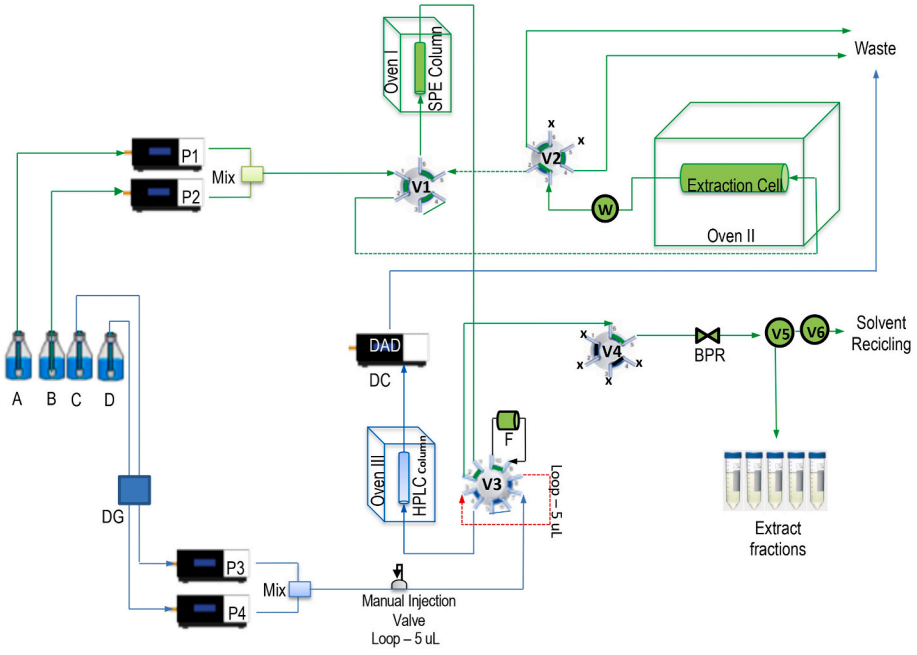

Fig. 1. Diagram of the 2D PLE × HPLC-PDA system configuration. A, B, C, and D: solvent reservoirs; P1 and P2: binary pumps (PLE-SPE); P3 and P4: analysis system binary pumps (HPLC); DG: degasser; DC: detector; W: heater exchanger; V1: selection valve; V2: depressurization valve; V3: interface valve; V4: automatic pressurization valve; V5 and V6: fraction collection valves; BPR: backpressure valve; F: filter; Oven I: oven for SPE column; Oven II: oven for extraction cell (PLE-SPE); Oven III: chromatographic oven (HPLC). Green: extraction module. Blue: HPLC module. Reprinted with permission from Viganó et al. [11]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

resveratrol 98% was supplied from Trafa Pharmaceutical Supplies, INC, Richmond, Canada.

Passion fruit (*P. edulis* sp.) bagasse and coffee husks (*Coffea arabica*) were chosen as a model of natural sample to validate the 2D PLE × HPLC-PDA system. The passion fruit bagasse was submitted to the same treatment described by Viganó et al. [15]; briefly, the sample was freeze-dried, ground, and submitted to supercritical fluid extraction (SFE) to remove the lipidic content. SFE was performed using a continuous flow of carbon dioxide at 15 g min^{-1}. Forty sample grams were loaded into a stainless-steel extraction cell (303 mm × 754 mm), and SFE was performed at 40 °C and 35 MPa. The process was stopped, and the system depressurized when the solvent-to-feed mass ratio (S F^{-1}) was equal to 80. After SFE, the solid sample reduced the lipidic content from 22 to 2 wt%. The mean particle diameter was 0.09 ± 0.01 mm, determined according to Tadini et al. [16]. The coffee by-product was composed of husk and defective beans dried at 60 °C for 24 h and ground. After that, the raw material presented 3.8 ± 0.1% and 0.44 ± 0.01 mm of moisture and mean Sauter diameter, respectively.

2.2. Online integration of PLE and liquid chromatography

2.2.1. System description

The 2D PLE × HPLC-PDA system was previously described by Viganó et al. [11]. It is divided into two modules: sample preparation and fractionation, which compose the first dimension, and chromatographic analysis, comprising the second dimension. A schematic diagram of the 2D PLE × HPLC-PDA system is provided in Fig. 1, from which green components and lines represent the first dimension, and blue components and lines compose the second dimension.

Pressurized liquid extraction (PLE) and solid-phase extraction (SPE) are the primary operations performed in the first dimension. The pumps (P1, P2; Waters, 1525, Milford, MA, USA) flow the solvent from the reservoir A and B to the selection valve (V1; Waters, Two-Position Ten-Port Selector Valve, Milford, MA, USA) that can be programmed to direct the solvent to the SPE column (50 × 4.6 mm, Waters, 1525, Milford, MA, USA) to activate and conditionate the adsorbent or to the extraction cell (111.5 mm × 20 mm, Citua, Campinas, SP, Brazil) to perform the PLE. Although the system allows to clean up the extract by performing SPE, this operation was not required in the current work. Consequently, instead of passing through the SPE column, the extract flows through a bypass route and is cooled at room temperature. It is important to note that the heat exchanger (W) was turned off. However, it can be necessary to cool the extract after PLE when working with higher extraction temperatures. After W, the extract flows to the depressurization valve (V2; Waters, Two-Position Six-Port Selector Valve, Milford, MA, USA) that is programed to direct the solvent to V1 and SPE column or to waste where depressurization takes place. The fluid that leaves the SPE column flows to the interface valve (V3; Waters, Two- Position Ten-Port Selector Valve, Milford, MA, USA). V3 can be programmed to inject an aliquot of extract in the second dimension or flow the extract to be collected and stored. V4 (Waters, Two-Position Six-Port Selector Valve, Milford, MA, USA) works to automatically pressurize the system during pressurization step while backpressure valve (BPR, Tescom Corporation, Elk River, Minnesota, EUA) maintain the pressure during the extraction. V5 and V6 (Waters, Two-Position Six-Port Selector Valve, Milford, MA, USA) are automatic valves for separating the extract fractions to be collected. In addition, SPE column and extraction cell are positioned inside oven I (Waters, 1525, Milford, MA, USA) and oven II (Memmert GmbH, UF55, Buechenbach, Germany), respectively.

