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Accelerated solvent extraction of phenolic compounds exploiting a Box-Behnken design and quantification of five flavonoids by HPLC-DAD in *Passiflora* species

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ABSTRACT

This work describes the development of a method for the extraction of phenolic compounds, mainly flavonoids, from species of *Passiflora* by employing accelerated solvent extraction (ASE) and using a Box-Behnken design with desirability functions for optimization. The optimal extraction conditions consisted of an extraction temperature of 80 °C, 64% (w/w) ethanol and five number of extraction cycles. A high-performance liquid chromatography-diode array detection (HPLC-DAD) method for the identification and quantification of orientin, isoorientin, vitexin, isovitexin and rutin flavonoids in the leaves of seventeen *Passiflora* spp. using gradient elution with acetonitrile (solvent B) and 0.2% (w/w) formic acid in water (solvent A) as the eluent mixture was also developed and validated. These five flavonoids were quantified with good linearity, LOQs, LODs, precision, and accuracy. Higher concentrations of isoorientin, orientin, vitexin, isovitexin and rutin were determinate in *P. edulis* f. *flavicarpa* (1.61 mg/g extract and 0.58 mg/g dry plant), *P. morifolia* (2.10 mg/g extract and 0.90 mg/g dry plant), *P. setacea* (2.48 mg/g extract and 0.97 mg/g dry plant), *P. setacea* (8.46 mg/g extract and 3.30 mg/g dry plant) and *P. galbana* (3.48 mg/g extract and 1.02 mg/g dry plant), respectively.

1. Introduction

The use of plants for therapeutic purposes is an ancient tradition of the cultures of various populations and is still a widely accepted alternative resource. For example, species popularly known as "maracujá" or passion fruit are utilized as medicinal plants in American, European, and Asian cultures. Maracujá belongs to the genus *Passiflora*, which comprises ca. 500 plant species of the family Passifloraceae. *Passiflora* is the most important genus of the family and is mainly distributed in tropical and temperate regions of South America [1]. In Brazil, approximately 142 *Passiflora* species have been reported, of which 83 are endemic [2]. Among these endemic species, the species most known and popularly employed in folk medicine are *P. alata* and *P. edulis*. The leaves of *Passiflora alata* have been included in the Brazilian Pharmacopeia since the first edition in 1926, containing a description of the drugs, fluid extract and tincture of this plant, and a monograph of *P. edulis* species was included in 2010 [3].

Reports of the chemical composition of leaf extracts of *Passiflora* species have described mainly flavonoids [4–8] and saponins [9–11], with the former representing the major group of active compounds present in these species and exerting healthy beneficial effects. The main flavonoids described for the genus *Passiflora* are *C*-glycosyl flavones derived from apigenin and luteolin (Fig. 1), and pharmacological studies of these plants in recent years have noted the putative involvement of this class of flavonoids in neuropharmacological activities [12–14]. Therefore, the easy and rapid quantification of these compounds is helpful in controlling the quality of *Passiflora* species as well as of the related phytotherapeutic drugs.

The traditional solvent extraction methods often used for the extraction of different bioactive compounds have certain drawbacks, such as long analysis times, laborious procedures, low selectivity, low extraction yields and large required amounts of toxic solvents. Recently, emphasis has shifted towards the use of sub- and supercritical fluids and GRAS (generally recognized as safe) solvents. Among these strategies, accelerated solvent extraction (ASE) is one of the most promising processes [15]. ASE offers several advantages including easy automation, faster sample analysis, better repeatability, low required solvent volume, low risk of exposure to solvents and maintenance of samples in an oxygen- and light-free environment. Furthermore, accelerated extraction systems also allow the operator to control the temperature, pressure, extraction time and number of extractions, which can increase

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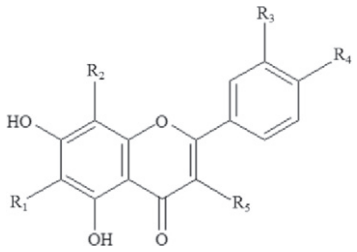

	Flavonoid	Aglycone	R_1	R_2	R_3	R_4	R_5
1	Isoorientin	Luteolin	Glu	H	OH	OH	H
2	Orientin	Luteolin	H	Glu	OH	OH	H
3	Vitexin	Apigenin	H	Glu	H	OH	H
4	Isovitexin	Apigenin	Glu	H	H	OH	H

Glu = glucose

Fig. 1. Structures of the flavonoids present in *Passiflora* spp.

the amount of compounds extracted from the plant when optimized [16].

Currently, the use of multivariate optimization strategies as analytical methodologies is widespread because these strategies reduce the number of experiments required to reach the optimal conditions and indicate the possible influences of some variables on others. Response surface methodology (RSM) is an approach to multivariate optimization used with several analytical procedures [17,18]. The Box-Behnken design as a model for the multivariate optimization of analytical procedures has higher efficiency than second-order designs such as the Doehlert Matrix and Central Composite Design, and its efficiency is equal to the Doehlert Matrix when three factors are studied [17].

