http://dx.doi.org/10.5935/0103-5053.20140130

J. Braz. Chem. Soc., Vol. 25, No. 8, 1472-1483, 2014.
Printed in Brazil - ©2014 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Article

Quantification of Harman Alkaloids in Sour Passion Fruit Pulp and Seeds by a Novel Dual SBSE-LC/Flu (Stir Bar Sorptive Extraction-Liquid Chromatography with Fluorescence Detector) Method

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Este trabalho descreve um método para quantificação dos alcaloides harmana e harmina na polpa e nas sementes dos frutos de maracujá azedo (*Passiflora edulis* f. *flavicarpa* O. Degener, Passifloraceae), por extração dual por sorção com barra magnética e cromatografia líquida de alta eficiência com detecção por fluorescência (SBSE-LC/Flu dual). Os parâmetros para SBSE foram otimizados usando planejamento fatorial e o método dual SBSE-LC/Flu foi validado seguindo os parâmetros do *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use* (ICH). A grande sensibilidade e o pequeno manuseio da amostra tornam o método SBSE-LC/Flu dual atraente para aplicação em análise fitoquímica ou de alimentos.

A method for the quantification of the alkaloids harmane and harmine in sour passion fruit (*Passiflora edulis* f. *flavicarpa* O. Degener, Passifloraceae) pulp and seeds by stir-bar sorptive extraction and high performance liquid chromatography with fluorescence detection (dual SBSE-LC/Flu) is described. The SBSE parameters were optimized using a fractional factorial design, and the dual SBSE-LC/Flu method was validated following the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines. The high sensitivity and minimal sample handling of the dual SBSE-LC/Flu method make it attractive for application in phytochemical analysis or in the food industry.

Keywords: dual SBSE-LC/Flu, fractional factorial design, harmane alkaloids, sour passion fruit

Introduction

The methods most frequently used for isolating and concentrating organic compounds involve organic solvent extraction, which is time-consuming. Moreover, excessive handling of samples may lead to serious errors, besides the fact that sample preparation is considered to be potentially the most polluting step of analysis. For this reason, alternative methods that use an extractant as fractionating and pre-concentrating agent, such as stir-bar sorptive extraction (SBSE), are being used increasingly in the study of trace compounds in several kinds of matrices. SBSE is a preparation technique with a high capacity for enriching solutes from aqueous samples: for example, in our previous studies about infusions of the leaves of the medicinal species *Passiflora alata* Dryander ("maracujá", in Portuguese), a method was developed and validated to determine pesticide residues through direct sampling by SBSE combined with gas chromatography-electron capture detector/flame photometric detector (GC-ECD/FPD) and gas chromatography-mass spectrometry (GC-MS).[1] SBSE requires small amounts of solvent and is based on the use of a stir bar incorporated in a glass tube coated with a stationary phase, the most well-known of which is polydimethylsiloxane (PDMS). The stir bar is placed in the aqueous sample and the analytes are distributed between this matrix and the PDMS phase during stirring.[2,3]

The fact that the SBSE technique reduces the total amount of solvent used in the analytical procedure and that stir bars are reusable are also major advantages from

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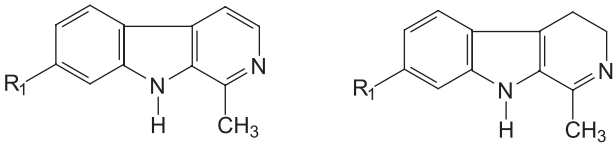
the standpoint of green analytical chemistry.[4] However, the combination of stir-bar sorptive extraction with liquid chromatography (SBSE-LC) is still underexplored, despite its potential applications in several fields such as biological and food analysis.[5,6]

PDMS is a non-polar material, and one of the most severe limitations of this SBSE coating is the poor recovery of highly polar analytes. On the other hand, combining SBSE with liquid desorption and high performance liquid chromatography (HPLC) may be an attractive approach for the analysis of alkaloids of intermediate polarity in complex samples, such as in food analysis. The literature offers several strategies to improve the extractability of polar compounds, including dual mode SBSE extraction,[5] which was utilized in this work. Ochiai *et al.*[7] optimized and validated a dual mode SBSE extraction multi-residue method to determine pesticides in vegetables, fruits and tea. In this method, two extractions were performed simultaneously: one extraction with 30% NaCl, mainly targeting hydrophilic (polar) analytes with log $K_{o/w} < 3.5$ (log octanol/water partitioning coefficient) and another, without the addition of NaCl, targeting hydrophobic (low-polarity) analytes with log $K_{o/w} > 3.5$.

Among SBSE applications, the analysis of substances naturally found in fruits has elicited increasing interest. *Passiflora edulis* f. *flavicarpa* O. Degener (Passifloraceae family; passion fruit or sour passion fruit) is a tropical fruit native to Brazil, the world's largest producer of this species, where it is known popularly as "maracujá" or "maracujá azedo" (sour passion fruit) and is widely cultivated, mainly for the use of its pulp in the food industry to produce processed juice and candies.[8]

Flavonoids are reported to be the major phytoconstituents of sour passion fruit. These compounds include *C*-glycosyl flavonoids such as isoorientin, orientin, vitexin, and isovitexin, which are also found in the leaves.[9-11] The flavonoid content in *P. edulis* pulp is reportedly quite significant in comparison with other sources of flavonoids, such as orange juice and sugarcane juice.[10] Zeraik *et al.* correlated the isoorientin content with antioxidant activity and suggested passion fruit as a possible source of natural antioxidants.[12] Sour passion fruit pulp showed a higher antioxidant capacity than sugarcane juice in both radical scavenging (2,2-diphenyl-1-picrylhydrazyl, DPPH$^{\bullet}$) and ceric reducing antioxidant capacity (CRAC) assays.[13] The analysis of sour passion fruit rind extracts also showed a high content of the flavonoid isoorientin and the radical scavenging (DPPH$^{\bullet}$) ability was higher than that of pulp extracts.[14] In short, also due to its high nutritional value and flavonoid contents, sour passion fruit can be considered a functional food.[8,12,14]

In addition to flavonoids, various indole harmane-derived alkaloids are reportedly present in *Passiflora* leaves.[15,16] Despite the popular use of passion fruit juice as an anxiolytic drug,[8] only one study has attributed this effect to the presence of alkaloids.[17] This report also shows the investigation of the alkaloid content in *P. edulis* Sims (0.012 mg kg^{-1}) juice and in *P. edulis* f. *flavicarpa* (0.7 mg kg^{-1}) juice by thin layer chromatography and densitometric quantification using Dragendorff's reagent, and the identification of harmane (**1**), harmine (**2**), harmol (**3**) and harmaline (**4**), Figure 1.[17]



