Advances in Sample Preparation 9 (2024) 100107

Contents lists available at ScienceDirect

Advances in Sample Preparation

journal homepage: www.elsevier.com/locate/sampre

Salt-assisted liquid-liquid extraction and on-column concentration for chromatographic determination of phenolic compounds in beer

Luís Claudio Martins [a,1], Maria Soledad M.S.F. Acevedo [a,1], Mariana R. Gama [b], Fábio R. P. Rocha [a,*]

[a] Center of Nuclear Energy in Agriculture - University of São Paulo, Avenida Centenário, 303, 13400-970 Piracicaba, SP, Brazil
[b] Institute of Chemistry - Federal University of Rio Grande do Sul, Avenida Bento Gonçalves, 9500, 91540-000 Porto Alegre, RS, Brazil

ARTICLE INFO

Keywords:
Sample preparation
Preconcentration
Liquid chromatography
Flavonoids
Phenolic acids

ABSTRACT

Salt-assisted liquid-liquid extraction (SALLE) and on-column concentration were evaluated to simplify the chromatographic determination of phenolic compounds in beer. Resveratrol, flavonoids (quercetin and kaempferol), and phenolic acids (caffeic, p-coumaric, and trans-ferulic acids) were used as model compounds based on their concentrations reported in craft beers. SALLE with acetonitrile was effective for sample cleanup, with recoveries ranging from 84 % to 112 %. The on-column concentration was carried out in high performance liquid chromatography by starting the gradient elution with acidified water (sample carrier stream) in a reversed-phase mode. Consequently, the analytes were retained in a narrow band, and highly polar/ionic potentially interfering species were flushed from the column. The effectiveness of the approach was demonstrated by linear correlations between the peak areas and the injected volumes from 10 to 200 μL ($r \geq 0.999$), without significant variations in peak width (< 10 %), retention times (< 0.7 %), and peak symmetry (tailing factors within the range of 0.8–1.1). For all analytes, a linear response was achieved from 100 to 1000 μg L^{-1} ($r > 0.999$), with limits of detection within the range of 9–82 μg L^{-1}. The approach was also effective for on-column concentrations of resveratrol, quercetin, and kaempferol in sequential injection chromatography (SIC), with injected volumes up to 750 μL and linear responses within 100–1000 μg L^{-1} ($r > 0.997$). The proposal reduced consumption of organic solvents (with only 770 μL required per determination in SIC) and avoided solid-phase extraction cartridges for analyte preconcentration.

Introduction

Beer has been consumed as beverage for more than 5000 years [1]. The widespread consumption has motivated studies on its nutritional effects as a source of carbohydrates, amino acids, minerals, vitamins, and other macromolecules [2]. Beer typically contains numerous secondary metabolites, such as phenolic compounds, with remarkable antioxidant properties [3].

The phenolic compound content depends on the raw material used in the beer manufacturing process, with barley and hops providing approximately 70 %–80 % and 20 %–30 % of the total phenolics, respectively [4]. In addition to their antioxidant properties, phenolic compounds contribute to the organoleptic characteristics of the final products, such as flavor, astringency, and haze, and affect the filtration process and stability of beers during aging, thus affecting their shelf life [5]. Despite these significant effects, phenolic compounds are typically found in relatively low amounts in beer, e.g., 0.3–2.2 μg kg^{-1} caffeic acid, 0.6–4.7 μg kg^{-1} p-coumaric acid, 7.2–15.8 μg kg^{-1} trans-ferulic acid, and 0.7–2.1 μg kg^{-1} quercetin [6]. Although kaempferol and resveratrol are less common in beer, their determination is relevant owing to their bioactive properties [7]. For example, 9.0 μg kg^{-1} trans-resveratrol, 3.1 μg kg^{-1} cis-resveratrol, and 0.5 μg kg^{-1} trans-piceid (the resveratrol glucoside) were determined in beers using liquid chromatography–tandem mass spectrometry (LC-MS/MS) [8].

Phenolic compounds in beer are usually determined using liquid chromatography (LC), with detection by either UV spectrophotometry [9–11] or mass spectrometry [6,8,12,13]. Owing to the complexity of the samples and the relatively low concentration of some phenolic compounds, these procedures typically require sample cleanup and analyte preconcentration. The need for analyte preconcentration was

* Corresponding author.
E-mail address: frprocha@cena.usp.br (F.R.P. Rocha).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.sampre.2024.100107
Received 31 December 2023; Received in revised form 15 February 2024; Accepted 20 February 2024
Available online 22 February 2024

recently demonstrated [11]. Without sample pretreatment, relatively high limits of quantification were achieved (1.2 mg L^{-1} quercetin and 2.8 mg L^{-1} kaempferol), which hindered the practical applications of the proposed procedure [11].

Although essential for chromatography, sample treatment techniques typically involve several steps, are time-consuming, and generate large amounts of waste. These drawbacks have motivated further studies focused on sample cleanup and analyte preconcentration methods. One promising approach is salt-assisted liquid-liquid extraction (SALLE), in which analytes are extracted from aqueous samples using water-soluble solvents (e.g., acetone, 2-propanol, ethanol, and majorly acetonitrile), with the addition of an inorganic salt (e.g., NaCl, Na_2SO_4, or $MgSO_4$) to promote the formation of a two-phase system [14]. As the saline medium reduces the solubility of moderately polar analytes in the aqueous phase, they are separated from highly polar, potentially interfering species. SALLE is usually carried out in a single closed flask, minimizing the exposure of the analyst to volatile solvents and the number of steps involved compared to conventional liquid-liquid extraction. Its applicability in the isolation of phenolic compounds from several complex matrices has been demonstrated [15–20]. Sample pretreatment by SALLE has also been applied to determination of toxins in beer using LC-MS/MS [18].

On-column concentration is an interesting alternative to time-consuming approaches for analyte preconcentration. In this strategy, analytes are accumulated on the head of the chromatographic column (or even on the guard column) by modulating the eluent strength. A relatively large volume of the sample in a low-elution strength solvent is directed towards the chromatographic column. Analytes with high retention factors are effectively retained in a narrow zone and further eluted using an appropriate mobile phase, with a higher chromatographic elution strength [21]. A similar approach has been used for sample stacking in capillary electrophoresis [22] and cold trapping in gas chromatography [23], but it has been less usual in high-performance liquid chromatography (HPLC) [24,25], including miniaturized LC [26]. The feasibility of on-column concentration was demonstrated using sequential injection chromatography (SIC) by exploiting its capability to handle samples, carrier, and mobile phase under programmable flow [27]. By injecting 5 mL of aqueous solution, enrichment factors (EF) as high as 435, 405, and 420 were achieved for methyl, ethyl, and propylparaben, respectively [21]. Another application aimed at determination of traces of the emerging contaminant salicylic acid in natural waters achieved a limit of detection (LOD) as low as 20 ng L^{-1} and an EF of 122, consuming only 1.75 mL of sample and 150 μL of organic solvent per determination [28].

This study presents the evaluation of SALLE for sample cleanup and on-column concentration of analytes to simplify the determination of phenolic compounds in beer and improve detectability in either HPLC or SIC. A stilbene (resveratrol, REV), flavonoids quercetin (QUE) and kaempferol (KAF), and phenolic acids, caffeic acid (CAF), p-coumaric acid (COA), and trans-ferulic acid (FER) were used as model compounds based on the concentrations reported in craft beers and to evaluate the efficiencies for species with significant differences in polarities.

