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Article

Targeted-Analysis of β-Carboline Alkaloids in Passionfruit ("Maracujá") by SBSE(PDMS)-LC/Flu and UHPLC-MS

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Samples of pulp and seeds of sour (*P. edulis*) and sweet (*P. alata*) passionfruit were subjected to targeted analysis to identify β-carboline alkaloids, using combined UHPLC-MS (ultrahigh performance liquid chromatography-mass spectrometry) and SBSE(PDMS) (stir bar sorption extraction using polydimethylsiloxane as stationary phase) for sample preconcentration. All the samples contained harmane and harmine; harmaline was found only in *P. alata* seeds and in *P. edulis* pulp, while harmalol was found only in *P. alata* seeds. An analysis of sweet passionfruit by SBSE(PDMS) combined with LC/Flu (liquid chromatography/fluorescence detection) indicated $1.03 \times 10^{-1} \pm 3.05 \times 10^{-3}$ μg harmane L^{-1} pulp and $7.44 \times 10^{-5} \pm 2.55 \times 10^{-6}$ μg harmane g^{-1} seeds, but harmine was not detectable by the quantitative method. Our findings underscore the need for further studies about passionfruit food safety, in view of the potential toxicity of β-carboline alkaloids.

Keywords: β-carboline alkaloids, *Passiflora alata*, *Passiflora edulis*, SBSE(PDMS)-LC/Flu, UHPLC-MS

Introduction

Some *Passiflora* species are considered traditional medicinal plants which have sedative/anxiolytic properties. In fact, their leaf extracts are included in reference documents such as the European and the Brazilian Pharmacopoeia, among others. Literature reviews have found that medicinal *Passiflora* species are studied extensively, and alkaloids and flavonoids are reportedly the most relevant classes of natural products when it comes to pharmacological effects on the central nervous system. However, the popular use of passionfruit juice as an anxiolytic is not supported by pharmacological studies published in the literature.[1]

On the other hand, commercial interest in the production of *Passiflora* fruits to be eaten raw or used as raw material in the food industry has grown around the world. Brazil is the world's largest producer of passionfruit, and about 95% of Brazil's crops are of "maracujá azedo" (sour passionfruit, yellow passionfruit, *P. edulis* Sims f. *flavicarpa* O. Deg., a species native to South America). The pulp of this fruit is highly appreciated in Brazil in homemade and industrialized juices and is used as raw material for fruit-based beverages (alcoholic drinks, isotonic sport drinks, etc.) and sweets (jellies, ice creams, yogurts, etc.). "Maracujá doce" (sweet passionfruit, *P. alata* Curtis) is usually eaten raw, i.e., fresh, because of the low acidity of its pulp, and its cultivation corresponds to the remaining 5% of commercial "maracujá" crops in Brazil.[2]

Given the intersecting medicinal and nutritional aspects involved in the use of *Passiflora* fruit and by-products, knowledge about the chemical composition of secondary metabolites may be relevant to the safe use of *Passiflora* fruits in human nutrition, based on scientific information. However, most of studies about the dietary use of "maracujá" fruits and by-products do not usually discuss or consider the presence of plant defense compounds such as the alkaloids and cyanogenic glycosides of *Passiflora*.[1]

In this context, an analytical method developed and described in a previous study[3] is herein referred to as the SBSE(PDMS)-LC/Flu (stir bar sorption extraction using polydimethylsiloxane as stationary phase-liquid chromatography/fluorescence detection) method. This method is based on a combination of stir bar sorption extraction using polydimethylsiloxane (PDMS) as stationary phase and liquid chromatography/ fluorescence detection. The analysis of the pulp and seeds of *P. edulis* fruit revealed the presence of the alkaloids harmane and harmine at μg levels. SBSE is a sample preparation technique with high capacity for enriching solutes from aqueous matrices, based on the use of a stir bar incorporated in a glass tube coated with a stationary phase. The most widely employed

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and well-known technique is still PDMS, mostly due to the lack of systematic studies using the other commercial coating (the copolymer polydimethylsiloxane ethylene glycol sold by Gerstel under the brand name EG-Silicone Twister®).[4] SBSE is compatible with automation and can meet the requirements of the food and beverages industry, including health and food safety analysis, which frequently requires the preconcentration of the analyte and elimination of matrix interferences.