	R_1		R_1
(**1**) Harmane	H	(**4**) Harmaline	OCH_3
(**2**) Harmine	OCH_3		
(**3**) Harmol	OH		

Figure 1. Harmane alkaloids reported in passion fruit pulp, according to Lutomski *et al.*.[17]

Probably due to the difficulty of analyzing alkaloids in complex samples, such as fruit pulp or juices, the alkaloids in passion fruit pulp are still a little investigated subject. On the other hand, a number of pharmacological activities affecting mainly the central nervous system have been attributed to these alkaloids, as well as the antioxidant action, immunomodulatory effects and cardiovascular action of harmane alkaloids.[18,19] Moreover, the literature on *Passiflora* chemistry also relates toxicity, probably due to cyanogenic glycosides and indole alkaloids, with *P. incarnata*, which is the most extensively studied species.[8] However, only one study about the toxicity of *P. edulis* leaves' aqueous extract has shown hepatobiliary and pancreatic toxicity in rodents and humans.[20]

Another reason for investigating the harmane alkaloid content in passion fruit is the growing applications of its by-products, since there are several ongoing technological efforts to reduce waste and add value to the by-products of passion fruit juice production. For example, pressing of its seeds yields oil, which is mostly used in cosmetics, and the seed press residue can also be used by the cosmetic industry in exfoliating products or as an ingredient in the production of animal feed. The commercial use of these by-products, however, requires a more in-depth knowledge about their chemical composition to avoid safety and toxicological problems.[21]

Several HPLC methods have been reported for the analysis of harmane alkaloids in *Passiflora*, but not

specifically for *P. edulis* pulp. Rehwald *et al.*[15] attempted to determine the concentration of harmane alkaloids in 17 different samples of *P. incarnata* by reverse-phase HPLC, but only one of their samples contained trace amounts of harmane (ca. 0.1 mg L^{-1}). Using HPLC with selective fluorimetric detection, Tsuchiya *et al.*[22] made a quantitative analysis of several β-carboline alkaloids in *P. incarnata*. The vegetative parts of greenhouse-cultivated *P. incarnata* contain 0.012 and 0.007% of harmane and harmine, respectively, while the content of these alkaloids in plants grown in the field has been reported as 0.005% and nil, respectively.[15,23] Abourashed *et al.*[24] analyzed harmane alkaloid contents in the leaves of 91 *Passiflora* species, among them *P. edulis* Sims, using accelerated solvent extraction (ASE), followed by acid-base and solid phase extraction on cation exchange resin before reverse-phase HPLC-UV/photodiode array detector (PAD) analysis. *P. edulis* Sims was found to contain concentrations of 0.02 mg L^{-1} of harmane and 0.11 mg L^{-1} of harmine.

This article describes the development and validation of a method for quantifying the alkaloids harmane and harmine (Figure 1, **1** and **2**, respectively) in sour passion fruit pulp and seeds through direct sampling by dual mode SBSE combined with liquid chromatography and fluorescence detection (SBSE/LC-Flu). The extraction was optimized by applying a fractional factorial design to investigate the relative contributions and interactions between the numerous experimental variables involved in SBSE. The dual mode was investigated with a view to enhancing the efficiency of the SBSE step. This is the first study involving the analysis of harmane alkaloids using SBSE combined with HPLC.

Experimental

Samples, chemicals and materials

A pool of around 10 kg of fruits of *Passiflora edulis* f. *flavicarpa* O. Deg. (sour passion fruit) were purchased locally in São Carlos, SP, Brazil, on April 2013. To prepare the pulp and seed samples, all the fruits were cut open with a knife and the pulp with seeds were removed with a spoon. The pulp without seeds was mixed in order to obtain a homogeneous sample and was stored at –20 °C prior to its use. The seeds were separated by using a domestic sieve and dried on a conventional laboratory oven without forced ventilation at 40-45 °C, until constant weight (around 72 hours). The dried seeds were triturated in a domestic blender and ground; only particles between 0.5-1.0 mm were utilized for the extractions.

Methanol and acetonitrile were of HPLC grade, both purchased from Tedia (Fairfield, OH, USA). Formic acid was purchased from Merck (Darmstadt, Germany). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Harmane, harmine, harmol, harmalol (98% purity) and harmaline ("purum" grade) standards were purchased from Sigma-Aldrich (Steinheim, Germany).

Commercial stir bars (Twister™) for sorptive extraction were supplied by Gerstel (Mulheim an der Ruhr, Germany) and consisted of glass-encapsulated magnetic stir bars, 20 mm long, with 0.5 mm film thickness, coated with 47 μL of PDMS (code GC 011444-001-00). Prior to their first use, the stir bars were conditioned for 72 h using methanol and dichloromethane (50:50 v/v). The solvent mixture was then discarded and the stir bars were dried in a desiccator at room temperature and heated for 2 h at 300 °C under a nitrogen stream.

Optimization of the SBSE process

Alkaloid extraction was optimized using an experimental fractional factorial design 2^{5-1} (16 trials plus 3 central points) and was performed in triplicate and in random order on three different days. Experiments were conducted to evaluate the influence and interaction of the five selected factors (pH, extraction time, percentage of NaCl, desorption time and percentage of methanol as desorption solvent). Table 1 lists the values of the levels used. MiniTab 16 software was used to calculate the effects and to plot the Pareto chart ($p = 0.05$) and the estimated response surface.

Table 1. Levels and factors used in fractional factorial design 2^{5-1}, utilized for the optimization of the extraction of the alkaloids harmane and harmine by SBSE

Factor	Level		
	–1	0	+1
pH	3	6	13
Extraction time / min	60	90	120
NaCl / %	0	36	50
Desorption time / min	10	20	60
Methanol as desorption solvent / %	10	50	100

Experiments were conducted separately using harmane and harmine standard solution (20 μg L^{-1}) of each alkaloid in Milli-Q water. After placing a clean stir bar into a 10 mL vial, the vial was capped and stirred at 1000 rpm at ambient temperature for the required time. After the extraction, the stir bar was removed from the sample solution, carefully dried with tissue paper, and completely immersed in an 150 μL vial with the desorption solvent.

