Amazonia

LC-ESI-MS Determination of Quassinoids Isobrucein B and Neosergeolide in *Picrolemma sprucei* Stem Infusions

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Infusões dos caules de *Picrolemma sprucei* (pseudônimo: *P. pseudocoffea*) são principalmente utilizadas como antimaláricos em toda região amazônica. Desta espécie foram isolados os quassinoides isobruceína B e neosergeolida, os quais apresentam atividade antimalárica e citotóxica. Neste estudo, apresentamos o desenvolvimento de uma metodologia analítica por LC-(+)-ESI-MS/MS visando a determinação dos principais quassinoides desta espécie. O método desenvolvido foi empregado para análise de uma formulação artesanal (infusão de 1 g de matéria seca - caules pulverizados de *P. sprucei* em 1 L de água fervente). Padrões previamente isolados de isobruceína B e neosergeolida foram utilizados para a determinação da linearidade na faixa de calibração entre 0,25 to 5 µg mL^{-1} e 0,5 a 10 µg mL^{-1}, respectivamente. Como padrão interno foi utilizada a substância fluoroglucinol na concentração de 4,0 µg mL^{-1}. Ambos os compostos apresentaram boa linearidade, precisão e exatidão e as concentrações de isobruceína B e neosergeolida obtidas nas infusões foram de 60,1 e 774 µg L^{-1}, respectivamente.

Infusions of the stems of *Picrolemma sprucei* (pseudonym: *P. pseudocoffea*) are used in the Amazon regions of Peru, Brazil and French Guiana as antimalarials among other uses. They contain the bitter quassinoids isobrucein B (**1**) and neosergeolide (**2**) that have important antimalarial and toxic properties among others. In this study, an LC-(+)-ESI-MS/MS method was developed and applied to the determination of **1** and **2** in a common remedy prepared by infusing 1 g of dry, powdered stems of *P. sprucei* in 1 L of boiling water. Isolated **1** and **2** were used in calibration ranges of 0.25 to 5 µg mL^{-1} and 0.5 to 10 µg mL^{-1}, respectively, with the internal standard phloroglucinol at 4.0 µg mL^{-1}. Good linearity, precision and accuracy were observed for both compounds. The concentrations of **1** and **2** in the stem infusions were found to be 60.1 and 774 µg L^{-1}, respectively.

Introduction

One of the classic goals of the Brazilian natural products research community is the search for new biologically active compounds.[1] More recently, new interests include investigations of daily and seasonal rhythms of secondary metabolite biosyntheses and quality control of medicinal

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plants.[2] *Picrolemma sprucei* Hook. f. (Simaroubaceae) is one of several medicinal plant species which are known in the Brazilian Amazon by the common name caferana (meaning "false-coffee", and giving rise to the often used pseudonym for this plant, *P. pseudocoffea* Ducke). It is widely distributed throughout the Amazon region from Peru where it is popularly known as sacha-café[3] to French Guiana where it is called café lane.[4,5] Root, stem and whole plant infusions of *P. sprucei* are used throughout

the Amazon region in the treatment of malaria,[6] gastro-intestinal problems and intestinal worms.[3] At higher doses, *P. sprucei* infusions have notable toxic effects and are used to provoke abortions.[7]

Recently, *P. sprucei* has been the subject of several studies whose results in general lend support to its uses in traditional medicine. For example, leaf and stem infusions and other extracts have been shown to possess *in vitro* antihelminthic activity.[8] Also, an ethnopharmacological study in French Guiana showed that alcohol extracts of *P. sprucei* root, stem and bark are used in local traditional medicine as a curative treatment for malaria in association with *Geissospermum* spp. (Apocynaceae) and *Quassia amara* (Simaroubaceae) or modern drugs.[5] In a subsequent study, Bertani *et al.*[9] demonstrated that a *P. sprucei* water extract can inhibit hemozoin formation and *in vitro* assays demonstrated the antimalarial activity of this extract against the chloroquine-resistant *Plasmodium falciparum* strain W2. In the western Brazilian Amazon, dried stems are commonly commercialized in local marketplaces by traditional healers or raizeiros and are typically used in the preparation of infusions for oral ingestion.