However, despite the growing applications of SBSE in food analysis, including trace compounds,[5] the analysis of bioactive food compounds with potential toxic activity such as β-carboline alkaloids (also known as harmane alkaloids) requires greater attention. Some β-carbolines are neuroactive in humans and may be implicated in human diseases such as cancer and Parkinson's disease. In fact, industrial processing of protein-rich foods may be a source of these compounds. On the other hand, industrial thermal processing of plants such as roasting during the production of chicory coffee enriched with other plant material (added to improve the aroma of the final beverage) may also result in the formation of β-carbolines, which justifies the proposal of analytical methods for their detection and quantification in foods.[6]

The evolution of liquid chromatographic techniques such as UHPLC-MS (ultrahigh performance liquid chromatography-mass spectrometry), which enable faster analysis using smaller amounts of solvents than conventional HPLC, coupled to mass spectrometry, is an important trend that justifies further studies of complex plant samples such as *Passiflora* extracts,[7] in order to identify or quantify plant compounds not yet recognized in the conventional phytochemical investigation of "maracujá" fruit. Notwithstanding their economic relevance, the literature lacks investigations into the alkaloids from *P. alata* and *P. edulis* fruits by LC-MS techniques. Lutomski *et al.*[8] identified alkaloids from *P. edulis* f. *flavicarpa* juices only by thin layer chromatography (TLC) combined with detection using Dragendorff's reagent.

Previous investigations into the alkaloids of these species using modern separation techniques were strongly motivated by their importance as phytomedicines, and thus focused on leaf extracts[1] or callus cultures of *P. alata*.[9] Moreover, LC-MS targeted analysis (i.e., analysis of specific metabolites) may be a powerful tool for plant metabolomics studies.[10]

This work focuses on targeted analysis leading to the identification of further β-carboline alkaloids from sweet (*P. alata*) and sour (*P. edulis*) pulp and seeds by UHPLC-MS techniques and the quantification of harmane and harmine in sweet fruits by SBSE(PDMS)-LC/Flu.

Experimental

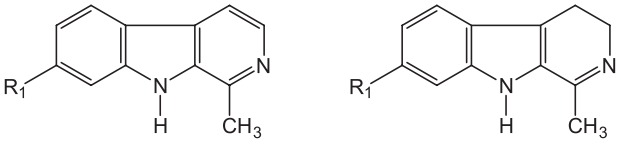
Plant samples, chemicals and materials

Commercial samples of sour and sweet passion fruits were purchased locally in fruit markets in the cities of São Carlos and São Paulo, São Paulo State, Brazil. The identification of these fruits is based on their morphological characteristics (size and shape of the fruit, color and texture of their rinds) and on organoleptic aspects (fragrance of the fresh whole fruit, flavor and fragrance of the pulp).[2]

The pulp was separated from the seeds by sieving (1.4 mm aperture), stored in glass jars, and frozen to –20 °C prior to its use. To prepare the pulp and seed samples, the fruits were cut open with a knife and the pulp and seeds removed with a spoon and gently mixed in a domestic blender. This procedure simulated the domestic preparation process normally used in Brazil. The pulp with seed was stored at –20 °C prior to its use.

HPLC-grade acetonitrile and methanol were purchased from Tedia (Fairfield, OH, USA), formic acid PA grade from Merck (Darmstadt, Germany), sodium chloride analytic grade from Spectrum (Gardena, CA, USA). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

The structure of the standards of alkaloids utilized in this work (harmane, harmine, harmaline, harmol and harmalol) are shown in Figure 1. All these standards were purchased from Sigma-Aldrich (Steinheim, Germany), with 98% of purity.



	R_1
1 Harmane	H
2 Harmine	OCH_3
3 Harmol	OH

	R_1
4 Harmaline	OCH_3
5 Harmalol	OH

Figure 1. Structures of β-carboline alkaloids standards.