Desorption was conducted by sonication for the required time. After desorption, the stir bar was removed and the methanol stripping solution was used directly for HPLC-Flu analysis. The response used for optimization was the area of the alkaloid's chromatographic peak. Recovery (R%) of each experiment was calculated by comparing the final concentration of the alkaloid, after the SBSE experiment ([experimental]) with a 20 μg L^{-1} alkaloid standard solution, using equation 1.

$$R\% = \frac{[experimental]}{20} \times 100 \quad (1)$$

Dual mode SBSE extraction

Dual SBSE was performed simultaneously on two aliquots of the same passion fruit sample, under different extraction conditions and using one coated stir bar *per* sample.

Analysis of passion fruit pulp

The first sample of 1 mL of passion fruit pulp was poured into a 10 mL vial with 2 mL of 0.1 mol L^{-1} NaOH (to ensure pH 13), 5.0 g of NaCl and 7 mL of Milli-Q water. In parallel, a second sample of 1 mL of passion fruit pulp was poured into another vial with 1 mL of 0.1 mol L^{-1} NH_4OH (to ensure pH 10), 5.0 g of NaCl and 8 mL of Milli-Q water. A stir bar was used in each vial to stir the samples for 120 minutes at 1000 rpm and room temperature. After sampling, the stir bars were removed, rinsed with purified water and dried with tissue paper. The stir bars were then desorbed by sonication for 60 minutes in the same vial with 150 μL of methanol. After desorption, the stir bar was removed and the methanol stripping solution was used directly for HPLC-Flu analysis.

Analysis of passion fruit seeds

The first sample of 1.0 g of passion fruit dried seeds was poured with 1 mL of 1.0 mol L^{-1} NaOH (to ensure pH 13), 5.0 g of NaCl and 9 mL of Milli-Q water. In parallel, a second sample of 1.0 g of passion fruit dried seeds was poured into another vial with 0.3 mL of 0.1 mol L^{-1} NH_4OH (to ensure pH 10), 5.0 g of NaCl and 9.7 mL of Milli-Q water. A stir bar was used in each vial to stir the samples for 120 minutes at 1000 rpm at room temperature. After sampling, the stir bars were removed, rinsed with purified water and dried with tissue paper. The stir bars were then desorbed by sonication for 60 minutes in the same vial with 150 μL of methanol. After desorption, the stir bar was removed and the methanol stripping solution was used directly for HPLC-Flu analysis.

HPLC analysis

Samples were filtered through a 0.45 μm Millex-HV PVDF membrane (Millipore, New Bedford, MA, USA) prior to HPLC analysis. HPLC analyses were carried out with a Waters Alliance 2695 liquid chromatograph (Waters, Milford, MA, USA) coupled to a Waters 2996 photodiode array detector (UV/PAD) and a Waters 2475 fluorescence photodiode array detector (Flu/PAD), controlled by Waters Empower software. The separation was performed using a Waters X-Terra® C_{18} column (250 mm × 4.6 mm i.d., 5 μm) preceded by an X-Terra® C_{18} guard column (2.0 cm × 4.0 mm i.d.; 5 μm), also from Waters. Chromatographic conditions were: 0.5% formic acid in acetonitrile (solvent A) and 0.5% formic acid in water (solvent B). The gradient was programmed from 20 to 34% A for 10 min, and 34 to 20% A for 18 min. The flow rate was 1 mL min^{-1}, the column temperature was 25 °C and the injection volume was 10 μL. To detect harmane, the fluorescence detector was set at $\lambda_{excitation}$ = 254 nm and $\lambda_{emission}$ = 425 nm, and to detect harmine, the detector was set at $\lambda_{excitation}$ = 254 nm and $\lambda_{emission}$ = 410 nm. The alkaloids were identified in passion fruit samples by direct comparison with standards (fluorescence and UV spectra and retention time).

Analytical validation

Validation of the dual SBSE/HPLC-Flu method was performed according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines,[25] which establishes the evaluation of the following parameters: specificity, linearity and range, accuracy, precision (repeatability and intermediate precision), limit of detection (LOD) and limit of quantification (LOQ). The dual method was validated using passion fruit pulp samples and seed samples, separately, which were spiked with harmane or harmine solutions, also separately.

To examine the specificity, the purity of the peaks was checked using fluorescence detector software. Sections of the spectra corresponding to the upslope and downslope of each peak were overlapped, and peaks were considered pure when the two spectral sections coincided.

The linearity was determined by the correlation coefficients of the analytical curves, which were built by analyzing the working solutions at five different concentration levels, spiking the samples of passion fruit pulp or seeds with volumes of the stock solution of harmane (100.0 μg L^{-1} in methanol) or harmine (100.0 μg L^{-1} in methanol). For the analysis of passion fruit pulp, final

concentrations were from 0.1 to 70.0 μg L^{-1} and for the analysis of the seeds, final concentrations were from 0.1 to 20.0 μg L^{-1}.

Recovery experiments were performed to evaluate the accuracy of the methods. Passion fruit samples were spiked with three concentration levels of the alkaloids (pulp samples: spiked with 0.1, 30.0 and 70.0 μg L^{-1} in methanol; seed samples: spiked with 0.1, 10.0 and 20.0 μg L^{-1} in methanol) prior to extracting the alkaloid. The spiked samples were analyzed in triplicate. Accuracy was expressed as the percentage of deviation between the amount of standard found by HPLC-Flu analysis and the amount added at the three concentration levels examined.

Repeatability was estimated by injecting in triplicate, on the same day, spiked samples containing three different concentrations of alkaloids (pulp samples: spiked with 0.1, 30.0, and 70.0 μg L^{-1}; seed samples: spiked with 0.1, 10.0 and 20.0 μg L^{-1}). Intermediate precision was determined by analyzing, in triplicate, the same solutions employed in the repeatability test on three consecutive days. Precision was expressed in terms of relative standard deviation (RSD).

LOD and LOQ were estimated experimentally by injecting standard solutions of harmane and harmine diluted in methanol until the signal-to-noise ratio for the standards reached a 3:1 ratio for LOD and 10:1 for LOQ.

Quantitative analysis of harmane and harmine in passion fruit samples

The alkaloids in the passion fruit samples were quantified by the standard addition method, using analytical curves constructed from samples spiked with a stock solution of harmane (100.0 μg L^{-1} in methanol) or harmine (100.0 μg L^{-1} in methanol) to reach a final concentration in the range of 0.1 to 70.0 μg L^{-1} each in the case of pulp samples and in the range of 0.1 to 20.0 μg L^{-1} each in the case of seed samples. This procedure was repeated in quintuplicate. The amount of harmane was calculated based on the peak area at $\lambda_{excitation}$ = 254 nm and $\lambda_{emission}$ = 425 nm and the amount of harmine was calculated based on the peak area at $\lambda_{excitation}$ = 254 nm and $\lambda_{emission}$ = 410 nm. Data were expressed as the mean ± standard deviation (SD).