Infusions of *P. sprucei* contain the quassinoids isobrucein B (**1**) and neosergeolide (**2**) (Figure 1). These compounds have been isolated previously from the stems and roots of *P. sprucei* on a milligram scale[10] and more recently on a gram-scale.[11] Also, **1** has been isolated from the leaves of this plant.[12] Recently, the antimalarial, antileukemic, antifeedant and leishmanicidal activities of **1** were the subject of a review.[13] Finally, **1** and **2** display significant cytotoxicity to human tumor cell lines, no hemolytic activity to mouse erythrocytes, and moderate larvicidal activity towards *Aedes aegypti* (hemorrhagic dengue fever vector).[14]

Considering the biological activities of *P. sprucei* and of the quassinoids isolated from it, together with the possible occurrence of seasonal and circadian variation, stimulates and justifies the development of analytical methods for the quantification of these active metabolites in infusions used in traditional medicine. Liquid chromatography (LC) coupled to mass spectrometry (LC-MS) has emerged as the most specific analytical method for qualitative and quantitative analyses of natural products.[15] Additionally, advances in the elucidation of electrospray ionization (ESI) processes have allowed for the analysis of metabolites with less competition from low oxidative potential processes through molecular ion dissociation[16] in addition to the well established acid-base and cationizing (anionizing) reactions.[17] For these reasons, LC-ESI-MS predominates in Brazil and around the world

as the technique of choice in the analysis of natural products of low-volatility in polar analytes of diverse origins.[1] Given the range of dosages of traditionally used infusions of *P. sprucei*, the toxic effects associated with larger doses, and the ability of ESI coupled systems to analyze natural products, the aim of the present study was to develop a new method for determination of **1** and **2** in infusions of stems of *P. sprucei*.
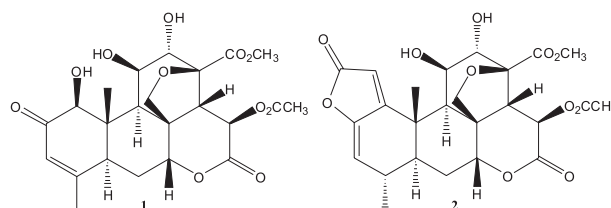

Figure 1. Quassinoids isobrucein B (**1**) and neosergeolide (**2**) present in infusions of *Picrolemma sprucei*.

Experimental

Collection of plant materials

Collection was performed in Silves, Amazonas State, Brazil, in April, 2003. Voucher specimens are on deposit at the UFAM Herbarium (Silva 5729 & 5730). Identification of *P. sprucei* Hook. f. was performed by Dr. Wayt Thomas.[18]

Chemicals and reagents

Methanol, HPLC grade, was purchased from J.T.Baker (Xalostoc, Mexico). Glacial acetic acid was of analytical grade and supplied by Merck (Darmstadt, Germany). Ultrapure water, obtained from a Milli-Q Plus System (Millipore, Bedford, MA, USA), was used in all analyses. **1** and **2** were isolated from *P. sprucei* as described previously[11,19] and used as reference standards. Their identity and purity were established based on 1D and 2D NMR, IR, HR-ESI-TOF-MS, HPLC-DAD and LC-ESI-MS analyses and comparison to literature data.[20]

Standard solutions

Stock standard solutions were prepared at 1 mg mL^{-1} in methanol and stored at -20 °C for a maximum of three months. Working solutions were prepared by appropriate dilution with methanol to have final concentrations in the ranges 0.25 to 5 µg mL^{-1} and 0.5 to 10 µg mL^{-1} for **1** and **2**, respectively. Working solutions had the internal standard (IS) phloroglucinol (1,3,5-trihydroxybenzene) at a concentration of 4.0 µg mL^{-1}.