This work reports the development of an extraction method for phenolic compounds, mainly flavonoids, from the leaves of seventeen species of *Passiflora* using a Box-Behnken design with desirability functions to determine the appropriate temperature, solvents, extraction time (how long the plant material is under pressure at the prescribed temperature) and number of extraction cycles for ASE. Then, a method for reversed-phase high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) was developed and validated for the quantification of flavonoids, vitexin, isovitexin, orientin, isoorientin and rutin, in standardized extracts of seventeen different species of *Passiflora.*

2. Experimental

2.1. General procedures

HPLC-grade acetonitrile (ACN) was purchased from Sigma-Aldrich, the formic acid 98–100% used in the preparation of the mobile phase was of analytical grade (Emsure). and ethanol was of analytical grade (Qhemis). Flavonoid standards of orientin (purity ≥95%) and isoorientin (≥98%) were purchased from Sigma, with vitexin (≥95%) from Fluka, isovitexin (≥95.2%) from Chromadex and rutin (≥96.37%) from HWI Analytik. Water was purified with a NANOpure Diamond™ system (Barnstead, Dubuque, Iowa, USA). Folin-Ciocalteu reagent (Spectrum), Na_2CO_3 (Ecibra) and gallic acid (≥98.99%, Haloquímica) were applied in the determination of total phenolics. For the determination of total flavonoids, quercetin (≥95%, Sigma-Aldrich), $AlCl_3$ (Riedel-de-Hain) and NaOH (Synth) were used. The mobile phases were filtered through nylon solvent filters (0.45 μm).

2.2. Plant materials

The leaves of *Passiflora* species were collected in Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) Cruz das Almas, Bahia, Brazil, in July of 2011. The plant material consisted of seventeen accessions of *Passiflora* from the active germplasm bank (BGP-EMBRAPA), maintained in its screen house and experimental fields (Table 1). The leaves of all species were air-dried at 30 °C for 7 days in an airflow oven and powdered; only particles between 0.5 and 1.0 mm were utilized for the extractions.

2.3. Extraction by ASE and sample preparation

Extracts of the dried and powdered leaves (6.0 g) of *Passiflora alata*, *P. capsularis*, *P. cincinnata*, *P. edulis* f. *flavicarpa*, *P. edulis* f. *edulis*, *P. galbana*, *P. gibertii*, *P. maliformis*, *P. malacophylla*, *P. morifolia*, *P. mucronata*, *P. quadrangularis*, *P. racemosa*, *P. setacea*, and *P. suberosa* and of 3.0 g of *P. vitifolia* and *P. tenuifila* were prepared by accelerated solvent extraction (ASE 100, Dionex Corporation, Sunnyvale, CA, USA). The plant material was extracted under the optimized conditions in five number of extraction cycles with 64% (w/w) ethanol at 80 °C, 1500 psi, and a static cycle timing of 10 min. Then, the cells were rinsed with fresh extraction solvent (100% of the extraction cell volume) and purged with N_2 gas for 60 s. The solvent was removed under reduced pressure at 55 °C to give the corresponding crude extracts, which were stored under refrigeration and protected from light. The extract yields (%) were obtained using the equation:

$$\text{Extraction yield } (\%) = W_2 \times 100/W_1 \quad (1)$$

Table 1
List of the *Passiflora* Brazilian accessions used in this study and present in the active germplasm bank of EMBRAPA (Cruz das Almas, Bahia, Brazil).

Code	Species	Origin — city, state
BGP08	*P. gibertii* N. E. Brown	Jaboticabal, São Paulo
BGP25	*P. edulis* f. *edulis* Sims.	Brasília, Distrito Federal
BGP32	*P. maliformis* L.	Londrina, Paraná
BGP77	*P. cincinnata* Mast.	Andaraí, Bahia
BGP104	*P. vitifolia* Kunth	Nova Odessa, São Paulo
BGP105	*P. tenuifila* Killip	Nova Odessa, São Paulo
BGP107	*P. morifolia* Mast.	Nova Odessa, São Paulo
BGP109	*P. galbana* Mast.	Nova Odessa, São Paulo
BGP114	*P. mucronata* Sessé & Moc.	Nova Odessa, São Paulo
BGP125	*P. capsularis* L.	Jaboticabal, São Paulo
BGP152	*P. suberosa* L.	Nova Odessa, São Paulo
BGP157	*P. quadrangularis* L.	Catu, Bahia
BGP163	*P. alata* Curtis	Alagoinhas, Bahia
BGP170	*P. malacophylla* Mast.	Nova Odessa, São Paulo
BGP172	*P. racemosa* Brot.	Nova Odessa, São Paulo
BGP205	*P. edulis* Sims. f. *flavicarpa* Deg.	Piracicaba, São Paulo
BGP237	*P. setacea* DC.	Lagedinho, Bahia

where W_2 is the weight of the crude extract and W_1 is the weight of the dried sample. Prior to injection into the HPLC, hydroethanolic crude extracts of each species of *Passiflora* were purified by solid-phase extraction (SPE) using the typical method with minor modifications [19]. A C_{18} cartridge (Agilent SampliQ, 3 mL/200 mg) was conditioned with 3 mL of methanol, followed by 1 mL of water. Next, 2 mL of a methanolic solution of the sample (5 mg/mL) was added, and the flavonoid fraction was obtained by elution with 3 mL of 60% (w/w) methanol. All samples were prepared and analyzed in triplicate.

2.3.1. Optimization of the ASE extraction procedure

The optimal ASE performance was determined by applying the Box-Behnken design to the independent variables: temperature (°C), ethanol concentration (% w/w) and number of extraction cycles. This design required fifteen experiments, which were performed in a random order to avoid any systematic error. The response of interest consisted of a combination of responses (dependent variables): yield of the extract (%), total phenolic content (mg GA/g sample) and total flavonoid content (mg QUE/g sample). A time cycle of 10 min and flushing of 100% (the cell washing volume) were fixed. All assays were performed with 3.0 g of plant material from *P. alata*. Table 2 shows the factors, levels and experimental matrix of the Box-Behnken design with the respective responses for each run.