Materials and methods

Reagents and solutions

All standards were acquired with analytical grade (purity ≥ 95 %) from Sigma-Aldrich, St Louis, MO, USA (caffeic acid ≥ 98 %, p-coumaric acid ≥ 98 %, trans-ferulic acid 99 %, and quercetin ≥ 95 %), Oakwood Chemical, Columbia, SC, USA (resveratrol, 99 %), and TargetMol, Boston, MA, USA (kaempferol, 99 %), and used without further purification. HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Stock solutions of each analyte (1000 mg L^{-1}) were prepared in ethanol and stored in dark at −10 °C. Working multispecies solutions within the concentration ranges of 10–1000 μg L^{-1} were prepared daily by diluting the stock solution with ultrapure water (resistivity > 18 MΩ cm^{-1}). Water was acidified to pH 2.5 with H_3PO_4 (Sigma-Aldrich), and used as the mobile phase, either in isocratic (SIC) or gradient elution (HPLC), with acetonitrile as the organic solvent.

Apparatus

HPLC

HPLC analyses were performed using a Shimadzu Prominence LC-20A system (Kyoto, Japan) equipped with an LC-20AT quaternary pump, CTO-20A column oven operating at 30 °C, SIL-20A autosampler, and SPD-M20A photodiode array detector with an 8-μL flow cell. Data were acquired and processed using the Chrom Perfect software version 5.93 (Shimadzu). All separations were carried out using a C_{18} chromatographic column (Shimadzu Shim-pack VP-ODS, 150 × 4.6 mm i.d., 5.0 μm particle size; 12 nm pore size) with a 5 mm long guard column.

Sequential injection chromatography

Low-pressure chromatography was performed using a SIChrom® equipment (FIAlab® Instruments, Bellevue, WA, USA) equipped with a 4.0 mL S17 PDP syringe pump (Sapphire Engineering Inc., Pocasset, MA, USA) and an 8-port high-pressure stainless-steel selection valve (C5H, Valco Instrument Co., Houston, TX, USA). Polyether ether ketone tubes (0.25 mm i.d.) were used for all connections in the SIC system and a 1.0 mL, 0.6-mm i.d. coiled polytetrafluoroethylene tube was used as the holding coil. The SIC system was controlled by the FIAlab® 5.9 software. Separations were carried out in a silica-octadecylsilane (C_{18}) superficially porous particle-packed column (Supelco Ascentis® Express, Sigma-Aldrich, 30 × 4.6 mm i.d., 2.7 μm particle size) coupled to a 5-mm long guard column.

The detection system was a multichannel CCD spectrophotometer (USB4000®, Ocean Optics, Dunedin, FL, USA), with a deuterium light source (DH-2000®, Ocean Optics) and optical fibers with a core diameter of 600 μm. The spectrophotometer was coupled to a 9-μL Z-shape flow cell with a 20 mm optical path (FIAlab® Instruments).

Sample cleanup

Four craft lager beer samples acquired at the local market were used to evaluate the SALLE procedure. These samples showed alcohol contents between 4.5 and 4.8 % v/v. After degassing under agitation for 2 min, the sample cleanup by SALLE was adapted from a previously described method to determine volatile phenols in beverages [29]. Briefly, 1.0 mL of each beer sample and 2.4 mL of acetonitrile were vortexed for 1 min in 15 mL Falcon® tubes. Phase separation was induced by adding 0.5 g NaCl to the mixture, which was further vortexed for 1 min and centrifuged at 2400 rpm (950 × g) for 10 min. All sample pretreatment steps were carried out at ambient temperature (25 ± 1 °C) and, after centrifugation, three distinct phases were observed: excess NaCl at the bottom, the aqueous intermediary phase, and the upper organic phase. For the on-column concentration, 1.0 mL of the organic phase was collected and the solvent was evaporated under N_2 flow. The solute was suspended in 1.0 mL of water acidified to pH 2.5 and subsequently analyzed by HPLC or SIC.

For the dilute-and-shoot approach in HPLC, beer samples were filtered through 0.22 μm PVDF filters and further diluted 1:1 v/v in deionized water.

On-column concentration procedure

HPLC

The on-column concentration was carried out with multispecies solutions and sample volumes of 10, 20, 50, 100, and 200 μL. Because of the capacity of the LC autosampler, the latter volume required two sequential injections of 100 μL. Gradient elution (A: water with pH adjusted to 2.5; B: acetonitrile) was performed according to the

following program: 0 % B (0–5 min), 25 % B (5–25 min), 40 % B (25–40 min), and 0 % B (40–45 min). The flow rate of the mobile phase was 0.4 mL min^{-1}. All measurements were performed in triplicate and based on the peak areas at 320 nm (CAF, COA, FER, and REV) and 360 nm (QUE and KAF).

Sequential injection chromatography

For the on-column concentration and separation of resveratrol, quercetin, and kaempferol, the SIC system (Fig. 1) was operated as follows: the syringe pump (SP) sequentially aspirated 250 μL of water (port 3, 50 μL s^{-1}) and 750 μL of the sample extract or reference solution (port 8, 10 μL s^{-1}) to the holding coil (HC). The flow direction was reversed and the sample zone was pumped through the chromatographic column (port 7) at 10 μL s^{-1} to retain the analytes at the head of the column. Subsequently, 2200 μL of the mobile phase (35 % v/v acetonitrile in water acidified to pH 2.5) was aspirated through port 4 to the HC and further dispensed through the chromatographic column at 10 μL s^{-1} for analyte elution. All measurements were carried out in triplicates at room temperature (25 ± 1 °C) and were based on the peak areas at 320 nm (REV) and 360 nm (QUE and KAF).

Analytical figures of merit

Calibration equations were defined using least-squares regression and linearity was evaluated by the lack-of-fit test at the 95 % confidence level. The limits of detection were estimated at a 99.7 % confidence level from $3xS_{y/x}/m$, where $S_{y/x}$ is the standard deviation of the y-residuals and m is the slope of the calibration curve [30]. Recoveries were estimated from beer samples spiked with either 0.25 or 1.0 μg mL^{-1} of each analyte. EF values were estimated from the ratio of the slopes of calibration curves obtained with (injected volumes of 200 and 750 μL for HPLC and SIC, respectively) and without on-column concentration (injected volume of 10 μL). Consumptive indexes (CI) were determined from the ratio of the injected volume to the achieved EF values [31]. Peak symmetry was evaluated from tailing factors calculated at 5 % of the peak height as recommended [32].

Results and discussion

On-column concentration

HPLC

The on-column concentration requires modulation of the elution strength for the retention of the analytes on the head of the chromatographic column and further elution without significant peak broadening. This approach is then compatible with gradient elution in HPLC, which was exploited in this study. As the separations were carried out in reversed-phase mode, the gradient started with water, which was acidified to pH 2.5 to favor the retention of the protonated forms of the analytes, majorly phenolic acids (pKa1 = 4.43 (CAF), 4.36 (COA), and 4.52 (FER)) [33]). In this step, acidified water was the carrier solution to avoid carryover of the sample and eluent zones.