Commercial SBSE stir bars, 20 mm long, with 0.5 mm film thickness, coated with 55 μL of PDMS (code GC 011444-001-00) were purchased from Gerstel (Mulheim an der Ruhr, Germany). Prior to their first use, the stir bars were conditioned according to the manufacturer's instructions 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.

Sample preparation by SBSE(PDMS)

Plant material was extracted by means of the SBSE(PDMS) dual method developed by Pereira *et al.*[3] This method consists of the parallel extraction of plant samples using two (SBSE)PDMS stir bars, at two different pH levels (pH = 10 and 13), and simultaneous chromatographic analysis of the analytes desorbed from the two stir bars. The general procedure of the SBSE(PDMS) dual method of sample preparation is explained in detail in the literature.[3] All the samples were filtered through a 0.45 μm Millex-HV PVDF membrane (Millipore, New Bedford, MA, USA) prior to UHPLC-MS or LC/Flu analysis.

Chromatographic analysis (UHPLC-MS)

For the UHPLC-MS analysis, samples of *P. alata* and *P. edulis* pulp and seeds were extracted separately by the SBSE(PDMS) dual method. The extracts obtained from each part of the fruit at the two pH levels (pH 10 and 13) were mixed and subjected to UHPLC-MS analysis. UHPLC-MS analysis were carried out with a Accela UHPLC System (Thermo Fischer Scientific, Waltham, MA, USA) coupled to a hybrid linear ion trap/Orbitrap® analyzer mass spectrometer (LTQ Orbitrap Velos system, Thermo Fischer Scientific,), controlled by Xcalibur® software (Thermo Fischer Scientific). The separation were performed using a Waters X-Bridge® BEH Shield RP_{18} column, 150 × 2.1 mm i.d.; 2.5 μm (Waters, Milford, MA, USA). The mobile phase was composed of 0.5% formic acid solution (A) and 0.5% formic acid in acetonitrile (B). The gradient was programmed from 80 to 66% A at 0-10 min and from 66 to 80% A at 10-18 min. The flow rate was 0.3 mL min^{-1}, the column temperature was 25 °C, and sample injection volume was 10 μL. The mass detection was performed in positive ion mode, using electrospray ionization, capillary voltage +3.7 kV and capillary temperature, 300 °C; nitrogen was utilized as nebulizer gas (35 arbitrary units) and sheath gas (10 arbitrary units). Full scan data were obtained in the range *m/z* 50-250; when necessary, data were also obtained by SIM mode and fragmentation analysis were performed by MS/MS, and accurate masses data were obtained within 5 ppm for the fragment ions.

Quantitative analysis of harmane by LC/Flu

Since harmine was not found into pulp fruit nor seeds extracts (see also Results and Discussion section), only harmane was quantified by LC/Flu analysis using the standard addition method, based on analytical curves constructed from samples spiked with a stock solution of harmane (100.00 μg L^{-1} in methanol), to reach a final concentration in the range of 0.1 to 3.0 μg L^{-1}; this procedure was repeated in triplicate. The general extraction conditions followed the procedure described for the SBSE(PDMS) dual method.[3] Plant samples were poured with water (1.0 mL pulp with 7.7 mL H_2O and 1.0 mg dried seeds with 8.7 mL H_2O), 5.0 g of NaCl and 1.0 mL of NaOH 1.0 mol L^{-1} to ensure final pH = 13.0 (see Table 1 for the amounts of stock solutions utilized for each point of the curves). The analytes were desorbed under sonication (60 min) with 150 μL of methanol.

Table 1. Amounts of stock solution utilized for each point in the quantification of harmane in samples of *P. alata* fruits by SBSE(PDMS)[3]

Final concentration of alkaloid[a]	Harmane stock solution[b] / μL	Methanol / μL
0.10	10	290
1.50	150	150
3.00	300	0

[a]Pulp samples (mg L^{-1}) or dried seeds samples (mg g^{-1}); [b]stock solution of harmane = 100.00 μg L^{-1} in methanol.