2.3.2. Determination of the total phenolic content

The total phenolic content (TPC) present in the extracts was determined by a modified [20] and adapted [21] Folin-Ciocalteu method. Briefly, 100 μL of the extracts (0.2 mg/mL in distilled water) was added to 6 mL of distilled water and 500 μL of Folin-Ciocalteu reagent. After a 1 min of equilibration, the mixture was neutralized with 2 mL of 15% (w/w) Na_2CO_3. After 30 min of reaction, the absorbance of the mixture was measured at 750 nm in a UV/Vis spectrophotometer. Gallic acid (2–20 μg/mL) was used as a reference standard, and the total phenolic content was calculated using the calibration curve for gallic acid. The results are expressed as mg of gallic acid equivalents (GA) per g of sample (mg GA/g sample). All samples were analyzed in triplicate.

2.3.3. Determination of the total flavonoid content

The total flavonoid content (TFC) of the crude extracts was determined using the aluminum chloride colorimetric method [22] with minor modifications. To 1 mL of the extract (0.2 mg/mL in methanol) was added 4 mL of distilled water and 200 μL of 5% (w/w) $NaNO_3$. After 6 min, 200 μL of 10% (w/w) $AlCl_3$ was added, and the mixture rested for 5 min. Then, 2 mL of 10% (w/w) NaOH was added, and the total volume was brought to 10 mL with methanol. The absorbance was measured after 30 min at 425 nm. Quercetin (2–26 μg/mL) was used as a standard, and the total flavonoid content was calculated using the calibration curve for quercetin. The results are expressed as mg of quercetin equivalents (QUE) per g of sample (mg QUE/g sample). All samples were analyzed in triplicate.

Table 2
Factors, levels and experimental matrix of the Box-Behnken design.

Factors				Levels		
				Low (−)	Mean (0)	High (+)
Temperature (°C)				40	60	80
Ethanol concentration (% w/w)				40	70	100
Number of extraction cycles				1.0	3.0	5.0
				Responses		
Run	T[a] (°C)	EC[b] (%)	Cycles	Yield (%)[c]	TPC[d]	TFC[e]	D[f]
1	−	−	0	39.68	463.89	49.22	0.35
2	+	−	0	47.73	502.78	41.46	0.00
3	−	+	0	11.07	452.78	110.89	0.00
4	+	+	0	24.85	241.67	101.61	0.00
5	−	0	−	26.86	555.56	72.60	0.52
6	+	0	−	37.13	538.89	79.43	0.64
7	−	0	+	37.46	587.50	65.78	0.59
8	+	0	+	45.12	611.11	84.53	0.79
9	0	−	−	40.44	502.78	58.54	0.49
10	0	+	−	19.38	259.72	85.83	0.18
11	0	−	+	54.26	461.11	60.21	0.54
12	0	+	+	20.76	347.22	80.31	0.33
13	0	0	0	38.64	462.50	60.57	0.47
14	0	0	0	39.25	494.44	63.70	0.52
15	0	0	0	40.57	408.33	60.89	0.44

[a] Temperature (°C).
[b] Ethanol concentration (w/w).
[c] Yield of the extract (%).
[d] Total phenolic content (mg GA/g sample).
[e] Total flavonoid content (mg GA/g sample).
[f] D: overall desirability.

2.4. Equipment and chromatographic conditions

HPLC fingerprint analysis was carried out using an LC system (Thermo Scientific Dionex Ultimate 3000; MA, USA) consisting of a Thermo Scientific Dionex Ultimate 3000 diode array detector (DAD), quaternary pump, on-line degasser and automatic sampler. The chromatographic separation of samples was achieved on a reversed-phase HPLC column (Waters XBridge™, BEH C_{18}, 100 mm × 3.0 mm I.D., 2.5 μm particle size). The column temperature was maintained at 30 °C, the injection volume was 10.0 μL, and a 0.6 mL/min flow rate was applied using a linear gradient of 0.2% (w/w) formic acid in water (solvent A) and acetonitrile (solvent B). The optimized gradients employed for the hydroethanolic extracts consisted of 5–20% B in A over 0–30 min. The re-equilibration duration between individual runs was 30 min. The flavonoids were detected at 337 nm, and the UV spectra of individual peaks were recorded within a range of 190–400 nm. Data were acquired and processed with Chromeleon software. All of the main flavonoids were identified by matching their retention times against those of standards. Quantification was achieved using the linear calibration curves of standards.

2.5. Optimization of the chromatographic conditions

To obtain a representative HPLC fingerprint of a plant, the developed method should furnish chromatograms with baseline resolution and high peak capacity. Thus, the developed fingerprint method featured good selectivity, reproducibility and feasibility for use with a large number of samples [23].

The chromatographic conditions were optimized to obtain the chromatographic fingerprint of *Passiflora* species. These analyses were first performed using *P. edulis* f. *flavicarpa* species and then applied to other samples. Throughout this process, the following chromatographic parameters were evaluated: injection volume, flow rate, column temperature, organic modifier, stationary phase, gradient range, and slope.