The effectiveness of on-column concentration for all the phenolic compounds investigated is shown in Fig. 2, in which an increase in the injected volume exhibit a negligible effect on the peak widths and retention times (RT). Therefore, the chromatographic resolution was unaffected even for the species eluted at the closest retention times (COA and FER), as shown in the inset Fig. 2. The peak areas (A) increased linearly with the sample volume (V) from 10 to 200 μL, as described by the following equations: $A_{CAF} = 14{,}353\ V - 11{,}317$ ($r = 0.9998$); $A_{COA} = 16{,}025\ V - 13{,}566$ ($r = 0.9998$); $A_{FER} = 13{,}720\ V - 103{,}537$ ($r = 0.9998$); $A_{REV} = 17{,}621\ V - 13{,}957$ ($r = 0.9999$); $A_{QUE} = 70{,}312\ V + 11{,}568$ ($r = 0.9986$); and $A_{KAF} = 8161\ V + 7845$ ($r = 0.9999$).

Linear calibration graphs were obtained for all analytes, $r > 0.999$ (Fig. 3), with on-column concentration (200 μL injected volume), as confirmed by the lack-of-fit test at 95 % confidence level. The coefficients of variation of the retention times were < 0.7 % for all analytes, and peak symmetry was not hindered. Tailing factors were between 0.8 and 1.1 for all analytes in the evaluated concentration range, with variations lower than 10 % by increasing the injected volumes, as shown in the Table 1. The separation efficiency was calculated for all analytes and showed values from 8016 plates (COA) to 36704 plates (REV) using injection volume of 200 μL (1000 μg L^{-1} of each analyte). The EFs were 20.7 (CAF and FER), 21.3 (COA and REV), 29.7 (QUE), and 15.7 (KAF), which were close to the expected values for the volume ratio of 20. The LODs were estimated to be 38 μg L^{-1} CAF, 9 μg L^{-1} COA, 26 μg L^{-1} FER, 29 μg L^{-1} REV, 18 μg L^{-1} QUE, and 82 μg L^{-1} KAF. These values demonstrate the efficiency of the proposed approach for preconcentration of the analytes.

Sequential injection chromatography

The feasibility of on-column concentration was also demonstrated in SIC, focusing on the rapid separation of resveratrol, quercetin, and kaempferol. Because of the low-pressure operation, short monolithic or superficially porous particle columns are required for this technique, and the selectivity of the stationary phase is decisive for successful separation. Therefore, a chromatographic column was selected among some options of silica-based monoliths and particle-packed columns with different surface chemistries (cyanopropyl, octadecyl, and pentafluorophenyl). Monolithic cyanopropyl and superficially porous pentafluorophenyl columns were evaluated based on the moderate polarity of the phenolic compounds; however, both yielded poor peak shapes and low chromatographic resolution, even with different acetonitrile/water mobile phase compositions. Enhanced chromatographic performance was achieved using a C_{18} superficially porous particle column. Isocratic elution with 35 % (v/v) acetonitrile in water acidified to pH 2.5 as mobile phase was effective in separating compounds with narrower peaks.

Similar to the HPLC procedure, water was selected as the carrier owing to its weak solvent strength in reversed-phase chromatography. This also contributed for removal of potentially interfering highly polar species simultaneously to the analyte retention. The effects of the carrier volume are shown in Table 2. Although the RT values were only slightly affected, the effects on the peak width and area were significant, demonstrating the relevance of the carrier stream in minimizing the overlap between the sample and eluent zones. Narrow peaks indicate the

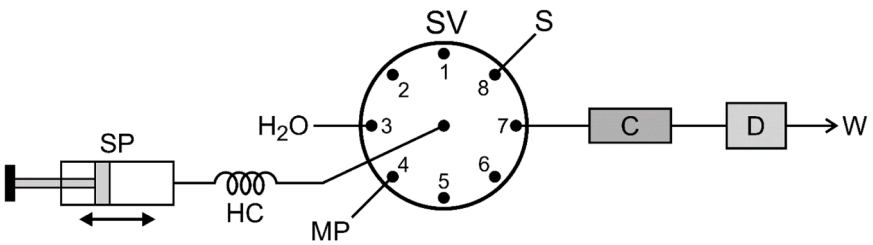

Fig. 1. Flow diagram of the sequential injection chromatographic system for on-column concentration of phenolic compounds. C: chromatographic column; D: spectrophotometric detector; HC: holding coil; MP: mobile phase; S: sample; SP: bidirectional syringe pump; SV: selection valve; W: waste vessel.

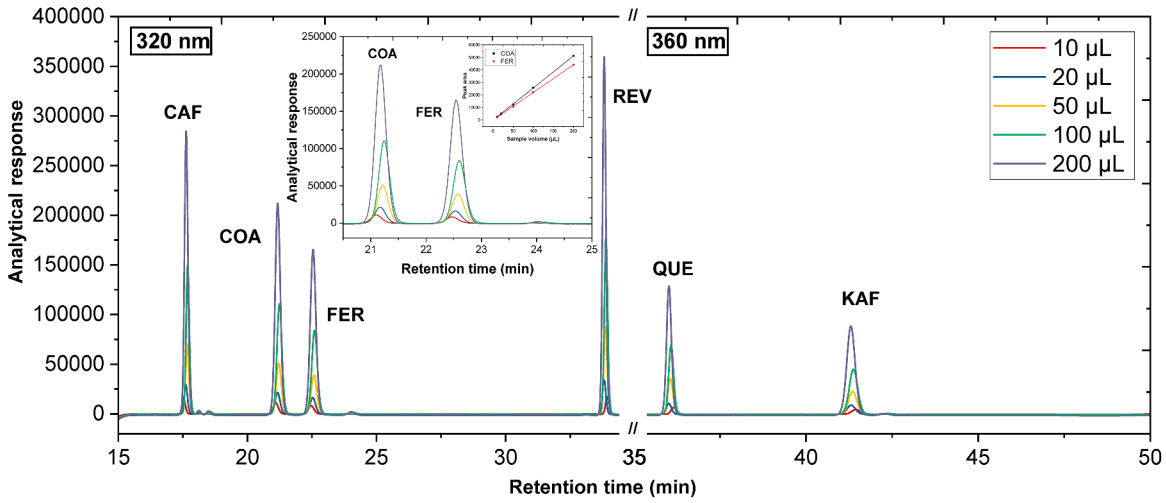

Fig. 2. On-column concentration from different sample volumes in HPLC. The inset shows a larger view of the separation of p-coumaric acid (COA) and trans-ferulic acid (FER) and the linear correlation of peak areas and the sample volume. CAF: caffeic acid, REV: resveratrol; QUE: quercetin; KAF: kaempferol. Analyte concentrations: 1.0 mg L^{-1}.