Gas chromatography-mass spectrometry (GC-MS)

To confirm the presence of alkaloids in passion fruit pulp samples, the extracts were analyzed by GC-MS. A Shimadzu GC2010 plus (Kyoto, Japan) gas chromatography system was used, coupled to a mass selective detector (Shimadzu MS2010 plus) operating in electron ionization (EI) mode (electron impact, 70 eV). The GC was fitted with a DB5 fused silica column of 30 m × 0.25 mm i.d. × 0.25 mm film thickness of 5% phenyl, 95% dimethylpolysiloxane (J & W Scientific, Agilent Technologies, Santa Clara, CA). The oven temperature was programmed from 50 to 300 °C at a heating rate of 5 °C min^{-1}. The injector and detector temperatures were held at 200 °C; injector split ratio was 1:3 and helium was used as the carrier gas under a pressure of 60 kPa. The interface temperature was 300 °C. The spectra were acquired in scan and selected ion monitoring (SIM) mode and, in the latter mode, harmane was monitored using *m/z* 182, 181 and 154 ions and harmine using *m/z* 212, 197 and 169 ions.

Results and Discussion

Passion fruit juice is usually prepared at home by beating the pulp and seeds in a domestic blender. In contrast, industrially processed juice uses only the pulp, which is separated from the seeds by depulpers through centrifugation and in batches to separate the pulp from the peel and seeds. Therefore, and also considering the possibility of applying the SBSE-LC method, for example, in routine analyses for quality control or safety inspection, the analytical method was developed using seedless passion fruit pulp and also the separated seeds.

Preliminary assays were performed by thin layer chromatography (TLC) to choose the harmane alkaloids for quantitative investigation in this work. The results of the TLC assays suggested the presence of harmane and harmine by direct comparison of the *P. edulis* fruit pulp and seed extracts with authentic commercial standards. The identity of these alkaloids was confirmed in the passion fruit extracts analyzed by HPLC-UV/PAD and Flu/PAD, by comparing their retention time (t_R) and spectra with those of commercial standards of harmane, harmine, harmol and harmalol. Using as criteria the coincidence of t_R, UV/PAD and fluorescence spectra, and also considering GC-MS data, two alkaloids - harmane and harmine (Figures 2 and 3) - were identified unequivocally in both pulp and seed extracts.

Different gradient programming, flow rate (0.8 and 1.0 mL min^{-1}) and mobile phase (organic solvent - methanol or acetonitrile - and their proportion) were also tested to determine an adequate resolution and shortest possible analysis time for the quantitative analysis of alkaloids. Although reverse-phase HPLC separation of basic alkaloids, such as the indole alkaloids, has been described as suffering from peak tailing,[15] the addition of 0.5% formic acid to water and to acetonitrile (pH ca. 3.5) resulted in good separation of all the analytes.

The performance of UV/PAD and Flu/PAD detection were compared and the latter was chosen for the analysis of *Passiflora* extracts due to its higher sensitivity, and also considering the good selectivity achieved by the adequate choice of $\lambda_{excitation}$ and $\lambda_{emission}$ for each alkaloid, which also provided high sensitivity.

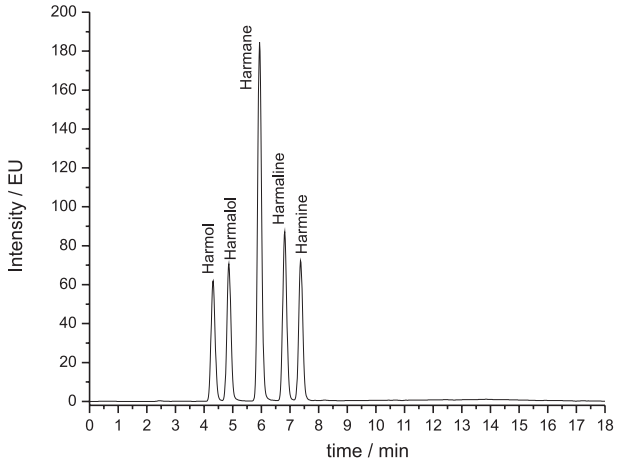


Figure 2. HPLC-Flu/PAD chromatogram ($\lambda_{excitation}$ = 254 nm, $\lambda_{emission}$ = 410 nm) of a mixture of commercial standards of harmane alkaloids. Chromatographic conditions were fully described in the Experimental section, HPLC analysis.

Optimization of the SBSE extraction

Experimental design, which considers several variables simultaneously, seems to be the most convenient approach in searching for optimal operating conditions in a reasonable number of runs.[26] Therefore, this methodology was used to optimize the extraction of harmane and harmine from passion fruit samples. For this optimization step, each alkaloid was investigated separately and the procedure described in the Experimental section "Optimization of the SBSE process" refers to the optimized conditions for each alkaloid.

Based on the literature,[1,27-29] five variables that could affect the SBSE extraction efficiency were selected to define the experimental field, using three different levels: NaCl percentage (m/v), pH, extraction time, desorption time and percentage of methanol as desorption solvent (Table 1). The