The chromatogram resolution of the compounds in the sample were tested and compared with different reversed-phase conditions using two analytical columns: Dionex C_{18} (100 mm × 2.1 mm, 5 μm) and Waters BEH C_{18} XBridge™ (100 mm × 3.0 mm, 2.5 μm). The mobile phase consisted of acetonitrile and 0.2% (w/w) formic acid in water. A low concentration formic acid was added to restrict the ionization of flavonoids and other carboxylic acids [24].

Orientin, isoorientin, vitexin, isovitexin and rutin were employed as chemical reference standards. Seven flavonoid standard solutions (1.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mg/L) were prepared from stock solutions of each flavonoid (200 mg/L).

2.6. Validation procedures

The proposed HPLC method was validated according to Brazilian legislation [25] and ICH guidelines [26] in terms of the following parameters: linearity, precision, limit of detection (LOD), limit of quantification (LOQ) and accuracy.

The linearity was determined by the external standard method; the calibration curve was built using standard solutions at seven different concentrations (1.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mg/L) of orientin, isoorientin, vitexin, isovitexin and rutin, measured in triplicate, plotting the peak area versus concentration. The linear regression coefficients (r) and analysis of variance (ANOVA) were obtained for the peak area against the concentration with correlation coefficients (R^2) >0.99, showing good linearity.

The accuracy was determined using a hydroethanolic extract (64% (w/w)) of *P. edulis* f. *flavicarpa*. The standard samples of orientin, isoorientin, vitexin, isovitexin and rutin were analyzed at three concentration levels (1.0, 7.5 and 15.0 mg/L) in the linear range. All samples were purified by solid-phase extraction (SPE) before analysis in triplicate. The accuracy was accessed by standard addition tests and expressed as recovery percentage in relation to each analyte. The LOD and LOQ were calculated based on the residual standard deviation of the regression line (SDb) and the slope of the calibration curve (S). The precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability demonstrates the correlation between successive measurements, termed repeatability conditions, such as various measurements of the same sample in the same laboratory in a short time period (same day, analyst and equipment). Meanwhile, the inter-day precision aims to verify if the method provides the same results in the same laboratory when performed by different analysts on different days. The precision was expressed in terms of the relative standard deviation (RSD). The repeatability was estimated by injecting in triplicate on the same day standard solutions of orientin, isoorientin, vitexin, isovitexin and rutin at three concentrations (1.0, 7.5 and 15.0 mg/L) in the linear range that had been subjected to five different preparations. The intermediate precision was determined by analyzing the same solutions employed in the repeatability test in triplicate on four non-consecutive days using different analyses.

2.7. Identification and quantification of the five flavonoids in the samples

Flavonoids are important compounds in *Passiflora*, possess many recognized medicinal properties, and can be used as chemical markers for quality control purposes. Chromatographic techniques in combination with DAD can be used for the reliable quantitation of individual flavonoid glycosides. In this study, the main *Passiflora* flavonoids were identified in the hydroethanolic extracts (64% (w/w)) of the leaves of seventeen species of *Passiflora* based on their retention times (t_R), co-injection of the samples with standards and comparison of their UV adsorption spectra. Quantification was performed based on external standards using the UV absorption peak area at 337 nm for all flavonoids.

3. Results and discussion

3.1. Optimization of the extraction procedures

In this work, a Box-Behnken design was applied to optimize the procedures for the extraction of phenolic compounds using ASE. The procedure is based on simultaneously obtaining the yield of the extract (%), total phenolic content (mg GA/g sample) and total flavonoid content (mg QUE/g sample) through the multi-response optimization in which these parameters are determined.

3.2. Total phenolic content

Folin-Ciocalteu reagent is commonly employed in assays for the determination of total phenols in the extracts of natural samples. This reagent consists of a mixture of phosphomolybdic and phosphotungstic acids. From reduction with certain reducing agents, molybdenum blue and tungsten blue are formed. The degree of the color change is proportional to the antioxidant concentration [20]. The total phenolic contents determined using Folin-Ciocalteu reagent are presented in Table 2. Gallic acid was used for the standard calibration curve, and the equation [$y = 0.0012x + 0.0006$ ($R^2 = 0.9982$)] was obtained for the curve. The total phenolic content was expressed as mg of gallic acid equivalents (GA) per g of sample (mg GA/g sample).

3.3. Total flavonoid content

The contents of total flavonoids were measured by the aluminum chloride colorimetric technique. $AlCl_3$ forms acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl groups of flavones and flavonols. In addition, aluminum chloride forms acid-labile complexes with the *ortho*-dihydroxyl groups in the A- or B-ring of flavonoids [22]. The total flavonoid contents determined using aluminum chloride are presented in Table 2. The total flavonoid contents were determined from the regression equation of the calibration curve ($y = 0.0032x + 0.0012$, $R^2 = 0.9983$) and expressed as mg of quercetin equivalents (QUE) per g of sample (mg QUE/g sample).