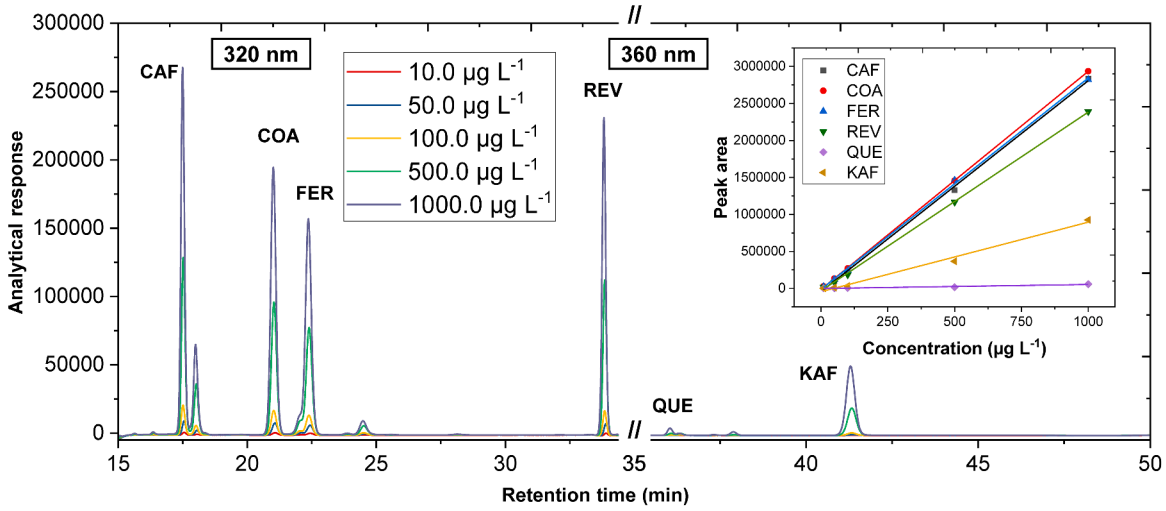

Fig. 3. On-column concentration in HPLC from solutions with increasing concentrations of the analytes (injected volume = 200 µL). The inset shows the corresponding calibration graphs. CAF: caffeic acid, COA: p-coumaric acid; FER: trans-ferulic acid; REV: resveratrol; QUE: quercetin; KAF: kaempferol.

Table 1
Tailing factors of the chromatographic peaks with on-column concentration of phenolic compounds by HPLC.

Concentration (µg L^{-1})	Injected volume (µL)	CAF	COA	FER	REV	QUE	KAF
10	200	1.0	1.0	0.9	1.1	–	1.1
100	200	1.1	1.1	1.0	1.1	0.8	1.1
1000	10	1.0	1.0	1.0	1.1	1.1	1.0
	20	1.1	1.0	1.0	1.0	1.1	1.0
	50	1.1	1.0	1.0	1.0	1.1	1.0
	100	1.1	1.0	1.0	1.0	1.1	1.0
	200	1.1	1.0	1.0	1.0	1.0	1.0

Tailing factors were calculated at 5 % of the peak height [32].

effective accumulation of the analytes on the head of the chromatographic column and that peak resolution was not impaired by the proposed approach.

The capability for handling higher sample volumes in SIC was investigated for on-column concentration of up to 750 µL injected volumes. The efficiency is demonstrated by the results shown in Fig. 4. Neither the peak widths nor RT changed significantly with the increase in the sample volume; the peak areas (A) increased linearly with the sample volume (V, µL), as described by the equations: $A_{REV} = 0.0137\ V + 1.18$ (r = 0.990), $A_{QUE} = 0.0151\ V + 1.61$ (r = 0.987), and $A_{KAF} = 0.0137\ V + 0.166$ (r = 1). The linear response decreased for sample volumes > 750 µL, which was attributed to the saturation of absorptive material in the column. In addition to analyte amount, this effect depends on the column size, particle diameter, and pore size; thus, it is more critical for superficially porous particle columns, as used in this study. For a sample volume of 750 µL, a suitable resolution (Rs = 1.8) for resveratrol (RT = 1.27 min) and quercetin (RT = 1.47 min) was achieved. Fig. 4 shows the fast chromatographic separation of the three analytes (approximately 3 min), a characteristic of SIC. Consequently, the consumption of mobile phase decreased (equivalent to 770 µL acetonitrile vs. 4400 µL in HPLC for each determination).

With a 750 µL injected sample, linear responses were achieved from 100 to 1000 µg L^{-1}, as verified by the lack-of-fit test at the 95 % confidence level, with coefficient of variation (CV) < 7 % for inter-day precision (n = 5). The LOD values were 89, 150, and 35 µg L^{-1} for REV, QUE, and KAF, respectively. The CV of the retention times and

Table 2
Effect of the carrier volume on the retention time and peak width of phenolic compounds in SIC (1.0 mg L^{-1} each).

Carrier volume (μL)	Retention time (s)			Peak area			Peak width (s)		
	REV	QUE	KAF	REV	QUE	KAF	REV	QUE	KAF
0	75.5	86.7	141.2	2.3	2.2	1.9	30.6	36.6	37.8
250	75.6	87.6	141.6	5.4	5.1	3.2	17.4	23.4	23.4
500	75.6	88.2	141.6	5.5	4.4	2.8	18.6	22.2	24.6
750	76.2	88.2	142.2	5.5	6.1	3.8	16.8	23.4	22.8
1000	75.6	88.1	141.6	5.4	6.1	3.8	15.1	22.2	21.1

REV: resveratrol; QUE: quercetin; KAF: kaempferol.

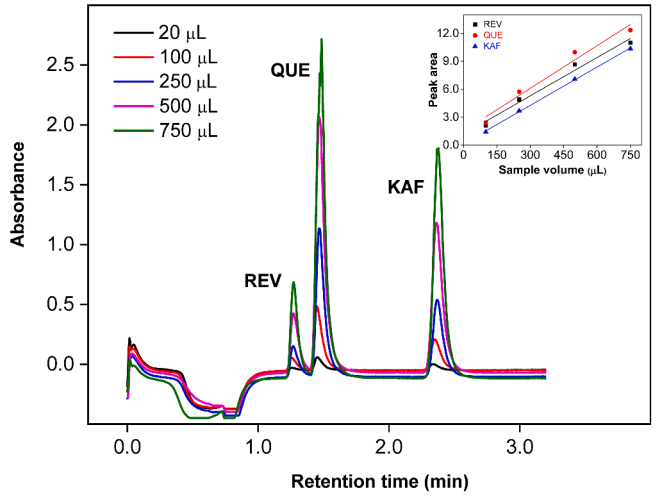

Fig. 4. Chromatographic separation of REV, QUE, and KAF (3.0 mg L^{-1} each) after on-column concentration from different injection volumes in SIC. The inset shows the increment of peak areas with the sample volume.

peak areas (0.45 and 2.1 %, respectively) were comparable to those previously obtained for separation using SIC [27,34,35].

Peak symmetry was more affected in SIC in comparison to HPLC. In fact, by increasing the injection volumes, tailing factors were between 1.2 to 1.8, 1.3 to 1.9, and 0.8 to 1.6, for REV, QUE, and KAF, respectively. This reflects the different operational conditions, including different column characteristics and mobile phase flow rates. However, the chromatographic resolution was not hindered (Fig. 4). The enrichment factors for REV, QUE, and KAF were 104, 79, and 67, respectively. These values are not comparable to previous works in SIC, which involved higher sample volumes (1.5 and 5.0 mL for preconcentration of salicylic acid [28] and parabens [21], respectively). Alternatively, this comparison may be based on CI [31], which demonstrates the efficiency of sample utilization in the preconcentration step. The achieved CI values (7.2, 9.5, and 11.2 μL for REV, QUE, and KAF, respectively) were comparable or superior than the previously reported values for on-column concentration in SIC (CI within 11.5–12.3 μL for parabens [21] and 14.3 μL for salicylic acid [28]), demonstrating the effectiveness of the approach for analyte preconcentration.