LC/Flu quantitative 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® C18 column (250 × 4.6 mm i.d., 5 μm) preceded by an X-Terra® C18 guard column (2.0 cm × 4.0 mm i.d.; 5 μm), also from Waters. The mobile phase was composed of 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. The fluorescence detector was set at $\lambda_{excitation}$ = 254 nm and $\lambda_{emission}$ = 425 nm.

Results and Discussion

Unlike previous studies of β-carboline alkaloids from passionfruit, which were based on conventional phytochemical studies,[1,8] this work focused on the use of modern analytical techniques (SBSE-LC, UHPLC-MS). The possibility of combining SBSE (a preconcentration technique) and correlated structural information (obtained

from MS analysis) with HPLC data opens up a wider range of possibilities for analysis of trace compounds or multi-samples such as those required in biotechnological studies (cell culture samples), agronomic or ecological studies, metabolomic analysis, etc.

The extracts obtained from *P. alata* and *P. edulis* pulp and seeds by SBSE(PDMS) were analyzed by UHPLC-MS. The analytical conditions were defined considering the data of Zhao *et al.*[11] about LC-MS analysis of β-carboline alkaloids in biological samples and also our preliminary tests, which were performed with commercial standards and with the fruit extracts obtained by SBSE(PDMS) from sour and sweet passionfruit. The development of the extraction by SBSE(PDMS), the so-called "dual SBSE method", is described in the literature,[3] including the evaluation of variables such as pH. The extracts obtained at different pH levels (pH 10 and 13) from each plant sample were combined in order to obtain MS spectra representative of the full range of the different compounds extracted in each pH condition. As expected, the positive ionization mode proved to be the best choice due to the presence of basic nitrogen groups in the β-carboline alkaloids. These compounds formed relatively stable $[M + H]^+$ ions, and therefore the UHPLC-MS/MS analysis provided information about product ions (Table 2), most of which were also reported by Zhao *et al.*[11] The product ions listed in Table 2 are compatible with the structure of β-carboline alkaloids, as follows: $[M + H - 15]^+$, related to the loss of a methyl group; $[M + H - 28]^+$, generated by the loss of CO; rearrangement of the $[M + H]^+$ ion leading to the loss of NH_3 and generation of $[M + H - 17]^+$ ion, and rearrangement of the $[M + H]^+$ ion resulting in the loss of C_2H_3N and generation of $[M + H - 41]^+$ ion, as proposed by Zhao *et al.*[11]

The β-carboline alkaloids in the passionfruit extracts were identified by comparing them with the retention times (t_R) of the commercial standards (when available) and monitoring $[M + H]^+$ and their corresponding product ions. The measured accurate mass data, together with the fragment losses forming the product ions, were also considered. Total ion current (TIC) and extracted ion chromatograms obtained by UHPLC-MS analysis of the *P. alata* extracts are shown in Figure 2 (pulp) and Figure 3 (seeds), and Table 2 summarizes the alkaloids identified in each fruit extract.

In the extracted ion chromatograms with peaks containing the targeted ions, both t_R and mass spectra data were adopted as criteria for identification of the compounds summarized in Table 2; the Supplementary Information gives representative mass spectra of all the alkaloids identified in this study. The extracted ion chromatograms at Figures 2b and 3b showed clearly the presence of harmane, $[M + H]^+ = 183$, due to the t_R of the main peak and its mass spectra. The peak with $t_R = 2.54$ min in Figure 3f was identified as harmaline, $[M + H]^+ = 215$, also considering both t_R and mass spectra. On the other hand, UHPLC-MS/MS analysis was required to identify harmol, $[M + H]^+ = 199$, and harmalol, $[M + H]^+ = 201$, in *P. alata* seed extracts, since other peaks at the same *m/z* of the targeted alkaloids were found in the extracted ion chromatograms (Figures 3c and 3d). These compounds were identified unequivocally considering accurate mass data of the product ions in the MS/MS spectra, adopting the criteria of ± 5 ppm to determine the elemental composition.[12] UHPLC-MS/MS also confirmed the absence of harmol, harmalol and harmaline in *P. alata* pulp, as already suggested by the extracted ion chromatograms at *m/z* 199, 201 and 215 (respectively, Figures 2c, 2d and 2f).