3.4. Optimization using a desirability function

To perform the multivariate optimization with multi-response treatment, a Box-Behnken design was applied using a mathematical-statistical tool developed by Derringer, which is based on the use of a desirability function. This feature allowed us to combine three distinguishing features of each studied response in a single response (overall desirability). Derringer and Suich [27] proposed the use of a desirability function for multiple response optimization experiments. To obtain the overall desirability, the individual desirabilities of all responses should be determined (in this case, the yield of the extract and total phenolic and flavonoid contents). Thus, each response y_i ($i = 1, 2, \ldots, m$) is transformed into a scale-free value, which is called an individual desirability function (d_i), where $0 \le d_i \le 1$, with 0 for an unacceptable response and 1 for a desirable response. The value of d_i increases as the desirability of the corresponding response increases. The individual desirability function was calculated according to Eq. (2), which was used to maximize the overall desirability (D); L and H are the lower and upper observed responses for each independent variable. The D value was calculated by determining the geometric mean of the individual desirabilities (Eq. (3)).

$$d_i = \frac{y_i - \mathrm{L}}{\mathrm{H} - \mathrm{L}} \tag{2}$$

$$D = (d_1 \times d_2 \times d_3 \ldots d_k)^{1/k} \tag{3}$$

where k is the number of responses (in this case, 3). $D = 1$ indicates a fully desired response, above which further improvements are not important.

The individual and overall desirability profiles were calculated according to Eqs. (2) and (3). Table 2 shows the matrix of the overall desirability values (D). Fig. 2 shows the predicted values and desirabilities analyzed using the coded values of the independent variables and the data processed by Statistica software [28] at a confidence level of 95%. The system produced a desirability close to 0.79 as a solution for the yield and total phenolic and flavonoid contents. The desirability parameter indicates the optimal region, taking into account all the variables, at the same time. The optimization desirability value was close to 0.8, which is considered a good and acceptable value, since it is composed of the geometric mean of individual desirabilities for each response in

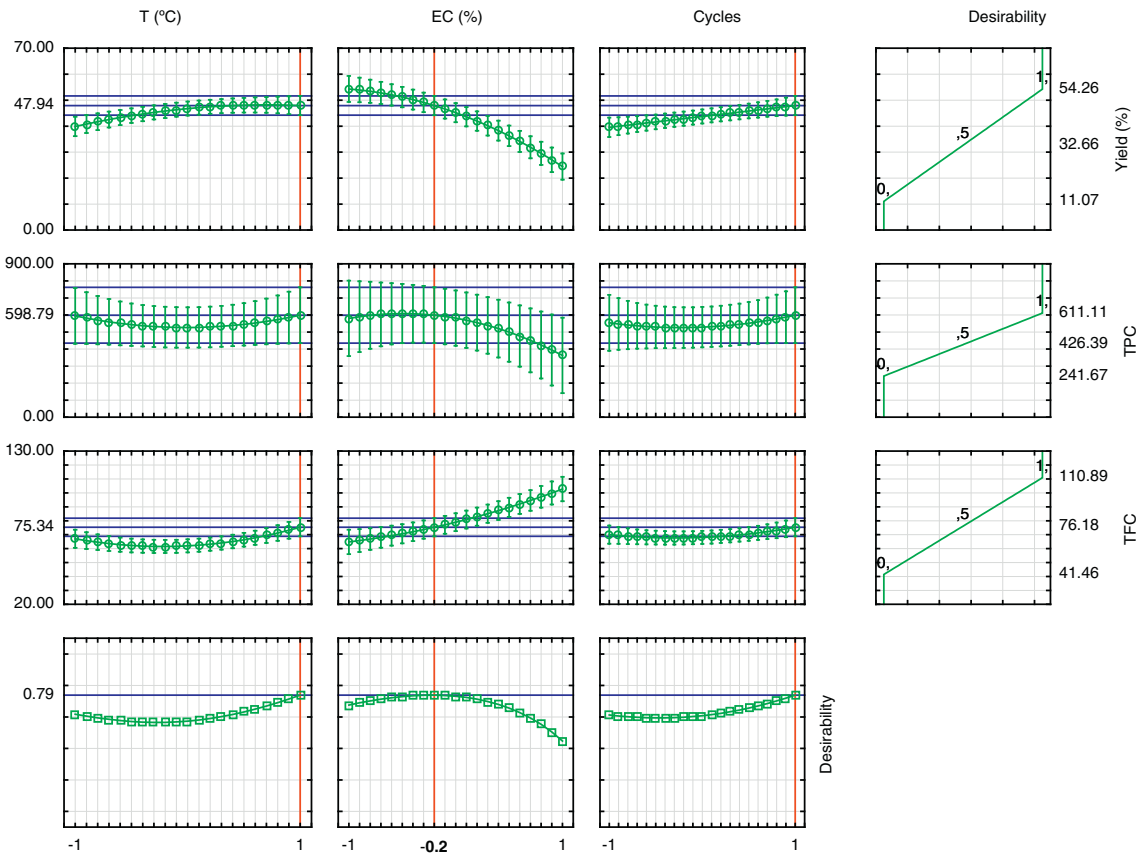

Fig. 2. Prediction and desirability profiles for the simultaneous optimization of the yield and phenolic (TPC) and flavonoid (TFC) contents. The vertical dashed lines indicate the values after optimization using a Box-Behnken design.

study. According to Derringer, desirability near of 1 should be accepted and close to 0 should be neglected. To calculate of the real value for each factor, Eq. (4) was applied, describing the relationship between the coded and real values:

$$C_i = \{(X_i - X_i^0)/(\Delta X_i)\}\alpha \quad (4)$$

where C_i is the coded value for the level of factor i, X_i is its real value in an experiment, Xi0 is the real value at the center of the experimental domain, ΔX_i is the step in the variation of the real value and α is the maximum coded value of the factor in the experiment (in this case 1). After the calculation, the real values found for each factor were as follows: temperature, 80 °C; ethanol concentration, 64% (w/w); and number of extraction cycles, 5. The prevision capacity of the mathematical function fitted to overall desirability was evaluated by ANOVA (Table 3) and by the correlation between normal probability plot of residuals (Fig. 3).