Salt-assisted liquid-liquid microextraction

Beer samples have complex matrices with diverse constituents in larger amounts than those of phenolic compounds. In addition, the composition may change significantly among different samples, particularly craft beers.

The chromatograms of 2-fold diluted samples (Fig. 5) show the difficulties in direct sample analysis. Despite the good resolution of some peaks, the determination of some of the investigated analytes was hindered by co-elution or baseline drift (sample 1: CAF and FER; sample 2: CAF and REV; sample 3: FER and REV; and sample 4: REV). In addition, direct sample injection may affect long-term chromatographic performance by introducing high amounts of concomitant species into the chromatographic column. Because loading a column with high amounts of foreign species is more critical for higher injected volumes, a previous sample cleanup is also beneficial for on-column concentration.

A simple procedure based on SALLE was then investigated for sample cleanup to isolate phenolic compounds from the sample matrix. This process is favored because of the higher solubility of analytes in polar organic solvents (such as acetonitrile) in comparison to water [36]. Although SALLE is not a selective extraction approach, it is effective for separation of moderately polar analytes from highly polar species and ions that tend to remain in the aqueous saline phase. The experimental conditions were established by varying the acetonitrile volume from 1.5 to 3.0 mL, for 1.0 mL of beer sample and excess NaCl (0.5 g). Superior phase separation and higher analyte recoveries (from 92 to 108 % for samples spiked with 1.0 μg mL^{-1} of each analyte) were achieved with 2.4 mL of acetonitrile. Sodium chloride was selected for SALLE based on previous results on the extraction of phenolic compounds [37]. Acetonitrile was evaporated from the sample extracts, and the solute was dissolved in acidified water (pH 2.5) to ensure that the injected solutions had a low elution strength medium needed for on-column concentration.

The effectiveness of this approach for the cleanup of beer samples is shown in Fig. 6, which demonstrates a superior resolution of the analytical peaks and elimination of baseline drifts, notably related to the more polar compounds eluted at the beginning of the chromatographic run (up to 15 min). This result was mainly reflected in the determination of the phenolic acids. As shown in Fig. 6, SALLE was poorly efficient in removing a matrix compound that eluted between COA and FER and affected analyte resolution (Rs = 1.1 for both COA-peak and peak-FER). However, for potentially co-extracted matrix compounds that are eluted close to resveratrol, higher peak purity is achieved after SALLE, as evaluated from the absorption spectra (see the resveratrol spectra in the inset of Fig. 6). As shown in Table 3, because of co-elution, the concentrations determined by the direct injection of 1:1 v/v diluted samples were 3.7 to 5.3-fold higher for CAF and 2-fold higher for COA and FER than those determined after SALLE. Although the discrepancies tended to be lower for REV, QUE, and KAF, the values determined without SALLE were 30 %–80 % higher for sample 4. SALLE also prevents the hindrance of loading matrix components by the direct injection of raw beer samples.

Under the established experimental conditions, the effectiveness of SALLE for the cleanup of beer samples was demonstrated by recoveries within 84 % and 112 %, except for REV in sample 4 (Table 4). For comparison, recoveries of 63 % (CAF), 88 % (COU), 92 % (QUE), and 100 % (FER) were achieved by SPE/HPLC-DAD [9].

Comparison to literature methods

Compared to previously described LC methods for the determination of phenolic compounds, the proposed method exhibits a lower consumption of organic solvents in both chromatographic separation (from 3.5 to 19.5-fold lower than previously described approaches, Table 5) and sample cleanup (e.g. up to 300 mL of toluene and cyclohexane are consumed per determination [38]), and a shorter analysis time,

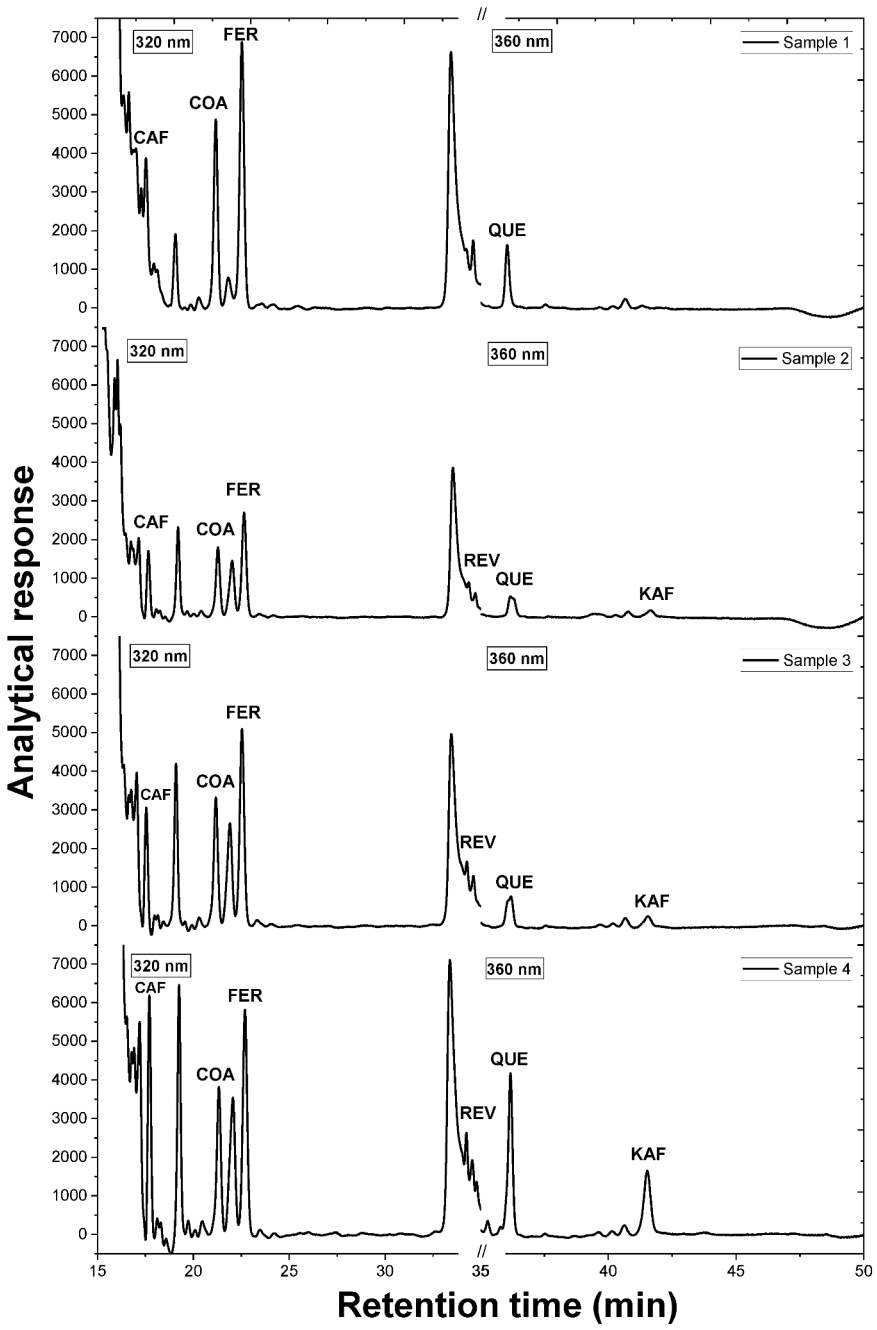

Fig. 5. Chromatograms obtained from direct injection of 1:1 v/v diluted beer samples in HPLC. Sample volume: 10 μL. CAF: caffeic acid, COA: p-coumaric acid; FER: trans-ferulic acid; REV: resveratrol; QUE: quercetin; KAF: kaempferol.