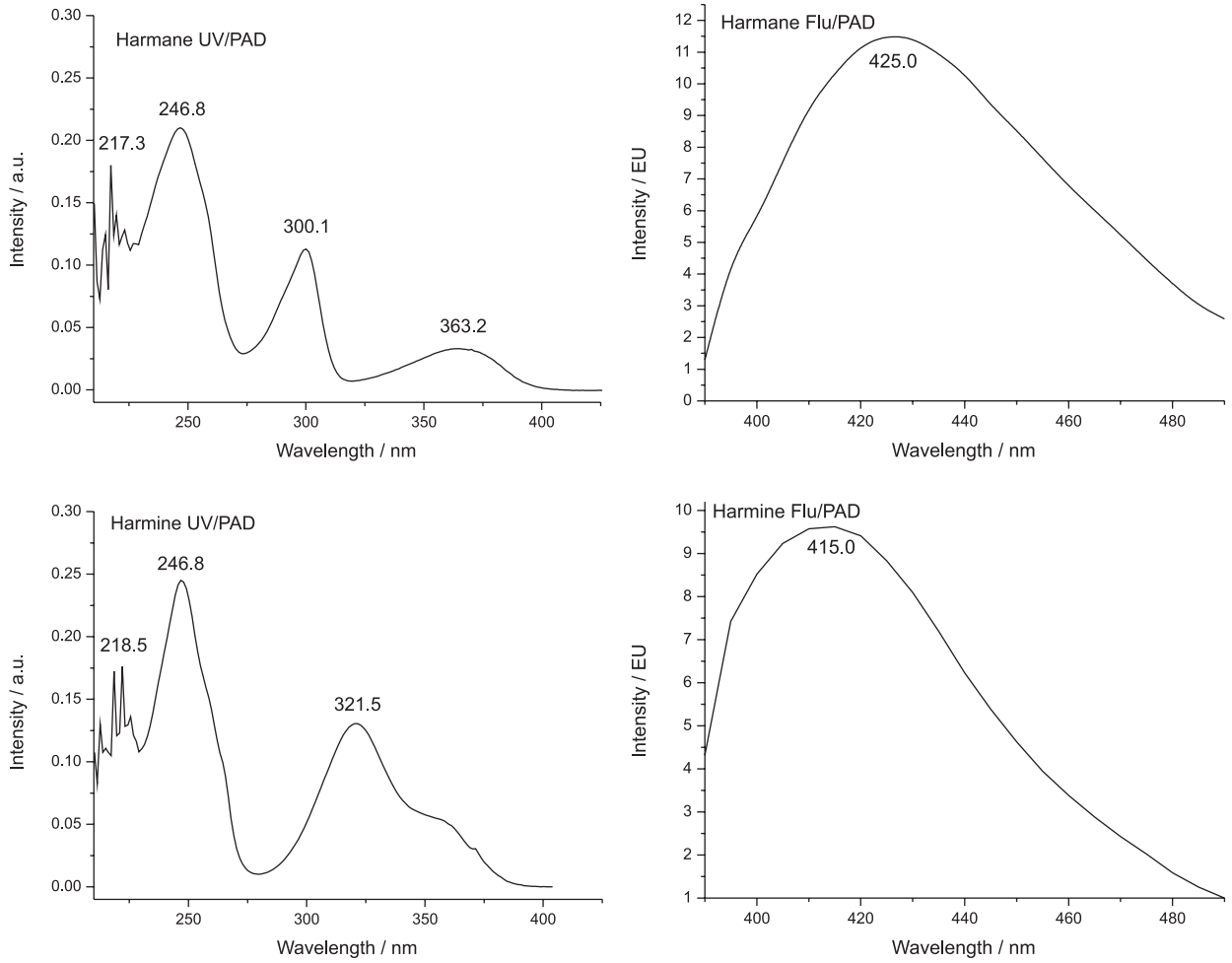


Figure 3. On-line UV/PAD and Fluorescence/PAD spectra of the alkaloids quantified in sour passion fruit pulp and seed, harmane and harmine.

sample volume (10 mL), stirring speed and stir bar type/size were kept constant during optimization. Preliminary experiments also indicated that the optimization should be conducted separately with harmane and harmine in order to achieve the highest extraction efficiency for each alkaloid.

The effect of the samples' pH on their extraction efficiency was investigated in the range of 3.0 to 13.0, considering that the extraction yield in SBSE is correlated with dissociation constants of the analyte[30,31] in aqueous solution, harmane and harmine may exist in three differently charged species, and the equilibria are governed by two pKa values (harmane: 14.50 and 8.6; and harmine: 14.43 and 8.0). The structure of harmane and harmine has two sites of dissociation - the weakly acid NH group of the indole ring and the basic nitrogen atom of pyridine.[32,33] Thus, at higher pH, alkaloids are expected to be in the neutral form and to exhibit lower water solubility, making them very suitable for SBSE extraction by the PDMS phase. For good reproducibility of the analytical method, the ionization state of the alkaloids must be controlled.

The Pareto chart shows that pH level has a significant effect on extraction efficiency (Figure 4). Notwithstanding recommendations to avoid too acidic (pH < 2) or too basic (pH > 9) conditions in order to prevent PDMS-phase degradation and to extend the lifetime of PDMS-coated stir-bars,[34] these problems were not observed since the robustness of the PDMS stir bar was confirmed in over 40 extractions with a minimum loss of extraction efficiency.

One of the difficulties in extracting alkaloids from passion fruit is their polarity: harmane has log $K_{o/w}$ = 3.1 and harmine has log $K_{o/w}$ = 3.56, which means they are considered intermediate polarity alkaloids. For polar analytes (log $K_{o/w}$ < 3.5), the percentage of recovery increases with the addition of inert salts because the extraction performance of SBSE for polar compounds depends strongly on the ionic strength of the sample matrix.[5] In the case of passion fruit samples, the ionic strength of the matrix was adjusted by adding 0 to 50% (m/v) of NaCl. It was observed that increasing the percentage of NaCl enhanced the extraction efficiency, possibly due to the salting-out effect and the electrostatic interactions between polar molecules and NaCl ions in sample solution. In the step of liquid desorption, the nature of the stripping solvent, desorption time and desorption volume are the most frequently studied variables.[5] To minimize the solvent volume, liquid desorption was performed in a vial containing a 150 µL glass insert. Since harmane and harmine are completely soluble in methanol, the percentage of methanol as desorption solvent was also studied (Table 1). The Pareto chart (Figure 4) shows the influence of each investigated factor on the response, as well as the possible cross-effects among these factors. Considering the chromatographic peak area of harmane or harmine as the response, the results demonstrated that the effects of pH and the percentage of NaCl and methanol are significant factors (p < 0.05). The percentage of NaCl *vs.* that of methanol interaction was found to be statistically significant (p < 0.05), as was pH *vs.* percentage of methanol and pH *vs.* percentage of NaCl. This interpretation can be confirmed by the response surface (Figure 5), which shows the optimal level for each variable. The highest response in harmane extraction was obtained with pH 13, 50% NaCl, 100% methanol, extraction time of 120 min and desorption time of 60 min, which resulted in a recovery rate of (98.76 ± 0.16)%.

In harmine extraction, all the effects are significant factors (p < 0.05). Figure 3 also shows the response surfaces of several interactions. The highest response in harmine extraction was obtained with pH 10, 50% NaCl, 100% methanol, extraction time of 120 min and desorption time of 60 min, which resulted in a recovery rate of (97.36 ± 0.11)%.