The column of responses generated from the application of the desirability function (Table 2) was analyzed with the Statistica software, and the ANOVA table shows that the data behavior can be explained by the mathematical model adopted because the lack of fit value (0.076711 > 0.05) is not significant at a confidence level of 95%. In addition, determination coefficient ($R^2 = 0.92524$) confirms the good quality of the model.

Table 3
ANOVA of the overall desirability (D) of the response.

	SS	df	MS	F	*p*
(1) T (°C) L + Q	0.017990	2	0.008995	5.5072	0.153677
(2) EC (%) L + Q	0.471721	2	0.235860	144.4043	0.006877
(3) Cycles L + Q	0.213851	2	0.106925	65.4645	0.015046
(1) * (2)	0.030625	1	0.030625	18.7500	0.049414
(1) * (3)	0.001600	1	0.001600	0.9796	0.426618
(2) * (3)	0.002500	1	0.002500	1.5306	0.341573
Lack of fit	0.059775	3	0.019925	12.1990	0.076711
Pure error	0.003267	2	0.001633		
Total SS	0.843293	14			

$R^2 = 0.92524$; Adj: 0.79068; Pure error = 0.0016333; $p = 0.05$.

3.5. Optimization of the chromatographic conditions and determination of flavonoids

The chromatographic conditions were analyzed, and the chromatographic resolution of each compound in the sample was compared

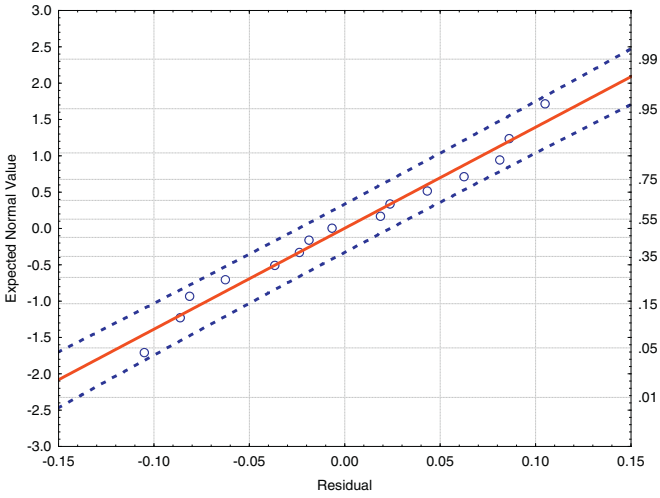

Fig. 3. Normal probability plot of residuals. The dashed line is the 95% confidence band.

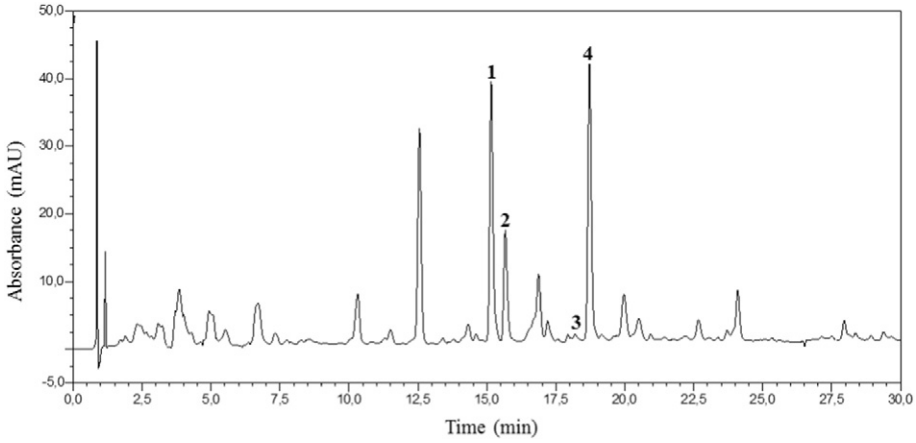

Fig. 4. HPLC fingerprint chromatogram (λ = 337 nm) of a hydroethanolic extract of *P. edulis* f. *flavicarpa*. Mobile phase: ACN (B):0.2% (w/w) formic acid (A) (0–30 min, 5–20% of B). Flow rate: 0.6 mL/min. Injection volume: 0.6 μL. Column temperature: 30 °C. Peaks: 1 — isoorientin; 2 — orientin; 3 — vitexin; 4 — isovitexin.

with different reversed-phase conditions using two analytical columns, Dionex C_{18} and Waters BEH C_{18} XBridge™. The ideal chromatographic conditions yielding the best selectivity, efficiency and resolution were obtained using the Waters BEH C_{18} XBridge™ column with 0.2% (w/w) formic acid in water (solvent A) and acetonitrile (solvent B). This stationary phase column with second-generation hybrid particles, BEH XBridge™ (BEH — ethylene bridged hybrid), provided good peak symmetry due to a reduced amount of residual silanols and therefore higher efficiency. In the gradient optimization, the time and range were taken into consideration, and the optimized gradient program ran from 0 to 30 min with 5 to 20% solvent B. The influence of the flow rate (1.0, 0.8 and 0.6 mL/min), injection volume (10.0, 0.8 and 0.6 μL), and column temperature (30 and 35 °C) were also examined. More detectable peaks were obtained, and the baseline was improved by approximately 337 nm, to which better flavonoid characteristics can be attributed. Fig. 4 illustrates an optimized chromatogram in which the chemical complexity of the hydroethanolic extract of *P. edulis* f. *flavicarpa* sample can be observed.

