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Validation of an analytical method for the determination of the main ayahuasca active compounds and application to real ayahuasca samples from Brazil

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ABSTRACT

Ayahuasca is a brew prepared from the water decoction of two Amazonian plants, which is legally used for religious, cultural or therapeutic activities. The potential use of ayahuasca as a natural or phytotherapeutic drug is directly linked to the action of its active compounds and their connection with the therapeutic efficacy of the beverage. In this context, the aim of the present study was to establish a selective, sensitive and reproducible analytical method for the quantification of the main active ayahuasca compounds. Thirty-eight samples from the state of São Paulo, Brazil, were analyzed and the simultaneous quantifications of *N*,*N*-dimethyltryptamine (DMT), tetrahydroharmine (THH), harmine (HME) and harmaline (HML) were performed. This study enabled the development of a fast validated analytical method with minimal matrix interference and high reproducibility for the tracing of active ayahuasca compound concentrations for the first time. This method is important as an auxiliary tool for the study of active compound effects in biological responses using different multi-omic platforms.

1. Introduction

Ayahuasca is a psychotropic Amazonian beverage formulated from the water decoction of *Banisteriopsis caapi* vines and *Psychotria viridis* leaves. This drink is legally used in Brazil, as well as some European countries and the USA [1], for religious, cultural and therapeutic purposes [2–4]. Its effects can be described as displaying potential efficacy within a model that involves biomedical, psychological, anthropological and theological elements [5,6]. It is believed that the psychoactive effects of ayahuasca may exhibit the potential for the treatment of mental disorders due to the effects of β-carboline-derived alkaloids present in vines and *N*,*N*-dimethyltryptamine (DMT) present in leaves [7,8]. Because of its effects on the nervous system, as well as the presence of alkaloids, ayahuasca is classified as psychoactive and has also been described as psychedelic or hallucinogenic [9].

Psychoactive substances, both natural and synthetic, were a significant object of study especially between 1950 and 1960, as they display therapeutic potential for the treatment of alcohol addiction, as well as other drugs of abuse [10,11]. Preliminary studies, for example, have demonstrated a positive effect of the action of substances such as LSD (lysergic acid diethylamide) in the treatment of alcohol addiction [11]. In the 1970s, however, LSD was considered a dangerous substance due to its recreational use and was prohibited [1,12]. Only in the 1990s did studies regarding this and other substances resume, boosted by

Abbreviations: DMT, *N*,*N*-dimethyltryptamine; THH, tetrahydroharmine; HME, harmine; HML, harmaline; UHPLC, ultra-high performance liquid chromatography; ANVISA, Brazilian National Health Surveillance Agency; UDV, União do Vegetal; IS, internal standard; SRM, selected reaction monitoring; ANOVA, analysis of variance; low (QCL), mean (QCM) and high (QCH), quality control (QC); CV, coefficient of variation; GC-NPD, gas chromatography coupled to nitrogen-phosphorus detector; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry

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success in treating anxiety and depression [11], such as, for example, the use of psilocybin in the treatment of anxiety and anxiety disorders in patients presenting potentially terminal diseases, like cancer [13–15].

A growing number of studies indicate the potential increase of ayahuasca in general addiction treatments, namely against alcohol, tobacco, and illicit drugs, such as cocaine and heroin [15–18], as well as in the treatment of depression and anxiety [19,20]. According to the Brazilian National Health Surveillance Agency (ANVISA), herbal drugs are obtained exclusively using active raw plant materials [21], and should be characterized concerning their efficacy and usage risks, as well as their quality reproducibility and constancy. Products comprising isolated active substances of any origin or their associations with plant extracts are not considered phytotherapeutic medicines [22].

In view of ANVISA recommendations and regulations, the establishment of reliable analytical methods for the quantification of active compounds present in ayahuasca beverages is essential for its application as a possible psycho-phytotherapeutic. In this context, the main active ayahuasca compounds, DMT, tetrahydroharmine (THH), harmine (HME) and harmaline (HML) were quantified by ultra-high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) operating in selected reaction monitoring (SRM) mode. SRM is a targeted mass spectrometry method, performed on triple quadrupole-like instruments, used to increase selectivity and sensitivity in the analysis of specific compounds, especially in complex matrices, such as ayahuasca. In the present study, a considerable number of samples from the state of São Paulo, Brazil, which are used in traditional União do Vegetal (one of the Brazilian ayahuasca religions) rituals, were investigated for the first time by a rapid and validated analytical method for the quantification of ayahuasca active compounds.