Theoretical recoveries can be predicted considering theoretical values of $K_{o/w}$ and the volume of PDMS, since

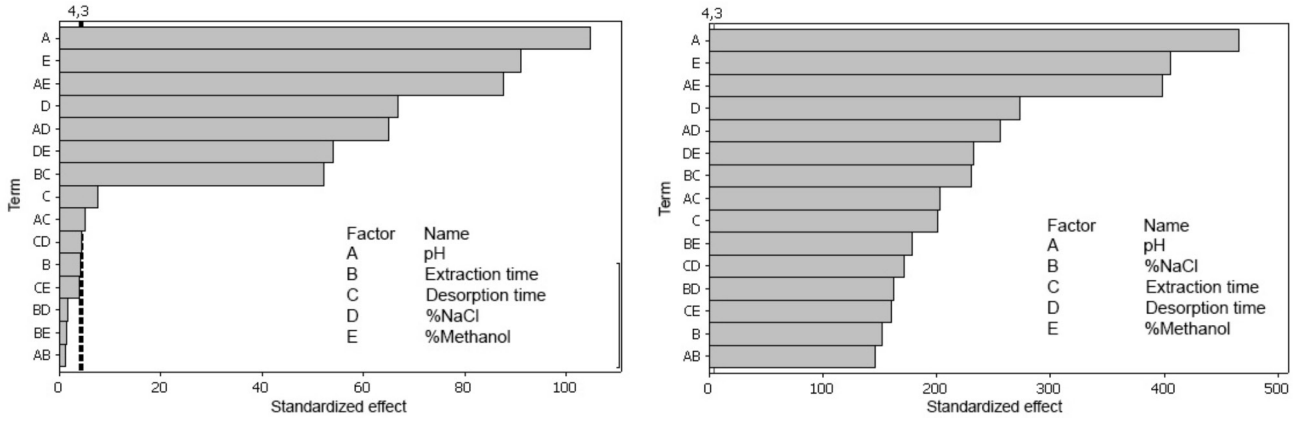


Figure 4. Pareto chart showing the values of effects from variables using the harmane area as response (left) and harmine as response (right).

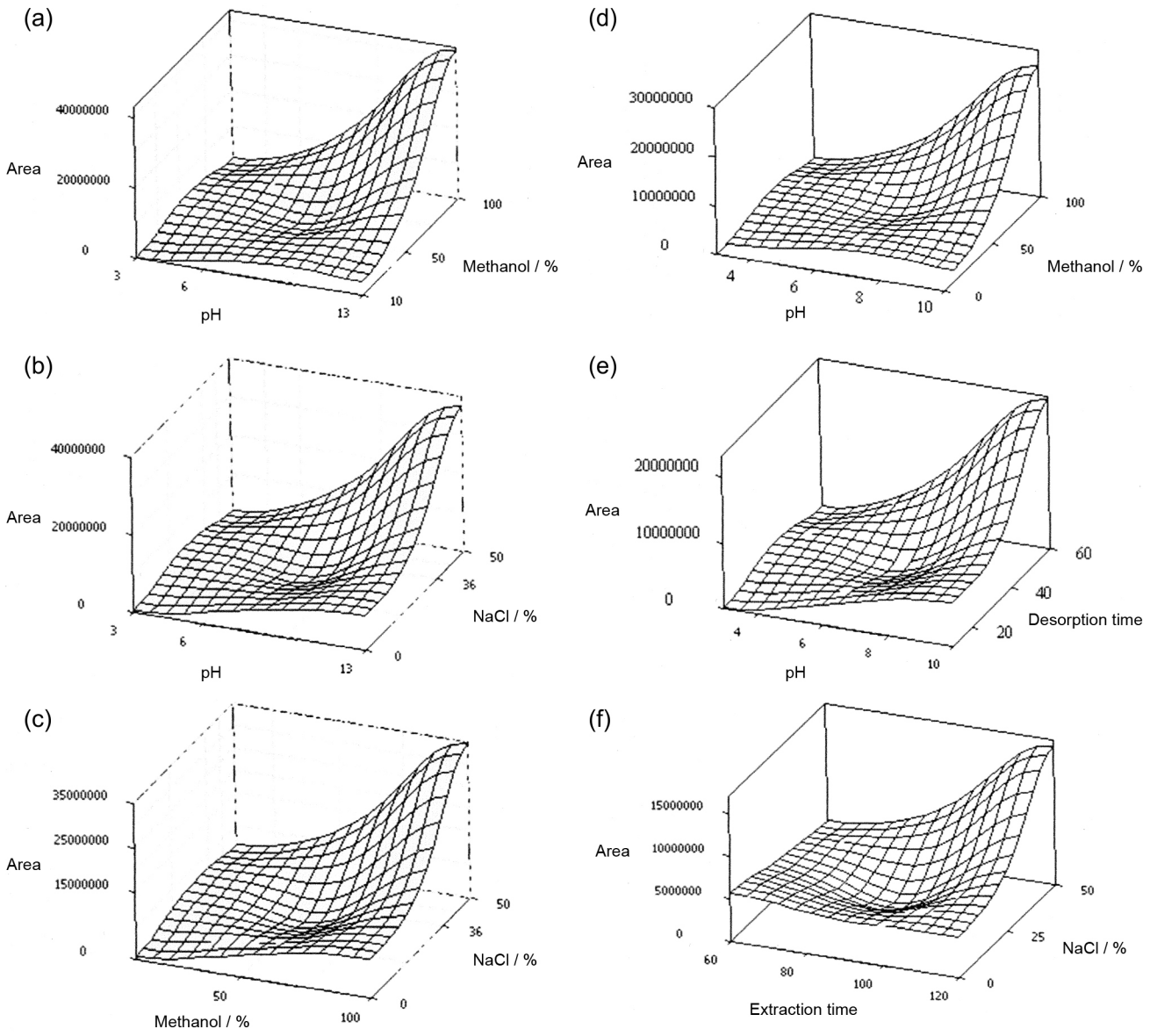


Figure 5. Response surface estimated from the fractional factorial design: For harmane: (a) methanol percentage *vs.* pH; (b) NaCl percentage *vs.* pH; (c) NaCl percentage *vs.* methanol percentage. For harmine: (d) methanol percentage *vs.* pH; (e) pH *vs.* desorption time and (f) NaCl percentage *vs.* extraction time.

sorption equilibrium, and therefore recovery, are also dependent on the β phase ratio (volume of the water phase divided by the volume of the PDMS phase).[35] Since the sample volume was 10 mL and the volume of PDMS phase was 55 μL, the theoretical value for recovery calculated using Twister Recovery Calculation™ was 94.5% for harmine and 85.5% for harmane. Thus, the experimental results exceeded the theoretical recovery values.

The extraction conditions were optimized individually for harmane and harmine and applied in the dual mode SBSE extraction. A major advantage of the dual mode is the possibility of simultaneous analysis using different extraction conditions without increasing the overall analysis time. In dual mode, the desorption stage of the two stir bars is performed in the same vial, allowing for the analysis and quantification of both alkaloids in a single chromatographic run while reducing the time and amount of solvent used.