Table 2. UHPLC-MS data on β-carboline alkaloids and summary of their identification in *P. edulis* and *P. alata* extracts

Alkaloid	t_R / min	$[M + H]^+$ *m/z*	Fragment ions *m/z*	Extract			
				P. alata (pulp)	*P. alata* (seeds)	*P. edulis* (pulp)	*P. edulis* (seeds)
Harmane	2.05	183.09114 ($C_{12}H_{11}N_2$ calcd. = 183.09167)	–	(+)	(+)	(+)	(+)
Harmine	2.65	213.10139 ($C_{13}H_{13}N_2O$ calcd. = 213.10224)	198 $[M + H - 15]^+$	(+)	(+)	(+)	(+)
Harmol	1.81	199.08603 ($C_{12}H_{11}N_2O$ calcd. = 199.08659)	171 $[M + H - 28]^+$	(–)	(+)	(–)	(–)
Harmalol	1.60	201.10165 ($C_{12}H_{13}N_2O$ calcd. = 201.10224)	184 $[M + H - 17]^+$ 160 $[M + H - 41]^+$	(–)	(+)	(–)	(–)
Harmaline	2.44	215.11699 ($C_{13}H_{15}N_2O$ calcd. = 215.11789)	200 $[M + H - 15]^+$ 174 $[M + H - 41]^+$	(–)	(+)	(+)	(–)

t_R: retention time of the commercial analytical standards; calcd.: calculated exact mass; (+): presence of the compound; (–): absence of the compound; Supplementary Information shows representative UHPLC-MS spectra of the β-carboline alkaloids.