2. Material and methods

A general analytical workflow is presented in Suppl. Fig. 1.

2.1. Ayahuasca preparation

In a broader context, there are many different preparation methods for ayahuasca. The preparation of ayahuasca within the perspective of Brazilian religions is – apart from ritual diversity – relatively similar. Since indigenous and mestizo preparations in Peru and elsewhere frequently include other plants, it is important to state that, in Brazilian religions, ayahuasca is prepared with only three ingredients: *B. caapi*, *P. viridis*, and water, using a decoction method. The preparation of ayahuasca will be briefly described within the context of the União do Vegetal (UDV). Ritualistic and anthropological details will not be included in this description and it is important to note that there are variations and styles depending on who is preparing the brew, so this is a very general description. The used plant material is preferably freshly harvested, usually close to where the brew is being prepared.

Typically, washed leaves of *P. viridis* and smashed stems of *B. caapi* are placed with water in large brew kettles and decocted for about an hour or two. After that, new batched of both plants are placed again in the kettle and the first decoction is added. From this point on there is a considerable variation in boiling time, concentration of the brew with evaporation and the reinforcements with new plant material. The subjective effects of the ayahuasca are tested by volunteers selected by the person who is responsible for the preparation in order to attain an expected range of the ayahuasca experience. After decoction, the plant material is discarded and only the liquid, which has a brown or caramel color, is kept. Metal, glass or PET containers are used to store ayahuasca still hot (around 70 °C) and keeping no air to avoid fermentation. The container type depends on the needs for transportation or storage. The UDV has a specific manual with hygiene recommendations for ayahuasca preparation and storage.

Therefore, it is important to note that there are different elements that add to the ayahuasca variability and that would have to be considered: alkaloid concentrations in the plant material, amount of water, decoction time and the number of reinforcements of the decoction with new plant material.

2.2. Ayahuasca samples

The ayahuasca samples ($n = 38$), coded and referenced according to their respective origins, were previously prepared for ritual consumption and provided by different UDV centers from several areas in the state of São Paulo, Brazil (Suppl. Table 1). Each sample was packed in completely filled 15 mL tubes, to avoid possible oxidative degradation. Subsequently, 1 mL aliquots were identified and conditioned at −80 °C until analysis. This work was registered at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge - SisGen (protocol A6E33A0).

2.3. Reagents

The commercially available analytical standards 98% harmine and 95% harmaline, as well as the internal standard (IS) 98% diphenhydramine hydrochloride were purchased from Sigma-Aldrich. Tetrahydroharmine and dimethyltryptamine were synthesized as previously described by Callaway et al. [23]. Samples, standards and mobile phases were prepared using methanol (HPLC grade, Tedia, Fairfield, OH, USA), deionized water at 18.2 mΩ cm resistivity (Millipore, Bedford, MA, USA) and 88% P.A. formic acid (JT Baker Chemical Company, Phillipsburg, NJ, USA).

2.4. Instrumentation

The analyses were performed by liquid chromatography tandem-mass spectrometry (LC-MS/MS) on a Waters Acquity™ chromatography system equipped with a binary solvent pumping system and an automatic sample injection system coupled to a triple quadrupole Micromass Quattro Micro™ API ESI mass spectrometer (ESi™ multi mode ionization) (Waters Corp., Milford, MA, USA).