demonstrating the advantage of on-column concentration. In the evaluation of the run times in Table 5, it should be considered that the reported studies have focused on analytes other than the six species evaluated in this study; primarily, those involving MS detection may yield diverse results, thus making the run times less relevant. Moreover, the extraction cartridges and drawbacks of conventional solid-phase extraction (time-consuming, risks of analyte loss, and contamination) were prevented. The precision achieved was better than that previously reported, and both the acquisition and operational costs were significantly lower than those for LC-MS/MS [6,8,13]. The LODs and linear working ranges were consistent with the analyte concentrations typically found in beers.

Conclusions

The effectiveness of two approaches for sample cleanup and analyte preconcentration in the chromatographic determination of phenolic compounds was demonstrated, contributing to a reduction in the analytical steps and operational costs involved in the chemical analysis of beers. SALLE was effective in extracting a stilbene (resveratrol), flavonoids (quercetin and kaempferol), and caffeic, p-coumaric, and trans-ferulic phenolic acids, resulting in improved chromatographic resolution and preventing baseline drift. On-column concentration was effective in both HPLC and SIC, which stood out because of its improved sensitivity/detectability and sample throughput/environmental friendliness. By avoiding the use of solid-phase extraction cartridges and the need for organic solvents in the washing step, on-column concentration

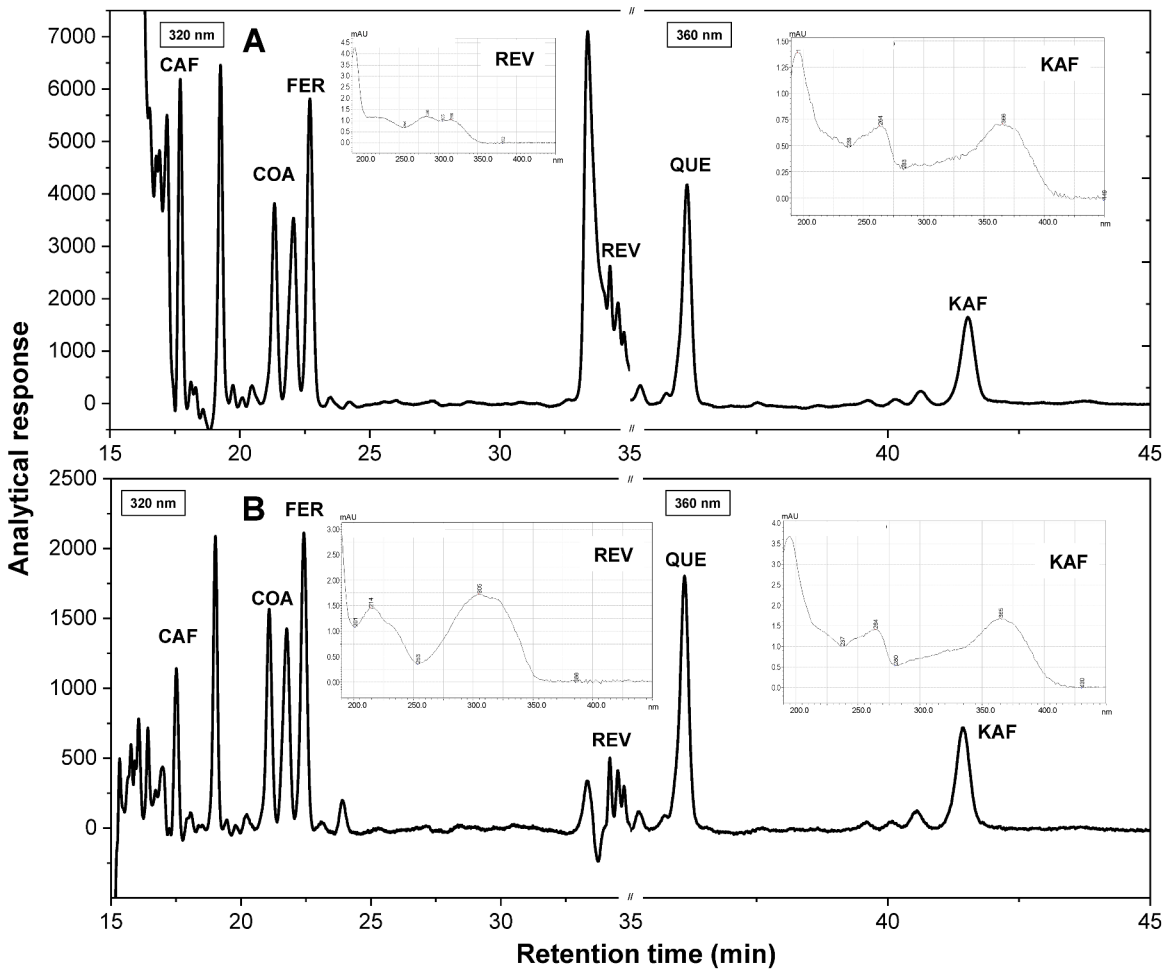

Fig. 6. HPLC chromatograms of a beer sample (sample 4, diluted 1:1 v/v) before (A) and after (B) SALLE. Sample volume: 10 μL. CAF: caffeic acid, COA: p-coumaric acid; FER: trans-ferulic acid; REV: resveratrol; QUE: quercetin; KAF: kaempferol.

Table 3
Concentrations of phenolic compounds (μg L^{-1}) in beers determined by HPLC with and without SALLE.

Sample	CAF		COA		FER		REV		QUE		KAF	
	Without SALLE	SALLE	Without SALLE	SALLE	Without SALLE	SALLE	Without SALLE	SALLE	Without SALLE	SALLE	Without SALLE	SALLE
1	374	70.6	870	451	1864	882	< LOD	< LOD	656	527	117	150
2	293	78.3	323	145	728	347	44.4	43.7	467	430	154	141
3	527	133	604	298	1365	638	62.5	54.7	553	481	183	170
4	1032	243	696	333	1562	687	98.2	73.8	1318	873	749	426

Table 4
Recoveries of phenolic compounds from beer samples determined by HPLC after SALLE. Samples spiked with 250 μg L^{-1} of each species.

Sample	Concentration, μg L^{-1} (recovery)					
	CAF	COA	FER	REV	QUE	KAF
1	254 (102 %)	239 (96 %)	257 (103 %)	243 (97 %)	261 (105 %)	191 (77 %)
2	264 (106 %)	249 (99 %)	252 (101 %)	239 (95 %)	252 (101 %)	236 (94 %)
3	250 (100 %)	250 (100 %)	275 (110 %)	200 (80 %)	257 (103 %)	238 (95 %)
4	241 (97 %)	238 (95 %)	255 (102 %)	167 (67 %)	257 (103 %)	256 (102 %)

also minimizes the risks of analyte loss and contamination by sample manipulation. Sample preparation was simplified compared to previous proposals, requiring only SALLE for effective isolation of the analytes with minimal solvent consumption. The combination of these approaches was successfully applied to complex matrices such as beer.