The results of the method linearity evaluation are presented in Table 4. The regression statistics were calculated from the calibration curves constructed for all of the studied flavonoids and showed satisfactory linearity with the correlation coefficient (R^2) >0.99 for all. Analysis of the linear models was realized using calculated *F* values of 2.23, 1.53, 1.31, 1.14 and 1.33 for the analytical curves of orientin, isoorientin, vitexin, isovitexin and rutin, respectively. These values were smaller than the tabulated *F* value ($F_{5,14} = 2.96$) at a confidence level of 95%, demonstrating that the linearity of the method was acceptable and the model is satisfactory. To validate the regression analysis, the calculated *F* values were 3388.20, 2864.85, 3145.88, 2825.61 and 3513.11 for the analytical curves of orientin, isoorientin, vitexin, isovitexin and rutin, respectively, which are higher than the critical *F* value ($F_{1,19} = 4.38$) at a confidence level of 95%, demonstrating that the regression was significant. As shown in Table 4, the LOD and LOQ values ranged from 0.710 to 0.848 mg/L and 2.368 to 2.827 mg/L, respectively. The LOD and LOQ obtained are in accordance with previous studies dealing with polyphenol compound analysis in bioproduct samples. They are lower than the observed for lignans employing similar detectors [28] and micellar electrokinetic chromatography with UV detection [29] and, expected higher than flavonoids and benzoic acids quantified by HPLC/MS/MS [30].

The precision of the developed HPLC method was evaluated based on the relative standard deviation (% RSD) of standard solutions of each flavonoid independently prepared at 1.0, 7.5 and 15.0 mg/L (Table 5). For both tests, the RSD was <5% for triplicate analyses at the three different concentrations, which aligns with the recommendation of the ICH protocols [27] and ANVISA [31].

The results obtained for accuracy are shown in Table 5. The acceptable recovery interval depends on the analytical complexity and the sample and is generally between 70 and 120% with an accuracy of ±20%. However, this range can be expanded to 50–120% with an accuracy of ±15% for more complex samples [32]. The recoveries and RSDs of each analyzed flavonoid demonstrate that this method is highly accurate for all the tested analytes at the tested three concentrations, with RSDs <5% and recoveries between 70 and 120%. These validation results indicate that the conditions used in the quantitative determination were satisfactory.

Table 4
Chromatographic and calibration parameters, LOD and LOQ.

Compound	Regression equation[a]	R^{2b}	LOD (mg/L)[c]	LOQ (mg/L)[d]
Orientin	y = 0.7581x − 0.3314	0.9975	0.848	2.827
Isoorientin	y = 0.7267x − 0.2944	0.9977	0.823	2.744
Vitexin	y = 0.6820x − 0.2793	0.9981	0.747	2.490
Isovitexin	y = 0.5395x − 0.2124	0.9980	0.750	2.499
Rutin	y = 0.3505x − 0.1531	0.9987	0.710	2.368

[a] In the regression equation y = ax + b, y refers to the peak area, and x refers to the concentration of the flavonoids.
[b] R^2: the square value of the correlation coefficient of the equation.
[c] LOD: limit of detection (3 × SDb/S).
[d] LOQ: limit of quantification (10 × SDb/S).

Table 5
Study of the precision (RSD %) and recovery (%) data for three different concentrations using the developed HPLC method.

		Precision		Accuracy	
Compound	Concentration level (mg/L)	Repeatability (1 day, $n = 3$) RSD (%)	Intermediate precision (4 days, $n = 3$) RSD (%)	Recovery (%)	RSD (%)
Orientin	1.0	2.21	2.37	97.99	1.69
	7.5	2.78	1.55	90.74	3.66
	15.0	1.51	2.32	82.62	1.08
Isoorientin	1.0	1.67	2.51	104.08	0.24
	7.5	2.52	1.82	91.38	3.51
	15.0	1.66	2.95	109.24	3.55
Vitexin	1.0	2.37	1.07	70.81	0.76
	7.5	3.12	1.77	107.93	1.01
	15.0	1.65	3.14	97.78	0.27
Isovitexin	1.0	1.99	1.17	102.73	0.55
	7.5	2.99	1.53	88.94	1.95
	15.0	1.73	1.94	108.49	0.49
Rutin	1.0	2.22	2.70	107.43	0.35
	7.5	2.95	1.83	70.12	0.54
	15.0	0.98	3.72	114.43	2.44

Table 6
Concentrations (mg/g extract and mg/g dry plant) of the compounds present in the hydroethanolic extracts of *Passiflora* species.