2.5. Standard solution preparation and analytical curve determinations

Stock solutions for each of the isolated analytes were prepared at different concentrations. Seven stock solutions were prepared for DMT (7.5, 18.0, 30.0, 42.0, 52.5, 63.0 and 75.0 mg/L), THH (17.5, 42.0, 70.0, 98.0, 123,147 and 175 mg/L), HME (30.0, 60.0,100, 150, 200, 250 and 300 mg/L), and HML (5.0, 7.5, 10.0, 13.5, 17.5, 22.5 and 27.5 mg/L), which correspond to each point of the prepared analytical curves. Thus, each of the points (final volume of 250 µL) consisted in 150 µL of methanol:water (1:1 v/v), 50 µL of the curve point stock solution and 50 µL of the IS solution at a final concentration of 2 mg/L (Suppl. Fig. 1B). The solutions were homogenized for 30 min by stirring and each of the points (Suppl. Table 2) was analyzed by LC-MS/MS to construct the analytical curves.

2.6. Sample preparation and LC-MS/MS analyses

The 38 ayahuasca samples were thawed, conditioned at room temperature and then centrifuged for 10 min at 10,000 rpm at 25 °C. The supernatants were then diluted in a 1:1 (v/v) methanol: water mixture in a volume equivalent to 2 mg/L of the IS (Suppl. Fig. 1A). Chromatography separation of samples was carried out on an Acquity™ UPLC BEH C18 analytical column (50 mm × 2.1 mm, 1.7 µm particle diameter) (Waters Corp.). After optimization of the chromatographic separation conditions, the mobile phases consisted in water (solvent A) and methanol (solvent B) and the linear gradient program comprised 0 min at 10% B, 4 min at 50% B, 5.5 min at 50% B and 6 min at 10% B,

Table 1
SRM conditions for the analysis of ayahuasca active compounds.

Analyte	Monitored transitions[a]	Cone voltage (V)[b]	Collision energy (eV)[b]
N,*N*-Dimethyltryptamine (DMT)	189 > 57 (1)	15	10
	189 > 144 (2)	15	10
Tetrahydroharmine (THH)	217 > 188 (1)	15	15
	217 > 200 (2)	15	10
Harmine (HME)	213 > 198 (1)	35	20
	213 > 170 (2)	35	25
Harmaline (HML)	215 > 174 (1)	35	20
	215 > 200 (2)	35	20
Diphenhydramine hydrochloride (IS)	256 > 152	15	35

[a] (1) Transition for quantification and (2) transition for confirmation.
[b] A specific condition was attributed according to each analyte.

at a flow rate of 0.35 mL/min and injection volume of 1 µL. The mass spectrometer source conditions were determined by evaluating the analyte signals through parameter variations. The final values used in the analyses were: electrospray in positive mode, 3 kV capillary voltage, source temperature 150 °C, desolvation gas temperature and flow of 400 °C and 100 L/h, respectively. The SRM conditions were set by selecting two specific transitions (precursor-product ion pairs with the highest intensities in the mass spectrum), referring to the molecular ions of each of the ayahuasca target compounds (Table 1), in order to quantify and confirm the identity of the analytes with high sensitivity and selectivity.

Data acquisition and processing for the 38 analyzed samples were performed using the TargetLynx™ application set in the MassLynx v. 4.1 software package (Waters Corp.). Calibration curves were constructed based on the internal standard calibration method. The mean values, corresponding to triplicate analyses of the samples, SD and CV were calculated using Microsoft Excel (version 2016), which was also the graphing software employed in this study.

3. Results and discussion

3.1. Analytical method optimization

Initially, procedures were performed to optimize the chromatographic conditions in order to guarantee better alkaloid and IS separation efficiency, as well as the establishment of short threshold times for elution using a C18 chromatographic column (BEH). The separation conditions were assessed both in the isocratic and gradient elution mode using different mobile phase compositions. Acetonitrile:water and methanol:water, with the addition (or not) of formic acid 0.1% (v/v) were assessed as eluents. Further mobile phase flows were tested between 0.2 and 0.5 mL/min. The final conditions displaying adequate results in terms of compromise between better peak resolution and shorter analyses times are described in the Material and methods section. The elution times for the different alkaloids were 1.30 min for DMT, 2.14 min for THH, 2.72 min for HML, 2.86 min for HME and 3.90 min for the IS, as can be seen in Fig. 1, which also presents a comparison of a representative chromatogram for ayahuasca active compounds separation using UHPLC-MS/MS for a standard mix (A) and for a real sample (B), indicating that matrix effect it is not significant. The method was then validated by determining the concentration ranges of the ayahuasca alkaloids.