LC and MS detection conditions

The liquid chromatography system consisted of an LC-10AD pump and a CTO-10AS column oven from Shimadzu (Kyoto, Japan). The separation of **1**, **2** and IS were performed on a reversed phase NST18 column, 250 mm × 4.6 mm ID, 5 µm particle size (Nano Separation Technologies, São Carlos, São Paulo, Br). A LiChrospher® 100 RP 18 column, 4 mm × 4 mm ID, 5 µm particle size, from Merck (Darmstadt, Germany) was used as guard column. The LC system was operated isocratically using mobile phase consisting of a mixture of methanol:water (50:50, v/v) and 2 % acetic acid which was pumped at a flow-rate of 0.8 mL min^{-1}. Injections of samples were performed manually through a 20 µL loop with a Rheodyne model 7125 injector (Rheodyne, Cotati, USA) and the column was kept in an oven set at 27±1 °C.

Detection was achieved with a Quattro Micro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray interface (ESI) source and tandem mass separation operated in the positive ion mode at a potential of 2.5 kV. The LC flow was split so that approximately 150 µL min^{-1} entered the mass spectrometer. The desolvation temperature was set to 250 °C and the source was set to 100 °C for LC-MS. The nitrogen desolvation and nebulizer gas flow rates were set to 250 and 20 L h^{-1}, respectively. Argon gas was used as collision gas. Cone voltage and collision energy were optimized for each analyte by performing full scan acquisitions. Optimization of MS conditions was achieved by direct infusion of standard solutions (10 µg mL^{-1}) prepared in the mobile phase and delivered by a syringe pump at a flow-rate of 10 µL min^{-1}. The cone voltage was set at 40 V for **1** and **2** and 20 V for IS, and collision energies of 20 eV for **1** and IS and 28 eV for **2** were used. For quantitation, MS/MS was operated in the multiple reaction monitoring (MRM) mode, monitoring the transitions 481>403, 505>225 and 127>81 for **1**, **2** and IS, respectively, with a dwell time of 1.0 s. Data acquisition and quantitative analysis were performed using a MassLynx (Micromass) data acquisition system, version 4.1.

Validation of the method

The method was rigorously validated in accordance with United States Food and Drug Administration (FDA) guidelines[21] by determination of the following parameters: linearity, range, precision, accuracy, limit of quantification (LOQ) for LC-MS/MS. For linearity and range, the calibration curves were constructed by injection of seven concentrations of **1** and **2**, ranging from 0.25 to 5.0 µg mL^{-1} and 0.5 to 10 µg mL^{-1}, respectively, in triplicate.

The analyte to IS peak area ratio was plotted against the respective standard concentrations and the linearity was evaluated by linear regression analysis. The acceptance criterion for each calculated standard concentration was a maximum 2% deviation from the nominal value, except for the LOQ, which was set as 10 % deviation. The precision of the method was determined by evaluating repeatability and LOQ was established using the criterion that responses of the analytes at the LOQ should be at least 5 times that of the blank response.

Sample preparation

5.00 g of dry, ground stems of *Picrolemma sprucei* were infused in 1.00 L of boiling water. The resulting mixture was covered, allowed to stand for 15 min, then immediately filtered. The filtrate was evaporated to dryness under vacuum which resulted in a residue. The residue was dissolved in methanol in an ultrasound bath which provided a solution (10.0 mL) which was filtered. The resulting filtrate or RSI (residue of stem infusion in methanol solution) was stored at –20 °C and IS was added prior to LC-MS analysis. The concentration of quassinoids was calculated from the linear regression equations.