According to Douglas *et al.*,[31] a property that differentiates harman alkaloids is the pKa values. The pKa value of harmaline is reported to be 10.0 and the equilibria of harmalol and harmol are governed by two pKa values. The pKa values for harmalol are 8.6 and 11.3; while for harmol they are 7.90 and 9.47. These differences were exploited in the optimization of the dual SBSE procedure, and the extraction conditions (one step at pH 10 and another at pH 13) were found to be favorable for obtaining the neutral (non-ionic) forms of harmine and harmane, respectively, which exhibit lower water

solubility, making them suitable for SBSE extraction. However, the PDMS phase does not promote the extraction of compounds with log $K_{o/w}$ < 3.5 (log $K_{o/w}$ harmol = 1.82; log $K_{o/w}$ harmalol = 2.19; log $K_{o/w}$ harmaline = 2.57). In a preliminary test, it was observed that less than 1% of harmol was extracted.

Like most microextraction techniques, the efficiency of SBSE extraction can also be affected by the composition of the matrix.[36] High levels of dissolved or suspended organic matter may compete with the analytes in the PDMS phase on the stir bar, thus possibly changing the extraction yield from one sample to another. Larger volumes of passion fruit sample volumes were found to reduce the extraction efficiency; a similar trend has also been reported in water samples.[37] After several tests, the use of a sample amount of 1.0 mL of passion fruit pulp or 1.0 g of dried seeds proved to be the best condition to ensure the high efficiency of the SBSE step.

The dual mode SBSE extraction of alkaloids standards yielded good results: the recovery rates of (98.87 ± 0.76)% for harmane and (97.82 ± 0.31)% for harmine were also higher than the recovery values calculated using Twister Recovery Calculation™. The results did not differ statistically (Student's *t*-test, $p = 0.05$) from those obtained in single mode extraction (discussed in the section Optimization of the SBSE extraction: recovery values of (98.76 ± 0.16)% for harmane and (97.36 ± 0.11)% for harmine). Moreover, considering that the two target alkaloids can be extracted simultaneously, thereby reducing the total analysis time, the dual mode was chosen for validation of the SBSE-LC/Flu method.

Analytical validation

The dual SBSE-LC/Flu method was validated following the ICH guidelines.[25] Linearity was checked based on the values of correlation coefficients of the analytical curves. All the curves showed a linear response with $r^2 > 0.99$ in the selected spiking range for each sample (Table 2). Fluorescence detection proved to be a highly selective and sensitive method for detecting alkaloids, but the fluorescence emission of harmane was found to be about 3-fold higher than the same amount of harmine. Harmane showed a LOD of 30 ng L^{-1} and LOQ of 100 ng L^{-1}, while harmine showed a LOD of 100 ng L^{-1} and LOQ of 400 ng L^{-1}.

The analytical performance data for the dual SBSE-LC/Flu method is given in Table 3. The precision of the dual method was estimated by measuring repeatability (intra-day, n = 3) and intermediate precision (in three days, n = 3) at three different levels. All the values of RSD in the repeatability and intermediate precision estimates were below 5%, as recommended by the ICH protocols.[25] The accuracy of the method was evaluated by recovery experiments, spiking the samples with the standards at three different concentration levels. Because the passion fruit samples already contained harmane and harmine, the area of their respective chromatographic peaks obtained in the analysis of non-spiked samples was subtracted in the alkaloid recovery calculations. The recovery values ranged from 83.61 to 105.90% and were also in line with ICH parameters, thus confirming the accuracy of the SBSE-LC/Flu dual method.

Harmane and harmine quantification in sour passion fruit

Due to the presence of several compounds in pulp and seed extracts, the matrix effect was observed and therefore the standard addition method was employed to quantify alkaloids. In addition, the peak purity of chromatographic peaks corresponding to harmane and harmine was determined by overlapping the spectra in different regions on the same peak, using the HPLC software. Figure 6 and 7, respectively, show typical chromatograms of the passion fruit pulp and seed samples obtained by the dual SBSE-LC/Flu method. Passion fruit seeds contained ($3.09 \times 10^{-2} \pm 5.87 \times 10^{-5}$) mg harmane g^{-1} dried seeds and ($8.11 \times 10^{-3} \pm 7.60 \times 10^{-4}$) harmine g^{-1} dried seeds, while pulp was found to contain (3.00 ± 0.04) mg harmane L^{-1} and (2.72 ± 0.02) mg harmine L^{-1}. These latter data are expressed as μg alkaloid L^{-1} pulp because the pulp could not be dried completely using either a conventional laboratory oven (40-45 °C, without forced ventilation) or by lyophilization.

These results indicate that both pulp and seed samples contain the two target alkaloids, and confirm the feasibility

Table 2. Parameters of the analytical curves utilized for quantification of harmane and harmine in sour passion fruit samples by dual mode SBSE/LC-Flu

Samples	Spiking range / (μg L^{-1})	Regression equation	r^2
Pulp spiked with harmane	0.1-70.0	$y = 1.22 \times 10^6 x + 8.46 \times 10^7$	0.990
Pulp spiked with harmine	0.1-70.0	$y = 3.42 \times 10^6 x + 2.59 \times 10^7$	0.994
Seeds spiked with harmane	0.1-20.0	$y = 7.40 \times 10^2 x + 1.54 \times 10^6$	0.998
Seeds spiked with harmine	0.1-20.0	$y = 3.15 \times 10^2 x + 1.72 \times 10^5$	0.999

Table 3. Analytical performance data for the dual mode SBSE-LC/Flu method applied to the quantitative analysis of harmane and harmine in the sour passion fruit samples

Samples	Standard spiked	Concentration levels / ($\mu g\ L^{-1}$)	Recovery (n = 3) / %	Repeatability (1 day, n = 3) RSD / %	Intermediate precision (3 days, n = 3) RSD / %
Pulp	Harmane	0.10	91.24	0.12	2.49
		30.0	93.05	0.35	0.82
		70.0	101.91	0.14	0.18
Pulp	Harmine	0.10	92.87	0.68	0.27
		30.0	104.60	1.12	0.41
		70.0	91.88	0.97	0.39
Seeds	Harmane	0.10	105.90	0.41	0.76
		10.0	99.34	0.35	0.16
		20.0	92.34	0.22	0.07
Seeds	Harmine	0.10	117.76	3.69	2.56
		10.0	95.45	0.68	1.17
		20.0	83.61	0.11	0.25