Due to the nature of the ayahuasca matrix, selectivity and matrix effects were the main evaluated parameters, in order to guarantee greater accuracy in the determination of the assessed analytes.

The analytical selectivity of a method can be assessed by either comparing an analyte-free matrix with a matrix spiked with analyte standards, in order to observe the occurrence of co-elution, or using modern and selective detectors, such as tandem mass spectrometers [24,25]. Mass spectrometers used as detectors add a new dimension to the analytical response, the mass-to-charge ratio (m/z). Then it is possible to know whether a compound with a determined m/z is eluting from the column or not. In this study, the SRM strategy was chosen to quantify specific ayahuasca active compounds due to its selectivity, even in the presence of interfering compounds [25].

The intensity of the matrix effect and interfering compound concentrations may differ from one matrix or sample to another. Commercial, analyte-free, matrix standards for complex matrices, such as plant extracts, are not available. In this case, the standard addition method [26] is recommended. Some authors believe that the matrix effect in the analytical response should be assessed by comparing the matrix superposition method with external standardization (standards prepared in solvents). The matrix interference can then be determined by overlapping the curves obtained by internal or external standardization with and without matrix superimposition. Parallel lines indicate that the matrix does not interfere with the analyte signal, but intersecting lines indicate matrix interference and quantification should be performed by the matrix superposition method [25].

Matrix effects can be also assessed by evaluating the relationship between solvent- and matrix-prepared curves [27]. In order to assess ayahuasca matrix effects, the actives were analyzed using a sample pool as matrix, consisting of a mixture of equal aliquots from a selected group of samples that contain the target analytes at low concentrations. Linearity was compared for the analytical curve of each alkaloid (DMT, THH, HME and HML) prepared in both the pooled sample and the solvent (Suppl. Fig. 2). The generated graphs indicated parallel curves, evidencing method selectivity. The matrix effect was less pronounced for DMT and THH compared to HME and HML. A lesser matrix effect can be associated to the ratio of the slope values from both curves [28], which ranged from 0.93 to 0.82, guaranteeing the quality of the analysis. Therefore, the internal standard calibration method was chosen for further analyses, since a large number of samples are involved in this study.

In order to calculate linearity, three analytical curves with seven points each were prepared, varying analyte concentrations according to the intervals described in the Suppl. Table 2. The equations of the lines, the correlation (r) and determination coefficients (R^2) and the range of the values obtained for the statistical residuals were then determined from the analytical curves constructed for the four analytes (Table 2). Based on the variance and linear regression, the linearity results were able to provide the concentration ranges for the determination of target analyte concentrations [26,27].

The low analyte dispersions were confirmed by residual plots. No significant trend was observed (Fig. 2), confirming the linearity of all curves. Homoscedasticity was verified according to the Cochran C test. C_{crit} value was 0.561 and C values for DMT, HME, HML and THH were 0.552, 0.444, 0.397 and 0.570, respectively. As C values for the analytes were lower than C_{crit} (with an exception for THH, which presented a value slightly higher than C_{crit}), it is possible to conclude that the observed variances are homogeneous.

The analysis of variance (ANOVA) data (Suppl. Table 3) demonstrate p-values below the established level of significance of 0.05 (5%), thus confirming the significance of the regression model and therefore the linearity of the method. The regression significance, determined by the F test, proposes that when $F \geq p$-value, a linear relationship between the variables must be present and the slope of the regression curve should not be zero, indicating a significant regression. However, if $F \leq p$-value, there is no indication of a linear relationship between variables x and y and there is no point in using a regression [29,30]. The obtained data indicate that the F values were always greater than the p-value for all the constructed curves. Thus, the curves for the four assessed alkaloids display adequate linearity in the determined working ranges and were used for the analyses.