Results and Discussion

Development of LC-ESI-MS conditions

In order to quantify **1** and **2** in an infusion of stems of *P. sprucei* used in Brazilian folk medicine, MS parameters were optimized in order to obtain more abundant precursor ions. Thus, the balance of protonated or deprotonated and cationized or anionized **1** and **2** or other adduct ion species of **1** and **2** were analyzed. Ideally, these species should be of high intensity to allow analytes to be detected at low concentrations in complex matrices. The intensity of ion species was therefore the main criteria for optimization. Initially, **1** and **2** were directly infused into the MS detector using ESI ionization. Both positive and negative ionization modes of the ESI source were tested by infusion of standard solutions of each compound at 10 µg mL^{-1}. The positive ion detection mode offered higher sensitivity than the negative mode using an acidic mobile phase. (+)-ESI-MS full scan spectra displayed protonated ions ([M+H]$^+$) with good intensities at *m/z* 481 and 505 for **1** and **2**, respectively.

During the optimization of the method, different LC conditions and solvent systems were evaluated. Addition of acidic modifiers such as acetic acid to the mobile phase was found to improve peak resolution. Furthermore, the positive ion detection mode [(+)-ESI] offered higher sensitivity than

negative ion mode [(-)-ESI] when acidic mobile phase was used. Also, addition of acetic acid or formic acid to the mobile phase led to significant enhancement of the $[M+H]^+$ ions and significant reduction of the cationized molecules. Table 1 presents the parent and fragment ions used for identification and quantification of compounds **1** and **2**.

Table 1. Protonated molecule and fragment ions obtained in ESI-MS/MS spectra. The most stable and intensive ions (*) were used for identification and quantification of isobrucein B (**1**) and neosergeolide (**2**) by LC-ESI-MS/MS

	Quassinoid	
	1	**2**
Protonated molecule $[M+H]^+$	**481**	**505**
Fragment ions	463	487
	445	463
	439	445
	421	427
	403*	417
	391	413
	375	398
	357	379
	356	367
	311	348
	301	339
	299	323
	284	321
	279	319
	214	227
		225*

Stem infusion in solution (RSI) was directly injected into the LC-ESI-MS/MS system. Figure 2 presents the representative MRM-chromatogram. Under the conditions described in the method, IS, **1** and **2** eluted at retention times of 3.5, 4.6 and 8.6 min, respectively, and elution of all components of the sample was complete within 10 min. Chromatographic profiles displayed good separation of the analyte components in a short period of time.

Method validation

The validation parameters were evaluated through estimation of linearity, precision (intra and inter day) and accuracy of the method. The linearity of an analytical method is its ability, within a definite range, to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample. The calibration curve for each analyte was based on 7 different concentrations by plotting
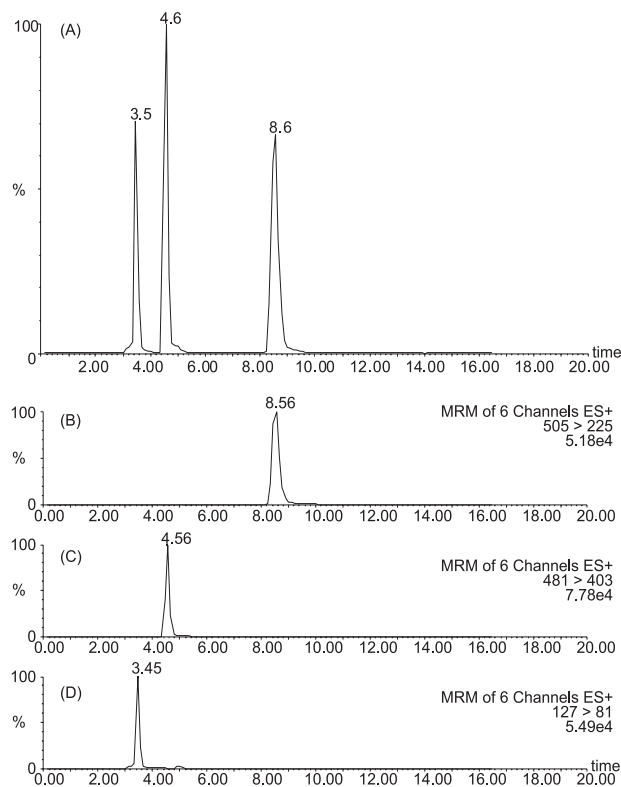