V3 plays an essential role in the system since it performs the automatic injections into the analysis module. Besides, an aliquot of extract is filtered in V3 through an in-line filter (F; 0.2 μm) and fills the 20 μL loop in each injection. In the second dimension, the pumps (P3, P4; Waters, 1525, Milford, MA, USA) flows the solvents from the reservoir C and D, that are degasified (DG; Waters, In-line Degasser-AF, Milford, MA, USA), and flow through V3 to the HPLC column positioned inside the oven III (Waters, 1525, Milford, MA, USA). After the chromatographic separation, detection is carried out by a PDA detector (DAD, Waters, 2998, Milford, MA, USA), providing the information to be recovered. The second dimension can also operate independently of the first dimension by operating the manual injector of 20 μL.

The components in the first and second dimensions are connected by stainless steel tubing of 0.040" and 0.010" intern diameter, respectively.

2.2.2. System validation

An ethanol solution (50 vol%) containing resveratrol (50 μg mL^{-1}) or chlorogenic acid and caffeine (50 μg mL^{-1}) was used to simulate the online analysis (identification and quantification). Resveratrol was chosen due to the similarity to piceatannol (the target compound in the passion fruit sample) and the large amount we had available. Chlorogenic acid and caffeine are the target compounds in coffee by-products samples. The solutions were pumped at 2 mL min^{-1} at 1500 psi and room temperature (oven I and II turned off). In the place of the extraction cell and SPE column were added bypasses. The pumping time took 40 min, and the V3 valve was programmed to do five automatic injections by the 20 μL loop. The same solutions were injected five times in the manual injector (20 μL). This step aimed to validate the online injection by V3, comparing the peak area with the manual injection (offline) since, for a real sample, the extracts are injected by V3, and standard solutions to compose the calibration curve are injected through the manual injector. Comparison between online and offline injections was performed by calculating the relative error (E), as presented in Equation (1); where, A_{online} and $A_{offline}$ are respectively the mean of the peak area obtained by online and offline injection.

$$E(\%) = 100 \times \frac{\left(A_{online} - A_{ofline}\right)}{A_{online}} \quad (1)$$

2.2.3. Online quantification with a real sample

PLE was performed at similar conditions reported by Viganó et al. [15]. In each run, 0.50 g of passion fruit bagasse or 0.50 g of coffee by-product sample was added to the extraction cell. A layer of glass wool was added before and after the sample bed in the extraction cell. Teflon monoliths (15 mm OD) with a central hole (3 mm) were inserted in the extraction cell to fill the free volume. The loaded extraction cell was inserted in the Oven II and connected to the pipelines. The SPE column was not employed; therefore, a bypass was used. The Oven II was turned on, and once it achieved 70 °C, 30 min were waited to heat the cell and start the system pressurization. The extraction module was pressurized (1500 ± 50 psi) using the automatic valve (V4) programmed as shown in Table SM1 (Supplementary Material). After reaching the desired pressure, 15 min (static extraction time) was spent before starting the dynamic PLE, which was performed with solvent composed of 75 vol% water (solvent A) and 25 vol% ethanol (solvent B), flowing at 2 mL min^{-1}. The Oven II temperature was 70 ± 1 °C and the backpressure was regulated to achieve 1500 ± 50 psi. During 125 min the PLE was run using the valve program shown in Table SM2 (Supplementary Material). The pipeline from the extraction cell to the exit started the process filled with pure solvent; therefore, the delay time to the interphase solvent/extract arriving at the exit was about 5 min. Such time was counted since it is important to program the automatic injections, the first injection was at 7.5 min, and the next was every 5 min for the passion fruit sample. The first injection for the coffee by-product sample was at 10.1 min, and the next was every 10.2 min, considering the difference in the chromatographic analysis time. In each injection, the command pulse

changes the V3 from position 1 to position 2 for 0.3 min, enough time to fill the in-line filter and the 20 μL loop. The automatic injections performed during the extractions will be called from here online injections. The extract that flows to the extract collector was separated in fractions every 5 min from 5 to 125 min for passion fruit and every 10.2 min from 5 to 127 min for the coffee byproduct sample. The 24 extract fractions recovered from passion fruit, containing 10 mL in each one, were centrifuged at 10,000 g for 10 min, and then injected in the manual injector (20 μL), which from here will be called offline injections. The 12 fractions collected from the coffee sample, containing 20 mL in each, were filtered in a nylon filter of 0.20 μm, then injected as performed for the passion fruit sample.

The PLE process online coupled with HPLC was repeated four times with 24 and 12 online injections in each run to extract the compounds from passion fruit bagasse and coffee byproduct, respectively. As previously described, the extracts collected in fractions were also analyzed offline. Additionally, the solid samples remaining in the extraction cell were recovered, submitted to a new extraction process (Section 2.4), and analyzed to assess whether the extraction had been complete.