Repeatability was verified by assessing three concentrations, termed

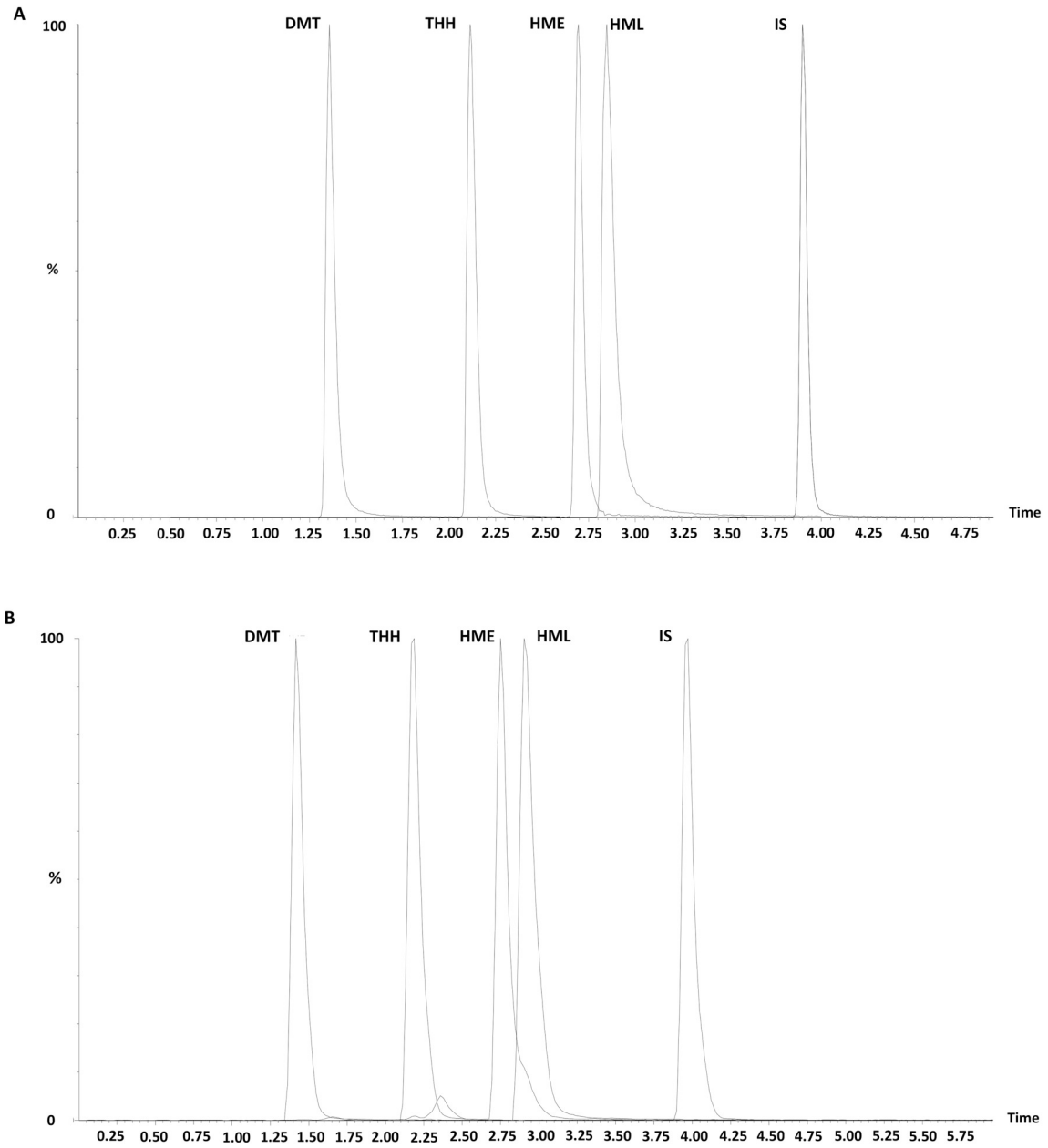

Fig. 1. Representative chromatogram for ayahuasca active compounds separation by UHPLC-MS/MS: (A) standard mix and (B) real ayahuasca sample.

quality control (QC), using internal standard calibration curves, according to ANVISA guidelines [27]. Internal standard calibration is often used to improve quantitative analysis, correcting variabilities due to sample preparation, storage and injection issues. Each of the four analytes at low (QCL), mean (QCM) and high (QCH) concentrations were included in the linear interval, in triplicate, and were then correlated to the calibration curve points, totaling 9 repetitions under the interval linear method (Table 3). QCL values corresponded to the second point of the curve, QCM, to the central point, and QCH, to the penultimate point.