Figure 2. Representative chromatogram. (A) Total ion count. (B) MRM of neosergeolide (**2**), (C) MRM of isobrucein B (**1**) and (D) MRM of the internal standard phloroglucinol (1,3,5-trihydroxybenzene). Chromatographic conditions: reversed phase NST18 column, 250 mm x 4.6 mm ID, 5 µm. Eluents were methanol:water (50:50, v/v) and 2% acetic acid. Flow rate was 0.8 mL min⁻¹. Injection volume was 20 µL. Column oven temperature was set at 27±1 °C.

the result of the linear regression analysis of the peak area ratio versus concentration.

Linear regression analysis provided the equations presented in Tables 2 and 3. For both **1** and **2**, the correlation coefficients (r^2) were evidence for a linear relationship between concentration and peak area ratio in the concentration range studied. The values of LOQ are also presented in tables 2 and 3 and were considered satisfactory for both analytes.

Application of the method

In order to check the applicability of the developed method, a stem infusion was evaluated. This particular preparation was chosen based on the wide availability of stems in marketplaces in the western Brazilian Amazon and the use of this preparation as an antimalarial in traditional medicine in this region. Concentrations of **1** and **2** in samples of stem infusion were calculated based on the integration of the $[M+H]^+$ peak area ratio of each of these analytes in the MRM chromatogram. By this process it is possible to select the compound of interest and quantification of the

Table 2. Confidence limits for quantification of isobrucein B with the method developed

Parameters	Isobrucein B		
Accuracy and Precision			
Concentration added (μg mL^{-1})	0.25	1.0	3.0
CV, n=6 (%)	1.1	1.2	1.6
Within-day precision, (CV, %)	1.5	1.7	1.8
Within-day accuracy[a] (%)	1.5	1.8	1.2
Linearity			
Range (μg mL^{-1})	0.25 - 5.0		
Linear regression	y = 0.8964x + 0.1958		
Correlation coefficient	0.9909		
Quantitation Limit			
Concentration (μg mL^{-1})	0.25		
Within-day precision, n=5 (CV, %)	5.2		
Within-day accuracy[a], n=5 (%)	4.6		

[a] Expressed as relative standard deviation, RSD

Table 3. Confidence limits for quantification of neosergeolide with the method developed

Parameters	Neosergeolide		
Accuracy and Precision			
Concentration added (μg mL^{-1})	1.5	3.0	5.0
CV, n=6 (%)	1.4	1.6	0.8
Within-day precision, (CV, %)	1.3	1.8	1.5
Within-day accuracy[a] (%)	0.9	1.3	1.4
Linearity			
Range (μg mL^{-1})	0.5 - 10.0		
Linear regression	y = 0.5972x + 0.9928		
Correlation coefficient	0.9948		
Quantitation Limit			
Concentration (μg mL^{-1})	0.50		
Within-day precision, n=5 (CV, %)	7.8		
Within-day accuracy[a], n=5 (%)	6.3		

[a] Expressed as relative standard deviation, RSD

individual compounds (considering different molar masses and fragment ions) can be performed without the need for complete chromatographic resolution, but in this case good chromatographic resolution was observed. By this method, **1** and **2** were determined to be present at concentrations of 0.601 and 7.74 μg mL^{-1}, respectively, in the sample solution of stem infusion (RSI) analyzed, which corresponds to concentrations of 60.1 and 774 μg L^{-1}, respectively, of these quassinoids in the infusion.

Conclusion

The LC-(+)-ESI-MS/MS method described herein proved suitable for the analysis of **1** and **2** in stem extracts and can be easily applied in routine analyses. Furthermore, the method may be used to certify the quality of popular medicinal uses of the extract and for ecological studies.

Acknowledgements

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