CRediT authorship contribution statement

Luís Claudio Martins: Conceptualization, Investigation, Validation, Writing – original draft. **Maria Soledad M.S.F. Acevedo:** Conceptualization, Investigation, Validation, Writing – original draft. **Mariana R. Gama:** Conceptualization, Writing – original draft. **Fábio R.P. Rocha:** Conceptualization, Supervision, Writing – review & editing.

Table 5
Comparison of chromatographic methods for determination of phenolic compounds in beer samples.

Detection	Run time (min)	Solvent consumption (mL)	Sample pretreatment	LOD (μg L^{-1})	CV (%)	Ref.
MS/MS	18	5.6[a]	SPE	28 (CAF), 11 (COU), 115 (FER), 58 (QUE)	1.7–6.8	[6]
DAD	42	19.3[b]	Filtration	220 (CAF), 80 (COU), 80 (FER), 350 (QUE), 830 (KAF)	5.7–7.7	[11]
PDA[a]/MS[b]	32	6.6[b]	LLE	20[a]/10[b] (QUE), 30[a]/12[b] (KAF), 30[a]/6[b] (FER)	8–14	[12]
DAD	57	15[a]	SPE	80 (CAF), 40 (COU), 10 (FER),110 (QUE)	2–6	[9]
DAD	35	15[a]	SPE	60 (CAF), 40 (COU), 70 (FER), 160 (QUE)	1–3	[10]
ESI-MS/MS	42	9.0[b]	SPE	—	—	[13]
PDA-ESI-MS/MS	58	11.6[b]	Filtration	60 (FER/COA/KAF), 70 (QUE)	—	[39]
MS/MS	6.0	2.7[c]	LLE and SPE	1 (REV)	2.5–13.6	[8]
DAD	45	4.4[b]	SALLE	38 (CAF), 9 (COU), 26 (FER), 28 (REV), 18 (QUE), 82 (KAF)	—	This work (HPLC)
DAD	2.5	0.77[b]	SALLE	89 (REV), 150 (QUE), 35 (KAF)	0.45	This work (SIC)

[a] methanol.
[b] acetonitrile.
[c] acetonitrile plus acetone, REV: resveratrol, QUE: quercetin, KAF: kaempferol, FER: ferulic acid, CAF: caffeic acid, COA: p-coumaric acid, SALLE: salt-assisted liquid-liquid extraction, SPE: solid-phase extraction, LLE: liquid-liquid extraction.

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.

Acknowledgements

The authors thank the fellowships and financial support from the Brazilian Agencies National Council of Technological and Scientific Development (CNPq, grant 315866/2021-7), São Paulo Research Foundation (FAPESP, proc. 2021/02309-5), and Coordination for the Improvement of Higher Education Personnel (CAPES), Financial code 001. This is a contribution of the National Institute of Advanced Analytical Sciences and Technology (INCTAA).

References

[1] J. Wang, L. Liu, T. Ball, L. Yu, Y. Li, F. Xing, Revealing a 5,000-y-old beer recipe in China, Proc. Natl. Acad. Sci. 113 (2016) 6444–6448, https://doi.org/10.1073/pnas.1601465113.
[2] C.W. Bamforth, Nutritional aspects of beer—a review, Nutr. Res. 22 (2002) 227–237, https://doi.org/10.1016/S0271-5317(01)00360-8.
[3] A. Martinez-Gomez, I. Caballero, C.A. Blanco, Phenols and melanoidins as natural antioxidants in beer. Structure, reactivity and antioxidant activity, Biomolecules 10 (2020) 400, https://doi.org/10.3390/biom10030400.
[4] C. Gerhäuser, H. Becker, Phenolic compounds in beer, in: Victor R. Preedy (Ed.), Beer Heal. Dis. Prev, Elsevier Inc. Academic Press, 2009.
[5] D. Šibalić, M. Planinić, A. Jurić, A. Bucić-Kojić, M. Tišma, Analysis of phenolic compounds in beer: from raw materials to the final product, Chem. Pap. 75 (2021) 67–76, https://doi.org/10.1007/s11696-020-01276-1.
[6] M. Cortese, M.R. Gigliobianco, D.V. Peregrina, G. Sagratini, R. Censi, P. Di Martino, Quantification of phenolic compounds in different types of crafts beers, worts, starting and spent ingredients by liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1612 (2020) 460622, https://doi.org/10.1016/j.chroma.2019.460622.
[7] M. Nardini, An overview of bioactive phenolic molecules and antioxidant properties of beer: emerging trends, Molecules 28 (2023) 3221, https://doi.org/10.3390/molecules28073221.
[8] G. Chiva-Blanch, M. Urpi-Sarda, M. Rotchés-Ribalta, R. Zamora-Ros, R. Llorach, R. M. Lamuela-Raventós, R. Estruch, C. Andrés-Lacueva, Determination of resveratrol and piceid in beer matrices by solid-phase extraction and liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 1218 (2011) 698–705, https://doi.org/10.1016/j.chroma.2010.12.012.
[9] A. Alonso García, B. Cancho Grande, J. Simal Gándara, Development of a rapid method based on solid-phase extraction and liquid chromatography with ultraviolet absorbance detection for the determination of polyphenols in alcohol-free beers, J. Chromatogr. A 1054 (2004) 175–180, https://doi.org/10.1016/j.chroma.2004.07.092.
[10] M. Dvořáková, P. Hulín, M. Karabín, P. Dostálek, Determination of polyphenols in beer by an effective method based on solid-phase extraction and high performance liquid chromatography with diode-array detection, Czech J. Food Sci. 25 (2008) 182–188, https://doi.org/10.17221/690-CJFS.
[11] M.C. Silva, J.P. dos Anjos, L.L.N. Guarieiro, B.A.S. Machado, A simple method for evaluating the bioactive phenolic compounds' presence in Brazilian craft beers, Molecules 26 (2021) 4716, https://doi.org/10.3390/molecules26164716.
[12] I. Marova, K. Parilova, Z. Friedl, S. Obruca, K. Duronova, Analysis of phenolic compounds in lager beers of different origin: a contribution to potential determination of the authenticity of Czech beer, Chromatographia 73 (2011) 83–95, https://doi.org/10.1007/s10337-011-1916-7.
[13] K.P. Cheiran, V.P. Raimundo, V. Manfroi, M.J. Anzanello, A. Kahmann, E. Rodrigues, J. Frazzon, Simultaneous identification of low-molecular weight phenolic and nitrogen compounds in craft beers by HPLC-ESI-MS/MS, Food Chem. 286 (2019) 113–122, https://doi.org/10.1016/j.foodchem.2019.01.198.
[14] C.F. Poole, Milestones in the development of liquid-phase extraction techniques. Liquid-Phase Extraction, Elsevier, 2020, pp. 1–44, https://doi.org/10.1016/B978-0-12-816911-7.00001-3.
[15] C. Rodrigues, A. Nicácio, I. Jardim, J. Visentainer, L. Maldaner, Determination of phenolic compounds in red sweet pepper (Capsicum annuum L.) using a modified QuEChERS method and UHPLC-MS/MS analysis and its relation to antioxidant activity, J. Braz. Chem. Soc. 30 (2019) 1229–1240, https://doi.org/10.21577/0103-5053.20190018.
[16] A.E. Nicácio, C.A. Rodrigues, I.C.S.F. Jardim, J.V. Visentainer, L. Maldaner, Modified QuEChERS method for phenolic compounds determination in mustard greens (Brassica juncea) using UHPLC-MS/MS, Arab, J. Chem. 13 (2020) 4681–4690, https://doi.org/10.1016/j.arabjc.2019.10.012.
[17] J. Xing, Y. Li, R. Zheng, H. Shen, X. Xu, L. Mao, X. Luo, J. Shen, W. Yao, Simultaneous detection of multiple phenolic compounds in fish by gas chromatography-mass spectrometry following a modified QuEChERS cleanup, Food Addit. Contam. Part A 39 (2022) 1136–1148, https://doi.org/10.1080/19440049.2022.2062058.
[18] L. Mariño-Repizo, H. Goicoechea, J. Raba, S. Cerutti, A simple, rapid and novel method based on salting-out assisted liquid–liquid extraction for ochratoxin A determination in beer samples prior to ultra-high-performance liquid chromatography coupled to tandem mass spectrometry, Food Addit. Contam. Part A 35 (2018) 1622–1632, https://doi.org/10.1080/19440049.2018.1486045.
[19] E.D. Tsochatzis, A. Mieth, J. Alberto Lopes, C. Simoneau, A salting-out liquid-liquid extraction (SALLE) for the analysis of caprolactam and 2,4-di-tert butyl phenol in water and food simulants. Study of the salinity effect to specific migration from food contact materials, J. Chromatogr. B 1156 (2020) 122301, https://doi.org/10.1016/j.jchromb.2020.122301.
[20] E.D. Tsochatzis, J.A. Lopes, H. Gika, T.K. Dalsgaard, G. Theodoridis, A fast SALLE GC–MS/MS multi-analyte method for the determination of 75 food packaging substances in food simulants, Food Chem. 361 (2021) 129998, https://doi.org/10.1016/j.foodchem.2021.129998.
[21] A.D. Batista, F.R.P. Rocha, On-column preconcentration in sequential injection chromatography: application to determination of parabens, Anal. Methods 7 (2015) 4371–4375, https://doi.org/10.1039/c5ay00425j.
[22] P. Gebauer, P. Boček, Electrophoretic sample stacking, Electrophoresis 30 (2009) 27–33, https://doi.org/10.1002/elps.200900053.
[23] S. Cho, Sample stacking with in-column silylation for less volatile polar compounds using GC–MS, J. Sep. Sci. 35 (2012) 661–665, https://doi.org/10.1002/jssc.201100920.
[24] M. Ávila, M. Zougagh, A. Escarpa, Á. Ríos, Determination of alkenylbenzenes and related flavour compounds in food samples by on-column preconcentration-