Accuracy is expressed as the standard deviation (CV) or coefficient of variation (CV) between measurements. The determined values

Table 2
Regression data for each ayahuasca analyte.

Linearity (n = 21)	Concentration range			
	DMT	THH	HME	HML
	(1.5–15.0 mg/L)	(3.5–35.0 mg/L)	(6.0–60.0 mg/L)	(1.0–5.5 mg/L)
r	0.9956	0.9982	0.9966	0.9969
R^2	0.9912	0.9963	0.9933	0.9939
Linear equation	y = 0.2644× + 0.4749	y = 0.0925× + 0.1756	y = 0.1798× + 1.282	y = 0.1608× + 0.0766
Residual range	−0.16 to 0.23	−0.07 to 0.18	−0.33 to 0.50	−0.029 to 0.032

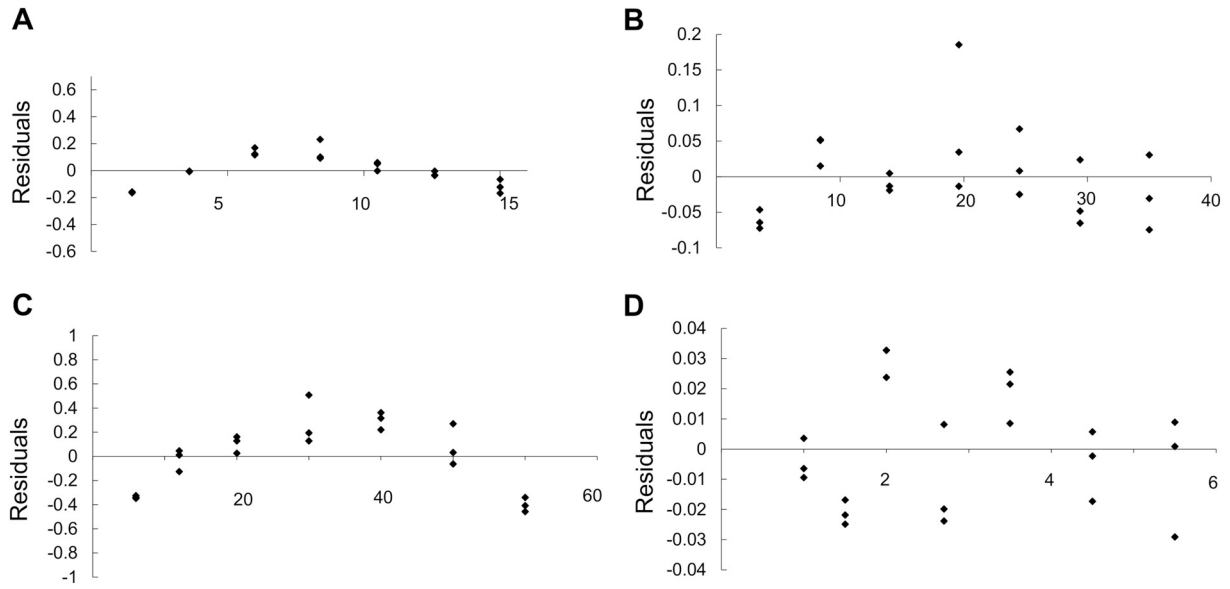

Fig. 2. Residual plots for the assessed analytes: (A) DMT, (B) THH, (C) HML and (D) HME. The X axis represents the concentrations of the analytes and the Y axis represents the residuals of the regression of the analytical curves constructed in ayahuasca matrix.

Table 3
Concentration values for the quality control (QC) range.

QC	DMT (mg/L)	THH (mg/L)	HME (mg/L)	HML (mg/L)
QCL	3.6	8.4	12	1.5
QCM	8.4	19.6	30	2.7
QCH	12.6	29.4	50	4.5

ranged between 0 and 3.5%, well within the established limit of 15% [27] (Suppl. Table 4). Precision was reported as a percentage of error in relation to the theoretical concentration values, and as observed for accuracy, were within the limit of 15%, ranging from 0 to 9.7%.