Species	Isoorientin		Orientin		Vitexin		Isovitexin		Rutin	
	mg/g extract	mg/g dry plant	mg/g extract	mg/g dry plant	mg/g extract	mg/g dry plant	mg/g extract	mg/g dry plant	mg/g extract	mg/g dry plant
P. alata	<LOQ	<LOQ	<LOD	<LOD	<LOQ	<LOQ	3.09 ± 0.019	1.49 ± 0.009	ND	ND
P. capsularis	<LOQ	<LOQ	0.94 ± 0.031	0.22 ± 0.007	4.05 ± 0.066	0.93 ± 0.015	<LOQ	<LOQ	ND	ND
P. cincinnata	1.39 ± 0.046	0.47 ± 0.016	<LOQ	<LOQ	<LOQ	<LOQ	7.68 ± 0.172	2.60 ± 0.058	ND	ND
P. edulis f. *flavicarpa*	1.61 ± 0.011	0.58 ± 0.004	0.74 ± 0.010	0.26 ± 0.004	<LOD	<LOD	6.17 ± 0.083	2.22 ± 0.030	ND	ND
P. edulis f. *edulis*	1.18 ± 0.047	0.46 ± 0.018	0.94 ± 0.029	0.37 ± 0.011	<LOD	<LOD	3.24 ± 0.007	1.26 ± 0.003	ND	ND
P. galbana	<LOD	<LOD	<LOD	<LOD	ND	ND	5.21 ± 0.199	1.53 ± 0.058	3.48 ± 0.110	1.02 ± 0.032
P. gibertii	ND	ND	<LOQ	<LOQ	<LOD	<LOD	<LOQ	<LOQ	ND	ND
P. malacophylla	<LOQ	<LOQ	0.58 ± 0.003	0.09 ± 0.000	<LOQ	<LOQ	<LOQ	<LOQ	ND	ND
P. maliformis	<LOQ	<LOQ	<LOQ	<LOQ	ND	ND	2.84 ± 0.013	1.09 ± 0.005	ND	ND
P. morifolia	<LOQ	<LOQ	2.10 ± 0.014	0.90 ± 0.006	<LOQ	<LOQ	5.67 ± 0.039	2.44 ± 0.017	ND	ND
P. mucronata	<LOD	<LOD	<LOD	<LOD	ND	ND	<LOQ	<LOQ	2.73 ± 0.029	0.94 ± 0.010
P. quadrangularis	ND	ND	<LOD	<LOD	<LOQ	<LOQ	2.60 ± 0.079	1.04 ± 0.032	ND	ND
P. racemosa	ND	ND	1.03 ± 0.006	0.30 ± 0.002	<LOD	<LOD	0.69 ± 0.006	0.20 ± 0.002	<LOQ	<LOQ
P. setacea	<LOQ	<LOQ	1.24 ± 0.061	0.48 ± 0.024	2.48 ± 0.142	0.97 ± 0.055	8.46 ± 0.393	3.30 ± 0.153	ND	ND
P. suberosa	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.57 ± 0.020	0.14 ± 0.005
P. tenuifila	<LOQ	<LOQ	<LOQ	<LOQ	ND	ND	<LOQ	<LOQ	ND	ND
P. vitifolia	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	6.90 ± 0.016	2.06 ± 0.05	ND	ND

ND = not detected.
The data represent the mean ± SD of at least three triplicates.

The flavonoids isoorientin, orientin, vitexin, isovitexin and rutin were quantified in seventeen species of *Passiflora* (Table 6) using the HPLC-UV/DAD method developed and validated in the present work. The HPLC fingerprints of the hydroethanolic extracts (64% (w/w)) of *Passiflora* leaves and of the standard flavonoid solution were obtained, and the peaks of each identified compound were assigned. In these LC fingerprints, the peaks were assigned as isoorientin (**1**), orientin (**2**), vitexin (**3**), isovitexin (**4**) and rutin (**5**).

Quantification of the compounds by HPLC-DAD showed a higher concentration the *C*-glycosyl flavonoid isoorientin in *P. edulis* f. *flavicarpa* (1.61 mg/g extract and 0.58 mg/g dry plant) and a lower concentration in *P. edulis* f. *edulis* (1.18 mg/g extract and 0.46 mg/g dry plant). The highest concentration of the *C*-glycosyl flavonoid orientin was found in *P. morifolia* (2.10 mg/g extract and 0.90 mg/g dry plant), and lower concentration was detected in *P. malacophylla* (0.58 mg/g extract and 0.09 mg/g dry plant).

The *C*-glycosyl flavonoid vitexin was only detected in *P. setacea* and was either not detected or appeared at trace levels in *P. capsularis*, *P. galbana*, *P. maliformis*, *P. mucronata* and *P. tenuifila*. The rutin was only quantified in two species with the highest concentration of compound in *P. galbana* (3.48 mg/g extract and 1.02 mg/g dry plant). Isovitexin was the *C*-glycosyl flavonoid most commonly detected in the tested *Passiflora* species and was present at the highest concentrations. *P. edulis* f. *flavicarpa*, *P. edulis* f. *edulis* and *P. setacea* contained the highest amounts of the quantified flavonoids.

4. Conclusions

In this study, an ASE extraction method was developed to simultaneously maximize the extraction yield, total phenolic content and total flavonoid content using a Box-Behnken design with a desirability function. The Box-Behnken design combined with multiple response treatments and a desirability function allowed effective optimization of ASE. The optimized extraction conditions consisted of an extraction temperature of 80 °C, 64% (w/w) ethanol and five number of extraction cycles. A reliable HPLC-DAD method was developed for the identification and quantification of five flavonoids (orientin, isoorientin, vitexin, isovitexin and rutin) in the extracts of the leaves of seventeen species of *Passiflora*. The LODs, LOQs, correlation coefficients, repeatability, intermediate precision and accuracy were in good agreement with the requirements for the developed method. The highest concentrations of isoorientin, orientin, vitexin, isovitexin and rutin were detected in *P. morifolia*, *P. edulis* f. *flavicarpa*, *P. setacea*, *P. setacea* and *P. galbana*, respectively.

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