2.2.4. Chromatographic conditions

The online and offline injections of extracts and offline injections of standard solutions were chromatographically separated using a C18 column (KinetexTM C18, 2.6 μm, 100 mm × 4.6 mm, Phenomenex Torrance, CA, USA). The compounds were quantified using Empower 2 software (Waters, Milford, USA).

The conditions employed to quantify the compounds from passion fruit bagasse were 40 °C, and a mobile phase composed of 80 vol% water (solvent C) and 20 vol% acetonitrile (solvent D) flowed at 1.5 mL min^{-1}. The run time was 8 min for resveratrol standard solution analysis and 4.5 min for extract analysis. Chromatograms were monitored between 210 and 400 nm and recovered at 306 nm. Piceatannol was quantified in the passion fruit extract using a calibration curve with concentrations from 0.77 to 197 μg mL^{-1} (y = 74456× – 63958; y: area, x: concentration; coefficient of determination (R^2) equal to 0.9996).

Chlorogenic acid and caffeine extracted from coffee husks were quantified at 50 °C using a flow rate of 1 mL min^{-1}. The mobile phase consisted of 1 vol% acetic acid (v v^{-1}) in water (solvent C) and 0.1 vol% acetic acid (v v^{-1}) in acetonitrile (solvent D). The following gradient was

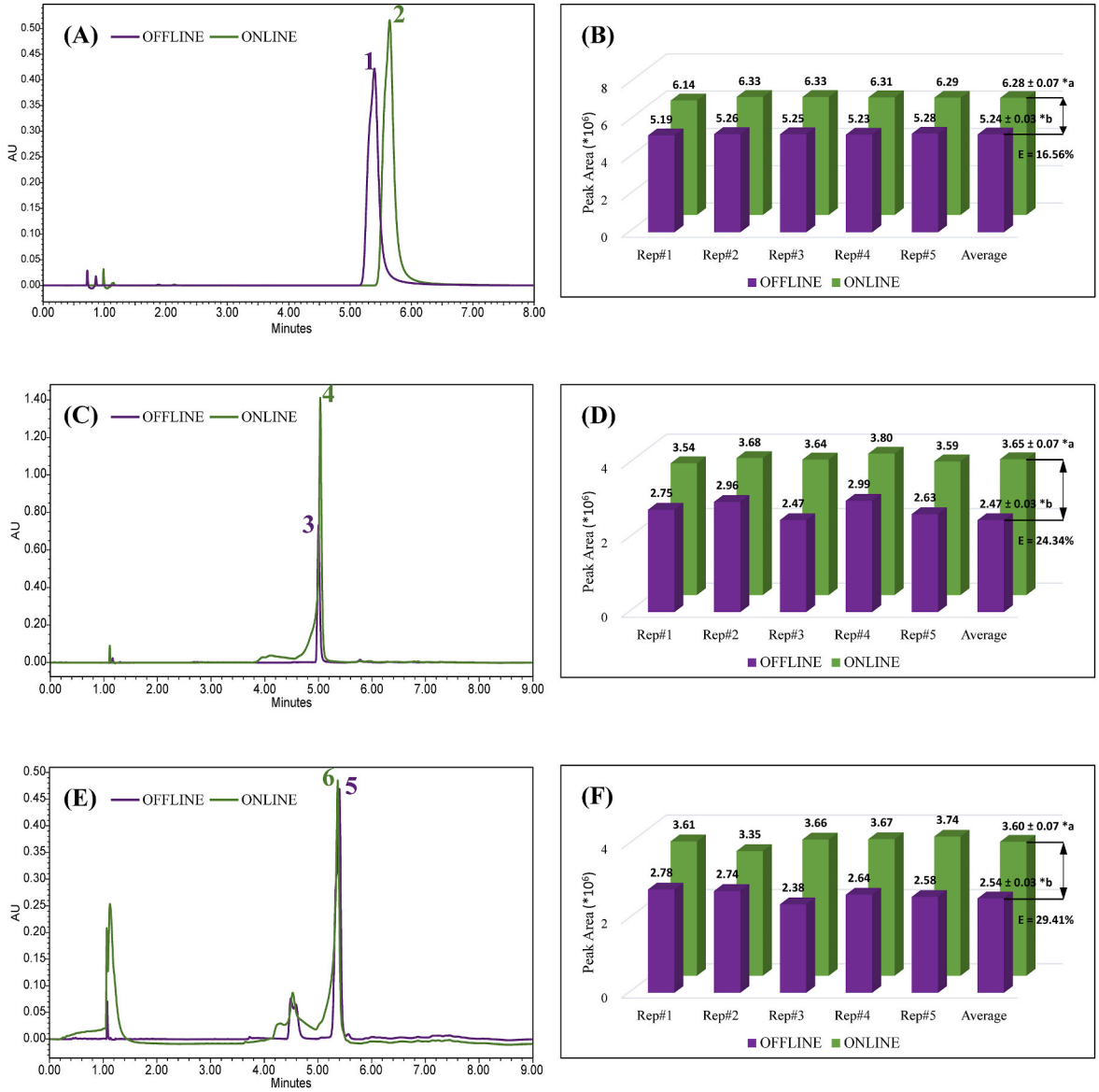

Fig. 2. Overlay of chromatograms (A, C, and D) and peak area (B, D, and F) obtained from online and offline injections of the solutions of resveratrol (peak #1 and #2; 306 nm), chlorogenic acid (peak #3 and #4; 325 nm), and caffeine (peak #5 and #6; 270 nm) at 50 μg mL^{-1}, respectively. *Different letters indicate a significant difference ($p < 0.05$). E: relative error calculated from Equation (1).