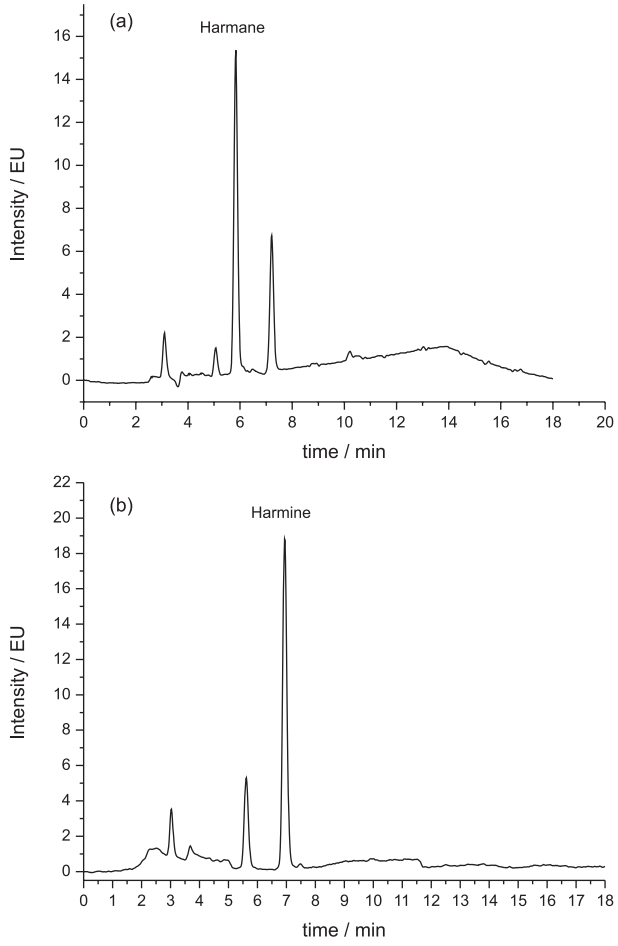


Figure 6. Typical HPLC-Flu/PAD chromatograms of the passion fruit pulp samples obtained by the dual SBSE-LC/Flu method: (a) detection at $\lambda_{excitation}$ = 254 nm, $\lambda_{emission}$ = 425 nm (best conditions for detection of harmane); (b) detection at $\lambda_{excitation}$ = 254 nm, $\lambda_{emission}$ = 410 nm (best conditions for detection of harmine).

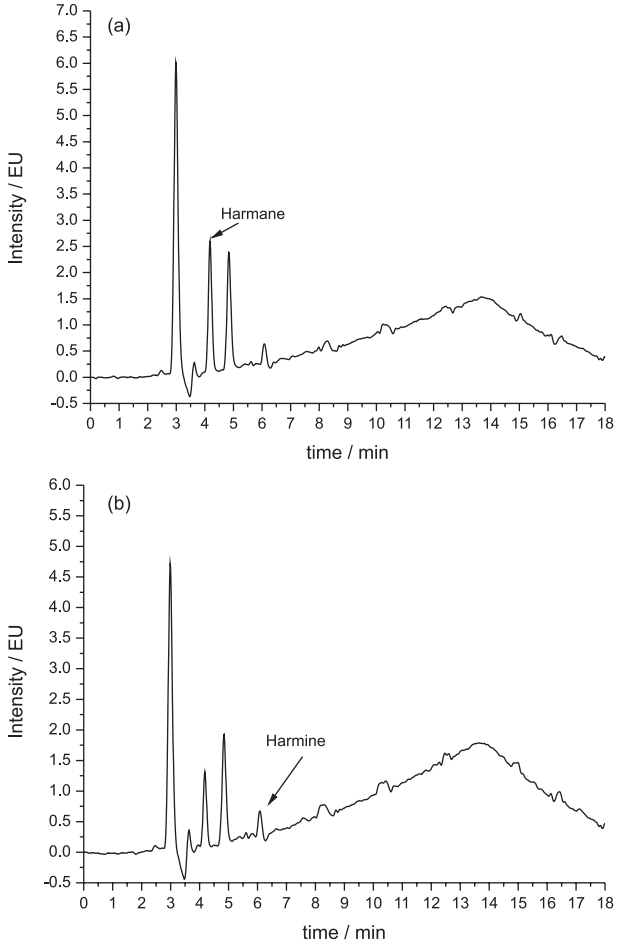


Figure 7. Typical HPLC-Flu/PAD chromatograms of the passion fruit seed samples obtained by the dual SBSE-LC/Flu method: (a) detection at $\lambda_{excitation}$ = 254 nm, $\lambda_{emission}$ = 425 nm (best conditions for detection of harmane); (b) detection at $\lambda_{excitation}$ = 254 nm, $\lambda_{emission}$ = 410 nm (best conditions for detection of harmine).

of applying the dual SBSE-LC/Flu method as a tool for further studies of passion fruit chemistry (e.g., seasonal or geographical variations in the content of harmane alkaloids).

The data indicating the presence of harmane and harmine in sour passion fruit pulp and seeds also suggests the need for further investigations considering the aspects of food and cosmetics safety. The high sensitivity of the fluorescence detector and the low LOD value achieved in this method is correlated with the structure and photophysical properties of harmane alkaloids. These characteristics are also correlated with the photosensitization process: e.g., recent reports show that some β-carboline alkaloids, including harmane and harmine derivatives, are efficient photosensitizers in response to UVA (365 nm) radiation.[38]

Conclusions

The results of this study indicate that SBSE combined with HPLC-fluorescence detection is a feasible method for the quantitative analysis of harmane alkaloids in passion fruit pulp. The analytical determination of alkaloids in fruits is not simple because of the complexity of the real matrices to be analyzed. However, the low LOD and minimal sample handling make the dual SBSE-LC/Flu method attractive. Moreover, in view of its possibilities of automation and speed, dual SBSE-LC/Flu is a candidate method for screening numerous plant samples in further agronomical or phytochemical studies of passion fruit alkaloids or even as a routine quality control procedure in the food industry. The data on the presence of harmane and harmine in sour passion fruit pulp and seeds also suggest the need for further investigations considering safety aspects of foods and cosmetics containing products derived from passion fruit.

Acknowledgements

The authors are grateful to Mr. Benedito M. dos Santos for technical assistance, and to Prof André Luiz Meleiro Porto and Mr. João Pedro de Freitas Lima for the GC-MS analyses. The authors also wish to thank the Brazilian Agencies FAPESP (Proc. 06/59457-6 and 2010/05711-4) and CNPq (Proc. 30669/2008-9) for fellowships and financial support to this work.

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Submitted: March 25, 2014
Published online: June 6, 2014

FAPESP has sponsored the publication of this article.