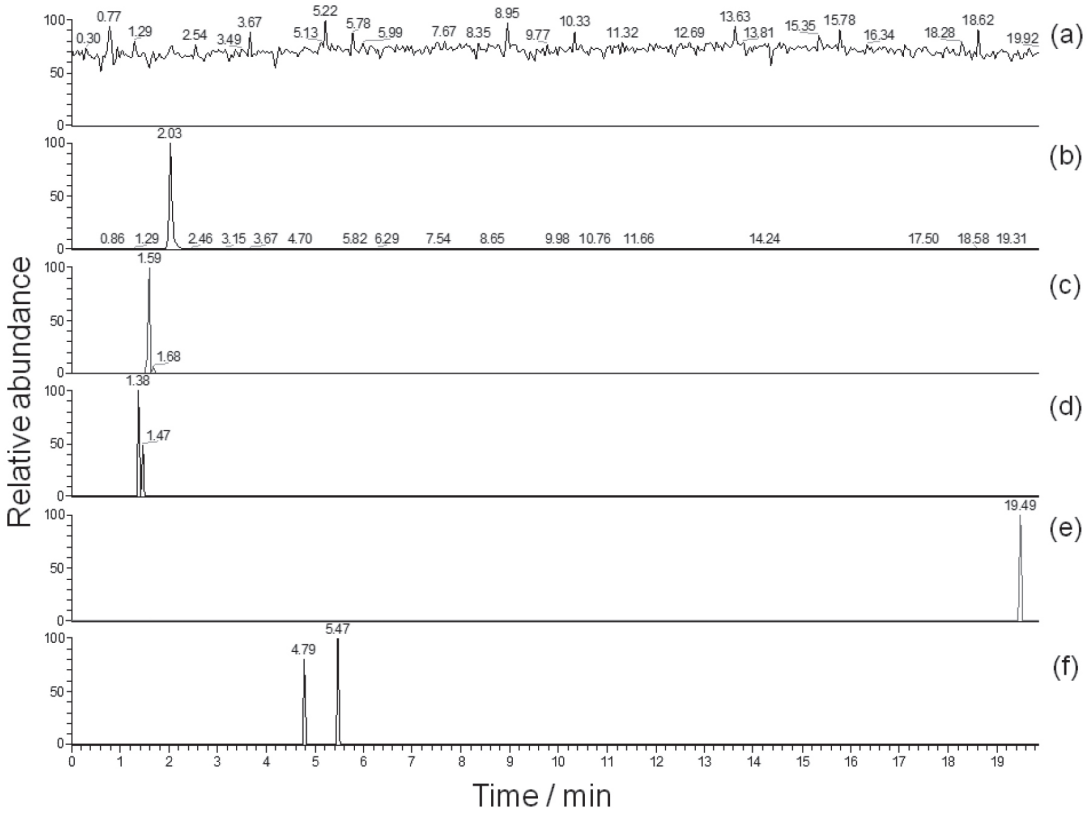


Figure 2. Representative chromatograms of the extracts from *Passiflora alata* pulp, obtained by UHPLC-MS. (a) TIC; extracted ion chromatograms: (b) *m/z* 183; (c) *m/z* 199; (d) *m/z* 201; (e) *m/z* 213; (f) *m/z* 215.

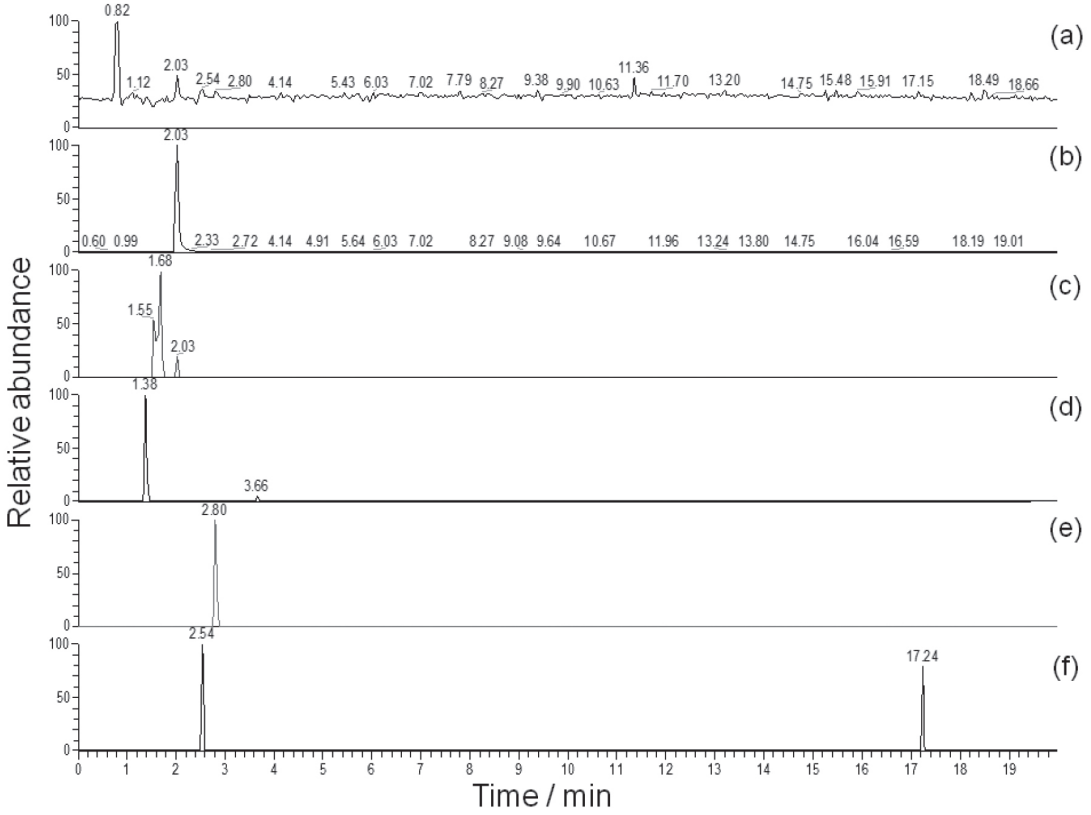


Figure 3. Representative chromatograms of the extracts from *Passiflora alata* seeds, obtained by UHPLC-MS. (a) TIC; extracted ion chromatograms: (b) *m/z* 183; (c) *m/z* 199; (d) *m/z* 201; (e) *m/z* 213; (f) *m/z* 215.