used: 0 min (95% C); 2 min (90% C); 2.5 min (85% C); 4 min (75% C); 5 min (55% C); 6 min (40% C); and 7 min (95% C) for 9 min. We added a 1 min delay 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 10 min. Chromatograms were monitored between 210 and 400 nm and recovered at 270 and 325 for caffeine and chlorogenic acid quantification, respectively. Chlorogenic acid and caffeine were quantified using the respective calibration curves ($y = 37984x + 153635$; y: area, x: concentration; R^2 of 0.9936) and ($y = 42746x + 56203$; y: area, x: concentration; R^2 of 0.9984) using concentrations from 0.625 to 200 μg mL^{-1}.

2.2.5. Extraction yield of target compounds

Concentrations of target compounds (piceatannol, chlorogenic acid, and caffeine) from online and offline injections were used to calculate the extraction yield according to Equation (2); in which C_i is the concentration of the target compound i (mg mL^{-1}), V_f is the volume of extract fraction (mL), and m is the mass of solid sample feed (g).

$$Target\ Compound\ Yield\left(mg\ g^{-1}\right) = \frac{C_i \times V_f}{m} \quad (2)$$

The target compound yield obtained from online and offline quantifications were compared by expressing the relative error described in Equation (3), in which $Y_{i,online}$ and $Y_{i,offline}$ are the yields of the target compound i (mg g^{-1}) obtained by online and offline quantifications.

$$E(\%) = 100 \times \frac{\left(Y_{i,online} - Y_{i,offline}\right)}{Y_{i,offline}} \quad (3)$$

2.3. Ultrasound-assisted extraction

Ultrasound-assisted extraction was selected to re-extract the remaining solid sample after PLE (waste) and to compare the extraction methods using different solvents. The ultrasonic bath (Elmasonic P60H, Germany) was set to 60 °C, 37 kHz, and 100 W, and the extractions took 60 min. The remaining PLE sample was added to 5 mL methanol 80 vol %. In contrast, the raw solid samples (0.50 g) were added to 10 mL methanol 80 vol% or 25 vol% ethanol. After 60 min of ultrasound-assisted extraction, the extracts were centrifuged (10,000 g; 10 min), supernatants were separated and once again centrifuged (10,000 g; 10 min). Next, the extracts were offline injected and analyzed as described in Section 2.3.4.

2.4. Statistical analysis

The results of the repetitions were expressed by the mean ± standard deviation of the mean. One-way analysis of variance (ANOVA) was used to evaluate the means statistically, and significant differences were analyzed by the Tukey's test (level of 95%, $p < 0.05$).

3. Results and discussion

3.1. System validation

The developed system (2D PLE-SPE × HPLC-PDA) is based on the online integration of solid sample preparation through PLE with chromatographic analysis on HPLC. Valve V3 is programmed to automatically inject an extract aliquot at certain intervals of time to be analyzed on the HPLC as described in Section 2.2.1. However, the system does not allow injecting the standard solutions with different concentrations through the V3 valve but using the manual injector. Given this issue, the system was validated by simulating the extraction through automatic injections of a standard solution through the V3 valve (online) and the manual injector (offline). The results of this analysis performed by online and offline standard solutions injections are shown in Fig. 2. Chromatograms of resveratrol, chlorogenic acid, and caffeine are respectively presented in Fig. 2(A), (C), and (E) and Fig. 2 (B), (D), and (F) show peak area from online and offline injections. The peaks' area of the three standard compounds obtained by online and offline injections solution differed significantly (p-values ≤0.00022), despite both loops (V3 valve and manual injector) presenting a 20 μL volume (Fig. 2). The area of the online injections was higher than the offline ones, presenting relative errors (E%) equal to 16.56, 24.34, and 29.41% (Fig. 2(B), (D) and (F)). Although the injection valves' manufacturers declare that the dead volume of both is low, the difference in the peaks comes from the volume between the valve ports, which we believe to be greater in V3 valve. Another essential feature is the difference in online and offline peak retention time presented in Fig. 2(A) for resveratrol. Such a difference was already expected since the pipe size (length) between the injection points and the chromatographic column are different for online