An analytical method is considered robust when it displays the ability to withstand small and deliberate variations in analytical parameters, leading to confidence during routine use. Thus, robustness assessments should be considered during methodology development. If the method is susceptible to variations in the analytical conditions, these should be controlled and precautions should be included in the analyses procedures. The robustness of a chromatographic method is verified by varying each of the analysis parameters at a time, such as pH and the ionic strength of the mobile phase, temperature programming, sample agitation and extraction variables, among others [25].

In this study, the flow rate of the mobile phase ranged from 0.35 mL/min to 0.30 mL/min and the column temperature ranged from 40 °C to 35 °C. Mobile phase flow variation values ranged between 93.3 and 110%, while temperature column variation values ranged between 87.5 and 106%, both within the established limit of 85–115% (Suppl. Table 5). These values are presented in terms of recovery in relation to the theoretical concentration values.

After determining these parameters, the analytical method was duly validated and applied to the ayahuasca samples.

3.2. Quantification of active ayahuasca sample compounds

Once validated, the method was applied in the analysis of the composition of the ayahuasca samples from the state of São Paulo regarding the presence of their main compounds (DMT, THH, HME and HML) and respective concentrations. The results are presented in Suppl. Table 6.

Important variations in the concentrations of the active compounds were observed. For DMT, the effective concentration range ranged from 75 to 150 mg/L, for THH, from 500 to 1500 mg/L and for HML, from 60 to 180 mg/L. A higher inter-sample concentration value dispersion was observed for HME (Fig. 3).

A study carried out by Callaway in 2005 [31] demonstrated variations in THH, HME and HML concentrations in 33 *B. caapi* samples obtained from different Brazilian regions on the same day and throughout the same period. The same procedure was performed for the determination of DMT in *P. viridis*, and significant variations in the concentration of this active compound were also observed in the studied plants, as we observed for ayahuasca samples. Another interesting comparison is that the ratio between HME and HML concentrations in the plants is similar to the one determined in ayahuasca.

The results of the present study were compared to the literature (Table 4), indicating very consistent data [31–36]. The variation of the active compounds in these samples is significant, in some cases reaching an order of magnitude of 10 times, when comparing the lowest and the highest concentration values for a certain analyte. Even higher variations are observed in the literature, albeit in very small samplings. It is interesting to note that the order of concentration of the alkaloids in the samples follows a trend of increasing order of concentration, namely HME ~ THH > DMT > HML, except in a gas chromatography analysis coupled to a nitrogen-phosphorus detector (GC-NPD), where HML was more concentrated [36].

4. Conclusions

In the present study, the LC-MS/MS technique allowed for the development, optimization and validation of an efficient and fast analytical method capable of simultaneously quantifying the four main active ayahuasca compounds with minimum sample preparation. Reliable quantification results for these active compounds in 38 samples from the state of São Paulo were obtained, and demonstrated the significant heterogeneity of this type of extract, which makes ayahuasca tea standardization for future medicinal use a major challenge.

Ayahuasca contains psychoactive compounds whose neurochemical and pharmacological bases are complex and, in general, not yet well known. Since the use of this compound in recent decades has expanded to the main Brazilian capitals, as well as the United States and Europe, an improved understanding of its mechanisms of action allows for optimized planning, creating new perspectives for the possible therapeutic use of this substance and/or its constituents. In addition, its chemical