In the case of the identification of alkaloids from *P. edulis*, all the compounds were identified using data from the UHPLC-MS/MS analysis, also adopting the criteria of ± 5 ppm to determine the elemental composition,[12] since the UHPLC-MS data were not conclusive due to the higher background signal (possibly caused by interference from other plant compounds not fully separated in the sample preparation step).

In order to obtain complementary quantitative data, the alkaloids harmane and harmine in the pulp and dry seeds of sweet passionfruit were analyzed by SBSE(PDMS)-LC/Flu. Despite the optimized conditions for the extraction of harmine (at pH = 10),[3] harmine in the extracts could not be quantified by LC/Flu because of the baseline interference of other co-eluted matrix compounds. Supplementary Information shows a typical chromatogram of passionfruit extracts, obtained by SBSE(PDMS)-LC/Flu. The alkaloids **3**, **4** and **5** were not quantified because during the optimization of SBSE(PDMS) extraction step, it was observed that PDMS stationary phase does not allow quantitative extraction of these compounds.[3] The results listed in Table 3 indicate that the total amount of harmane in sweet passionfruit does not seem to be important in absolute values. Nevertheless, considering that, according to Dutra *et al*.,[13] one *P. alata* fruit weighing an average of 263 g contains about 57 mL of pulp and 10 g of fresh seeds, the amount of harmane ingested upon eating an average size whole sweet passionfruit (pulp + seeds) is approximately 7 ng *per* fresh fruit.

Table 3. Quantification of alkaloids in sweet passionfruit (*P. alata*) by SBSE(PDMS)-LC/Flu

Alkaloid	Pulp / (μg L^{-1})	Seeds / (μg g^{-1})
Harmane	$1.03 \times 10^{-1} \pm 3.05 \times 10^{-3}$	$7.44 \times 10^{-5} \pm 2.55 \times 10^{-6}$
Harmine	n.d.	n.d.

n.d.: not detected.

Sweet passionfruit appears to contain fewer alkaloids than sour passionfruit, according to a direct comparison with reports in the literature. Pereira *et al*.[3] reported finding $3.09 \times 10^{-2} \pm 5.87 \times 10^{-5}$ μg harmane g^{-1} dried seeds and 3.00 ± 0.04 μg harmane L^{-1} pulp. Harmine was also found in the same sour passionfruit samples, which contained $8.11 \times 10^{-3} \pm 7.60 \times 10^{-4}$ μg harmine g^{-1} dried seeds and 2.72 ± 0.02 μg harmine L^{-1} pulp. However, comparisons of data on sweet and sour passionfruit must be cautious, since external factors may affect the production of *Passiflora* metabolites. For instance, an earlier study found that the level of the elements Fe, B and Cu in soil is inversely correlated with the total flavonoid content in *P. incarnata* leaves.[14]

Conclusions

This paper provides additional data about β-carboline alkaloids in sour passion fruit (*P. edulis*) and represents the first report of the β-carboline alkaloids in sweet passion fruit (*P. alata*) pulp and seeds identified by UHPLC-MS techniques. This is also the first report of quantification of alkaloids in sweet passionfruit pulp and seeds, by SBSE combined with LC/Flu.

Our findings also confirm the promising possibilities afforded by combining SBSE(PDMS) with UHPLC-MS in the targeted analysis of plant compounds, illustrating the potential of modern phytochemical analytical approaches in the study of food plants and bioactive compounds. The estimation of the amount of harmane contained in the raw pulp and seeds of one "maracujá" fruit, combined with the structural identification of further β-carboline alkaloids described herein, also points to the need for additional studies about the food safety of both passionfruit species (including toxicological studies), given the potential toxicity of alkaloids. These considerations include potential dangers resulting from the growing worldwide trend to search for alternative food ingredients (including fruits) and for the use of exotic and alternative non-conventional food plants in the context of gastronomic novelties.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgments

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