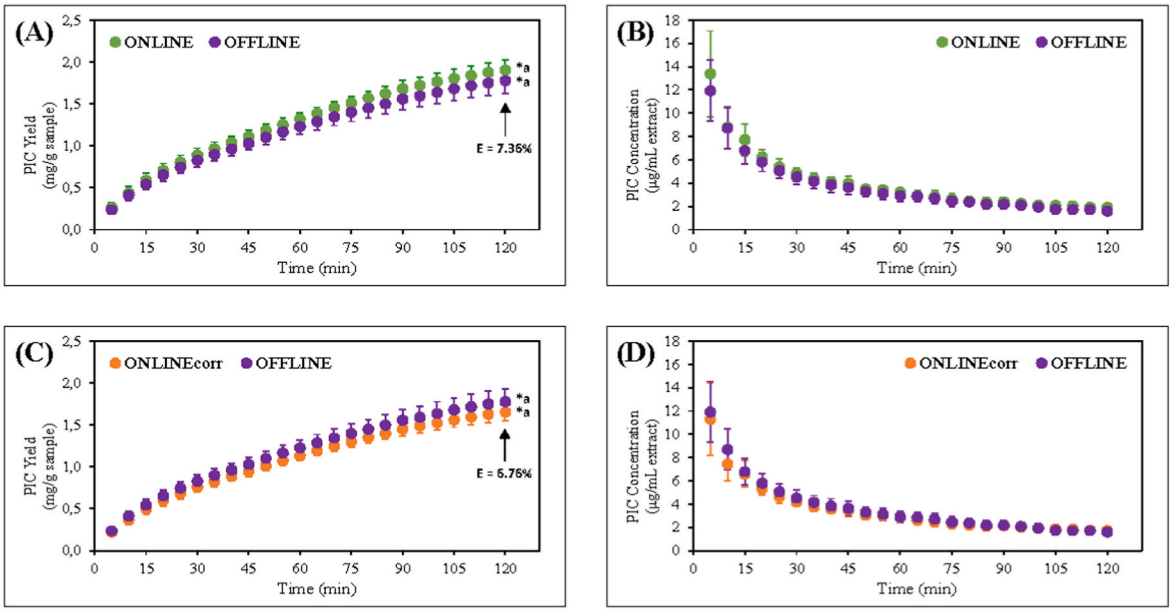

Fig. 3. Comparison of piceatannol (PIC) yield (A and C) and PIC concentration in the extract (B and D) from online, online-corrected (ONLINEcorr), and offline quantification. *Different letters indicate significant difference ($p < 0.05$). E: relative error calculated from Equation (3).

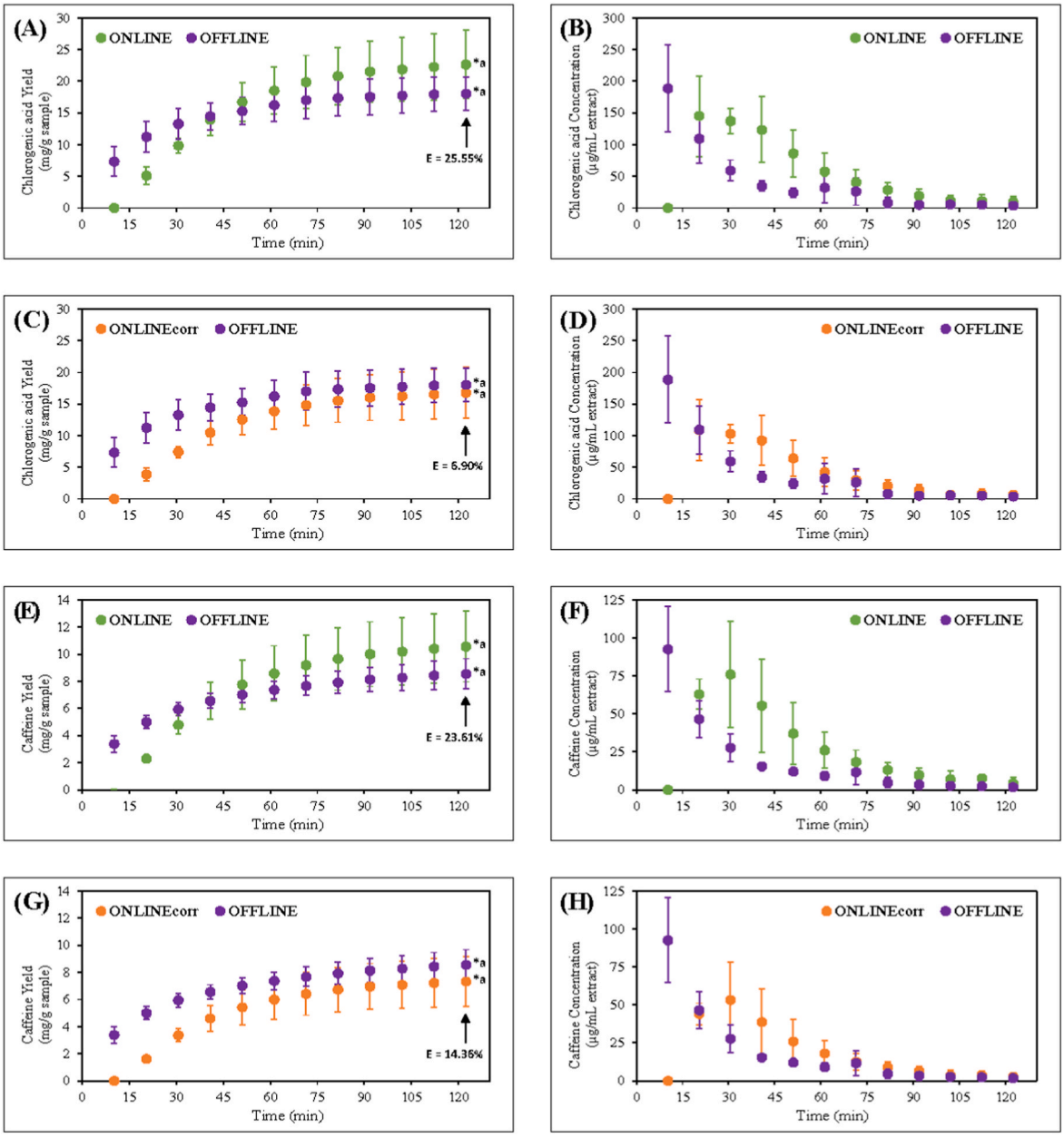

Fig. 4. Comparison of chlorogenic acid and caffeine yield (A, C, E, and G) and their concentration in the extract (B, D, F, and H) from online, online-corrected (ONLINEcorr), and offline quantification. *Different letters indicate significant difference ($p < 0.05$). E: relative error calculated from Equation (3).

and offline injections. However, the difference in the retention time for the caffeine and chlorogenic acid standards was not observed. Although the solvent used to dilute the three standards are the same (50 vol% ethanol), the chromatographic methods employed different separation temperatures and mobile phases. Therefore, the reduction in the disparity of retention times observed for chlorogenic acid and caffeine between online and offline injections (as depicted in Fig. 2(C) and (E)) can be attributed to the specific characteristics of the employed chromatographic method.