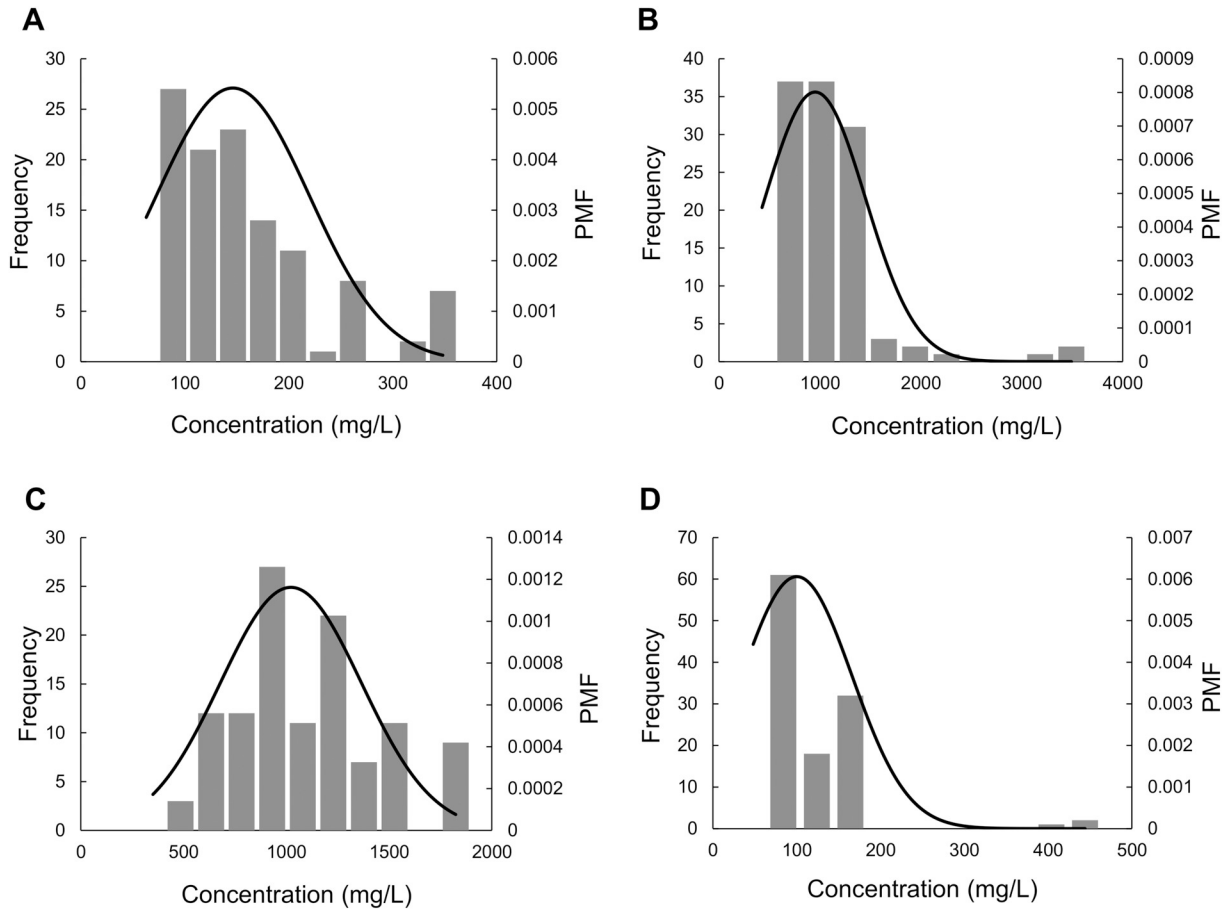

Fig. 3. Distribution of ayahuasca analyte concentrations: (A) DMT, (B) THH, (C) HME, and (D) HML. The concentrations were plotted on distribution charts in order to obtain a grouping trend for the analyzed samples. PMF: probability mass function, calculated with basis on the cumulative normal distribution.

Table 4
Comparison of the active ayahuasca compound concentrations obtained by different techniques.

Reference	DMT (mg/L)	THH (mg/L)	HME (mg/L)	HML (mg/L)	Number of samples	Analytical technique
Callaway [31]	160–5840	450–5260	450–6250	100–900	20	HPLC-FD
Pires et al. [32]	420–730	210–670	370–830	640–1720	8	GC-NPD
Gambelunghe et al. [33]	240	–	340	60	1	GC–MS
Mcllhenny et al. [34]	120–3190	1222–11,900	910–16,000	54–1550	6	LC-MS/MS
Gaujac et al. [35]	100–1810	–	–	–	7	GC–MS
Lanaro et al. [36]	402–2070	850–2053	295–2894	28–181	9	HPLC-DAD
This study	62–340	402–3308	414–1816	44–420	38	LC-MS/MS

characterization opens new perspectives for technique optimization regarding other ayahuasca constituent screening and assessments that may be used as tools for possible information policies associated with the consumption of this psychedelic beverage.

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