capillary liquid chromatography, J. Chromatogr. A 1216 (2009) 7179–7185, https://doi.org/10.1016/j.chroma.2009.08.053.
[25] G.I. Baram, I.N. Azarova, A.G. Gorshkov, A.L. Vereshchagin, B. Lang, E. D. Kiryukhina, Determination of bis(2-ethylhexyl) phthalate in water by high-performance liquid chromatography with direct on-column preconcentration, J. Anal. Chem. 55 (2000) 750–754, https://doi.org/10.1007/BF02757910.
[26] M. Frommberger, P. Schmitt-Kopplin, G. Ping, H. Frisch, M. Schmid, Y. Zhang, A. Hartmann, A. Kettrup, A simple and robust set-up for on-column sample preconcentration-nano-liquid chromatography-electrospray ionization mass spectrometry for the analysis of N-acylhomoserine lactones, Anal. Bioanal. Chem. 378 (2004) 1014–1020, https://doi.org/10.1007/s00216-003-2400-5.
[27] D. Šatínský, P. Solich, P. Chocholouš, R. Karlíček, Monolithic columns—a new concept of separation in the sequential injection technique, Anal. Chim. Acta 499 (2003) 205–214, https://doi.org/10.1016/S0003-2670(03)00625-1.
[28] P.A.F. Souza, M.K. Sasaki, F.R.P. Rocha, Exploitation of a short monolithic column for in-line separation and preconcentration: environmental friendly determination of the emerging pollutant salicylic acid in natural waters, J. Sep. Sci. 43 (2020) 1232–1239, https://doi.org/10.1002/jssc.201901017.
[29] I.M. Valente, C.M. Santos, M.M. Moreira, J.A. Rodrigues, New application of the QuEChERS methodology for the determination of volatile phenols in beverages by liquid chromatography, J. Chromatogr. A 1271 (2013) 27–32, https://doi.org/10.1016/j.chroma.2012.11.026.
[30] J.N. Miller, J.C. Miller. Statistics and Chemometrics For Analytical Chemistry, 5th ed., Harlow Pearson Education, 2005.
[31] Z. Fang, Flow Injection Separation and Preconcentration, 1st ed., Wiley VCH, Weinheim, 1993.
[32] US Pharmacopeia, USP 35, Chromatography (621), 2012, 258–265.
[33] J.L. Beltrán, N. Sanli, G. Fonrodona, D. Barrón, G. Özkan, J. Barbosa, Spectrophotometric, potentiometric and chromatographic pKa values of polyphenolic acids in water and acetonitrile–water media, Anal. Chim. Acta 484 (2003) 253–264, https://doi.org/10.1016/S0003-2670(03)00334-9.
[34] M.R. Gama, F.R.P. Rocha, Solventless separation of underivatized biogenic amines by sequential injection chromatography, Microchem. J. 156 (2020) 104839, https://doi.org/10.1016/J.MICROC.2020.104839.
[35] A.D. Batista, P. Chocholouš, D. Šatínský, P. Solich, F.R.P. Rocha, On-line hyphenation of solid-phase extraction to chromatographic separation of sulfonamides with fused-core columns in sequential injection chromatography, Talanta 133 (2015) 142–149, https://doi.org/10.1016/j.talanta.2014.07.056.
[36] National Center for Biotechnology Information (NCBI), PubChem database.
[37] W. Chen, X. Tu, D. Wu, Z. Gao, S. Wu, S. Huang, Comparison of the partition efficiencies of multiple phenolic compounds contained in propolis in different modes of acetonitrile–water-based homogenous liquid–liquid extraction, Molecules 24 (2019) 442, https://doi.org/10.3390/molecules24030442.
[38] V. Jerkovic, F. Nguyen, A. Timmermans, S. Collin, Comparison of procedures for resveratrol analysis in beer: assessment of stilbenoids stability through wort fermentation and beer aging, J. Inst. Brew. 114 (2008) 143–149.
[39] R. Petrucci, P. Di Matteo, G. De Francesco, L. Mattiello, G. Perretti, P. Russo, Novel fast identification and determination of free polyphenols in untreated craft beers by HPLC-PDA-ESI-MS/MS in SIR mode, J. Agric. Food Chem. 68 (2020) 7984–7994, https://doi.org/10.1021/acs.jafc.0c02802.