3.2. Validation of the online quantification with real samples

The areas obtained from the online injections were corrected considering the relative error presented in Section 3.1, which were 16.6, 24.3, and 29.4% respectively for resveratrol, chlorogenic acid, and caffeine. As reported in Section 2.2.3, twenty-four and twelve online injections were performed over the 125 min of PLE, and the same fractions of extract were collected for offline analysis, i.e., injected in the manual injector. Therefore, all areas obtained online for resveratrol, chlorogenic acid, and caffeine were corrected by a correction factor (correction factor = (100 - E) 100^{-1}) of 0.8344, 0.7566, 0.7058, respectively. Figs. 3 and 4 compare the compounds yields and extract concentration, respectively from passion fruit bagasse and coffee husks, regarding online *vs.* offline and online-corrected *vs.* offline injection. Note that the relative error between the online and offline injections, regardless of the compounds, decreased when the correction factor was applied (ONLINEcorr). Another important feature observed in Figs. 3 and 4 is no significant difference between the online, online-corrected with the offline quantification ($p > 0.05$). Even so, the correction factor use is strongly advised to minimize the difference between the results obtained from both injection modes and, therefore, to obtain more accurate and confident results for sample quantification.

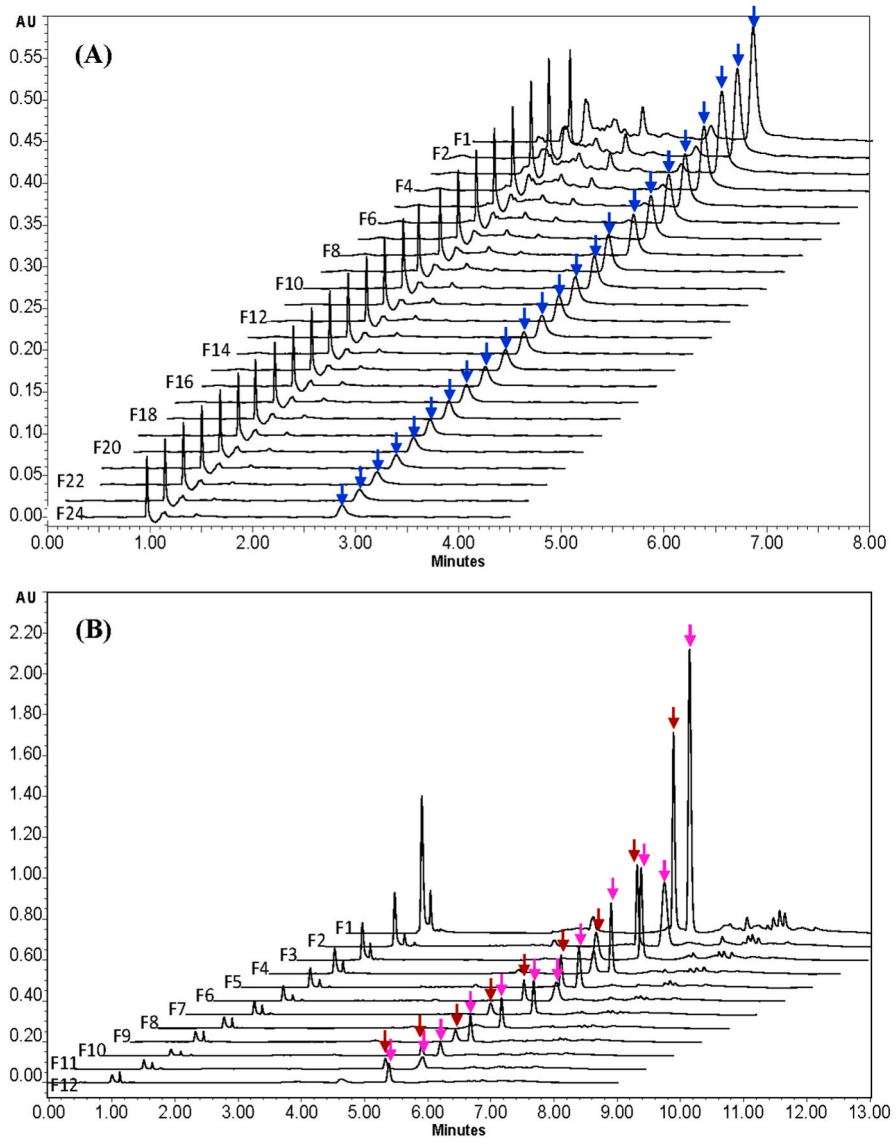

Fig. 5. Chromatograms obtained from online injections: (A) 24 automatic injections of passion fruit bagasse extract for piceatannol quantification recovered at 306 nm and (B) 12 injections of coffee husks extract for chlorogenic acid and caffeine quantification recovered at 270 nm. Blue, red, and pink arrows indicate piceatannol, chlorogenic acid, and caffeine, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Besides the use of the correction factor, some precautions in the 2D PLE-SPE × HPLC-PDA operation must be taken to achieve injections with higher representation of the collected extract fraction:

i) The time for performing the first online injection must be previously tested individually for each sample. The presented study used a similar time for the first online injection (5 min plus half-time of the chromatographic method) for both extract samples (passion fruit bagasse and coffee). However, the online method did not detect compounds in the first online fraction from coffee extract. Otherwise, the time of the first injection was consistent with the result obtained offline for the passion fruit sample. Therefore, one can observe through these two examples the importance of programming the first injection to coincide with the extraction time corresponding to the half fraction collected. Such synchronism allows a higher similarity between the online and offline injections.

ii) The injection must be done in the average time of each fraction. If the fractions are collected every 5 min, the online injection must be done in 2.5 min. This care is essential throughout the entire kinetics, but it is more impactful in the first minutes of extraction when the extract concentration decreases at a faster rate.

iii) The faster the drop in extract concentration, the quicker the chromatographic method should be since faster chromatographic methods allow more injections. Thus, the number of injections must be sensitive enough to read the variation in concentration. The analysis of piceatannol was faster than that of chlorogenic acid and caffeine, allowing a lower error between the results obtained online and offline. Therefore, analytical equipment that allows high-speed chromatographic methods (<2 min) would make the online analysis system even more accurate.

iv) The sample mass and extraction solvent flow rate must be chosen considering that dilutions are not made before the chromatographic analysis. Thus, these two variables must be selected to achieve the first extract fraction with concentration compatible with the chromatographic method. Moreover, 2D PLE × HPLC-PDA system can be further improved to minimize this trouble by implementing inline dilutions.

Fig. 5 presents the sequence of chromatograms obtained over the

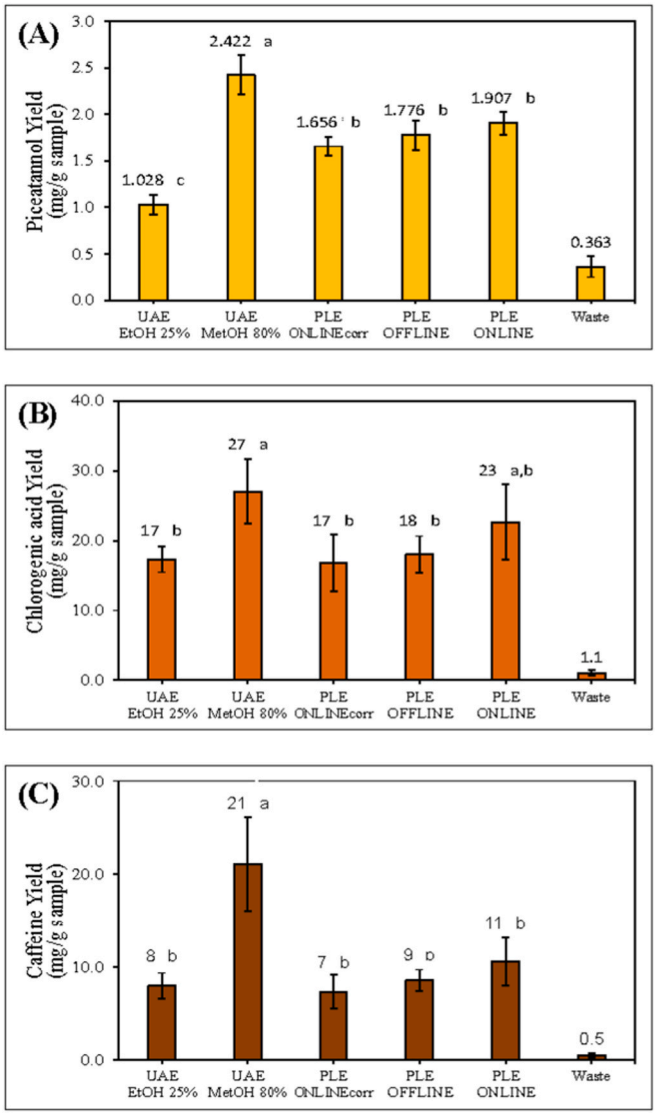

Fig. 6. Effect of different extraction methods on piceatannol, chlorogenic acid, and caffeine extraction yield and quantification of these compounds remaining in the PLE sample solid waste. Different letters between bars in the same graph indicate significant difference ($p < 0.05$).

125 min of PLE for passion fruit bagasse extract (Fig. 5(A)) and coffee husks extract (Fig. 5(B)) respectively corresponding to 24 and 12 online injections. The extraction rate profiles were different between the two raw samples. The peak's intensity from passion fruit bagasse decreased very slowly from the second half of the process (from F12 in Fig. 5(A)), showing that the adopted extraction method would take longer to exhaust the piceatannol from the sample. Otherwise, the chlorogenic acid and caffeine were more quickly extracted from the coffee husks at the same extraction conditions (75 vol% water and 25 vol% ethanol; 70 °C). This difference is due to several factors, such as the affinity of the compounds for the solvent, the interaction type with the raw material, and their solubility in the extraction solvent.

The non-depletion of the passion fruit sample can also be seen in Fig. 6. The PLE residue from the four replicates was re-extracted and the extract analyzed, presenting a remaining content of 0.4 ± 0.1 mg PIC g^{-1} initial sample. This value represents 22% of the content obtained by the online corrected quantification. Contrastingly, the remaining chlorogenic acid and caffeine in the coffee husks represented 4.6% (1.1 ± 0.1 mg chlorogenic acid g^{-1} initial sample) and 4.9% (0.5 ± 0.2 mg caffeine g^{-1} initial sample) of the content obtained by online corrected quantification, respectively. At this point, it should be emphasized that the PLE method was not optimized; an optimization process could achieve temperature, sample mass, solvent flow rate, solvent composition, heating time, and static time conditions that reduce the time required to obtain the solid sample depletion. In addition, some details need to be considered:

i) The relationship between sample mass and solvent flow rate must be adjusted so that the peak area of online injections is within the range of the calibration curve.
ii) The extraction temperature must allow the extraction and solubilization of target compounds without affect its stability.
iii) The extraction solvent must be compatible with chromatography mobile phase to produce chromatograms with high peak resolution. For instance, in chromatographic methods where the mobile phase primarily consists of water, utilizing a subcritical water extraction method can be very interesting.
iv) Samples with complex compositions may necessitate a cleaning step for their extracts. In such cases, employing SPE becomes necessary. Therefore, during method development, it is crucial to thoroughly study and optimize the operating conditions of the SPE process.

3.3. *Extraction comparison between different techniques*

Fig. 6 shows the global yield (mg g^{-1} sample) of (A) piceatannol, (B) chlorogenic acid, and (C) caffeine obtained from passion fruit bagasse and coffee husks using different extraction methods. In addition, the extraction yields from the PLE waste re-extracted in ultrasonic bath are also presented. The UAE using 80 vol% methanol allowed a greater extraction of piceatannol and caffeine than the other methods. Otherwise, statistically, this procedure allowed the equivalent chlorogenic acid extraction yield. Mixtures of organic solvents such as methanol and ethanol with water are chemically efficient for extracting polyphenols such as stilbenes and alkaloids such as caffeine. However, it is important to acknowledge that the use of these solvents is not without drawbacks. They can be toxic and pose risks to natural resources if not properly disposed of. Additionally, it is worth noting that solvents like methanol are derived from non-renewable sources. Therefore, it is crucial to highlight that while these solvents have been employed in this research, their usage should be minimized, and efforts should be made to find greener alternatives.

Furthermore, in this proposal, when in-line analysis is performed, the extraction solvent must be compatible with the chromatography mobile phase. In this sense, the great challenge of using high percentages of organic solvents for the extraction, in this proposed system, is the quality of the chromatographic peaks for further quantification. During tests previously performed to the presented work, we did not acquire chromatograms with well-resolved peaks using 75 vol% ethanol because the mobile phase comprised 80 vol% water. Therefore, the PLE needs to be optimized, and the chromatographic method should be short and allow solvent compatibility. Alternatively, variables other than extraction solvent such as temperature or application of ultrasonic power to assist PLE could be used and optimized to improve desorption of target compounds from the solid sample. Additionally, it is crucial to validate the complete method in accordance with international guidelines when developing new methods that integrate online extraction and analysis.

Furthermore, PLE using 25 vol% ethanol allowed higher piceatannol yields and the same chlorogenic acid and caffeine yields as UAE with ethanol (25 vol%), demonstrating the importance of the raw material and compound characteristics as well the extraction solvent and technique. The system allowed the online quantification of piceatannol, chlorogenic acid, and caffeine representing a low human intervention due to automation. However, reducing the total process time and the consumption of solvents is still challenging. It is noteworthy that in the 125 min of extraction, 250 mL of solvent were used, i.e., 62.5 mL of

ethanol and 187.5 mL of water against 10 mL of methanol in UAE 80 vol %. Despite being considered a green solvent, six times more ethanol than methanol was used. Therefore, considering the increasing demand for environmentally friendly techniques, it is crucial to emphasize the significance of improving the method's speed, minimizing solvent consumption, and replacing the use of organic solvents in the future works. Moreover, the exploration of alternative solvents such as eutectic solvents and ionic liquids holds great potential for further advancements in this field.

4. Conclusions

The 2D PLE × HPLC-PDA system was efficient for real-time extraction and quantification of piceatannol from passion fruit bagasse and chlorogenic acid and caffeine from and coffee husks. Therefore, the proposed system represents an alternative to integrate online the PLE and HPLC to analyze biocompounds from solid samples. The extraction yields obtained by online operation and corrected by correction factor were statistically equal as the extraction yields achieved after the extract fractions collection (offline injections). Therefore, showing the system potential to quantify in real-time biocompounds from solid samples. However, the system presents some limitations that need to be considered, which were defined in this work; the choice of the time of the first inline injection must be evaluated for each individual sample; the mass of raw material and the extraction solvent flow rate must consider the target compound content and the range of the calibration curve; a shorter time chromatographic method allows lower differences between online and offline quantifications, so shorter methods are preferable; the compatibility of the extraction solvent and mobile phase must be considered. Hence, the 2D PLE × HPLC-PDA system demonstrates significant potential for real-time quantification of extracted compounds. However, further refinement of the technology and the exploration of environmentally friendly methods are essential to minimize both the use of organic solvents and the overall processing time.

CRediT authorship contribution statement

Juliane Viganó: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Monique Martins Strieder:** Investigation, Methodology, Writing – original draft. **Rodrigo S. Pizani:** Investigation, Methodology, Writing – original draft. **Letícia S. Contieri:** Investigation, Methodology, Writing – original draft. **Leonardo M. de Souza Mesquita:** Investigation, Methodology, Writing – original draft. **Mauricio A. Rostagno:** Writing – review & editing, Supervision, Project administration, 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 data

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