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New Insights into the Chemical Composition of Ayahuasca

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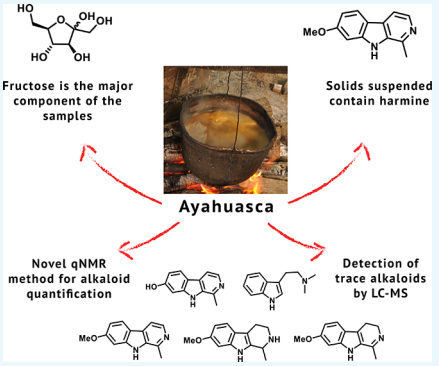
ABSTRACT: Ayahuasca is a psychedelic beverage originally from the Amazon rainforest used in different shamanic settings for medicinal, spiritual, and cultural purposes. It is prepared by boiling in water an admixture of the Amazonian vine *Banisteriopsis caapi*, which is a source of *β*-carboline alkaloids, with plants containing *N,N*-dimethyltryptamine, usually *Psychotria viridis*. While previous studies have focused on the detection and quantification of the alkaloids present in the drink, less attention has been given to other nonalkaloid components or the composition of the solids suspended in the beverage, which may also affect its psychoactive properties. In this study, we used nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) to study the composition of ayahuasca samples, to determine their alkaloid qualitative and quantitative profiles, as well as other major soluble and nonsoluble components. For the first time, fructose was detected as a major component of the samples, while harmine (a *β*-carboline previously described as an abundant alkaloid in ayahuasca) was found to be present in the solids suspended in the beverage. In addition, *N,N*-dimethyltryptamine (DMT), harmine, tetrahydroharmine, harmaline, and harmol were identified as the major alkaloids present in extracts of all samples. Finally, a novel, easy, and fast method using quantitative NMR was developed and validated to simultaneously quantify the content of these alkaloids found in each ayahuasca sample.



INTRODUCTION

Ayahuasca, commonly translated from the Quechua language as "vine of the spirits" or "vine of the dead", is a psychedelic beverage originally from the Amazon rainforest used in different shamanic settings for a variety of medicinal, spiritual, and cultural purposes.[1] It is prepared by boiling in water an admixture of the vine *Banisteriopsis caapi*, which is a source of *β*-carboline alkaloids, and other plants containing *N,N*-dimethyltryptamine (DMT), usually *Psychotria viridis* (Figure 1) or *Diplopterys cabrerana*, (where the preparation name is usually referred to as *yagé*).[2]

Almost four decades ago, ayahuasca traveled from its traditional uses in the Amazon basin to religious, therapeutic, and spiritual centers with a worldwide distribution.[3,4] Brazilian churches that use ayahuasca as part of their religious practices, such as the *Santo Daime* and *União do Vegetal*, as well as shamanic practices involving the ingestion of the beverage in group rituals, have expanded globally within the psychospiritual transnational networks.[4−7] Recently, ayahuasca has captured the attention of the scientific community as part of the "renaissance of psychedelic studies".[8−10] Several reports highlight its potential therapeutic applications in clinical and nonclinical settings for the treatment of depression,[11−14] grief,[15] eating disorders,[16] and substance use disorders (SUDs).[17−22] In addition, preclinical studies have shown that DMT, *β*-carbolines, and ayahuasca preparations present antidepressant-like effects in animal models[23−25] and that administration of the beverage can block ethanol preference in an animal model of dependence.[26]

The chemical composition of the psychoactive alkaloids present in ayahuasca is variable and has been extensively studied.[10] Generally, the main *β*-carboline alkaloids that are present in the drink are harmine (**1**) and (+)-tetrahydroharmine (**2**), while harmaline (**3**) and other demethylated variants such as harmol (**4**) are found as minor components (Figure 1).[27] The quantity of *β*-carbolines can be variable since different varieties of *B. caapi* (e.g., caupuri and tucunacá)[28] or species of *Banisteropsis* (e.g., *B. longialata*, *B. lútea*, *B. martiniana*, and *B. muricata*) can be used for the preparation of ayahuasca.[29−31] DMT (**5**) content in the brew is also variable and depending on the plant used, other minor tryptamines can also be found (e.g., *N,N*-dimethyl-5-hydroxitryptamine found in *D. cabrerana*, and *N*-methyltryptamine, and 2-methyl-1,2,3,4-tetrahydro-*β*-carboline found in *P. viridis*).[32−35] Recently, adulterants such as the monoamino oxidase (MAO) inhibitor moclobemide, psilocin, and yuremamine have been detected in European samples of ayahuasca.[36]

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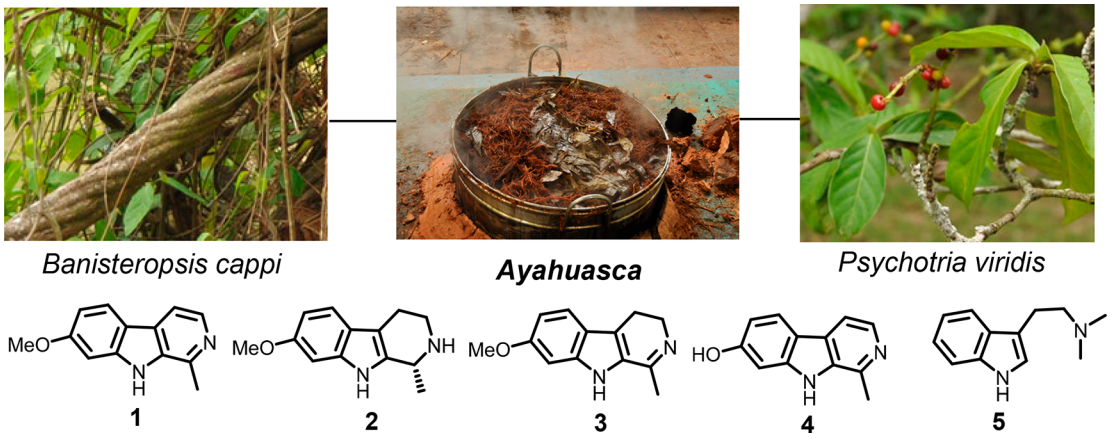

Figure 1. Ayahuasca prepared from *B. caapi* and *P. viridis*. Chemical structures for harmine **1**, tetrahydroharmine **2**, harmaline **3**, harmol **4**, and *N,N*-dimethyltryptamine **5**. Photographs courtesy of Dr. Juan Scuro and Dr. Ismael Apud, published with permission of the authors.

These mixtures of plants and drugs may present a higher toxicity profile than authentic preparations which can be problematic for users.

From a pharmacological point of view, the DMT present in the beverage works as a potent agonist of the 5-HT_{2A} receptor (which is known to mediate psychedelic effects) and also interacts with several other serotonin receptors in the nanomolar range, the sigma-1 receptor, and trace amine-associated receptor 1 (TAAR1).[37] On the other hand, β-carbolines are reversible inhibitors of monoamino oxidase A (MAO-A), and some of them are also inhibitors of the serotonin plasmatic transporter, SERT, such as tetrahydroharmine.[24] DMT is known to be inactive when ingested orally due to deamination by intestinal and hepatic MAO, so β-carbolines present in the ayahuasca brew allow for the inhibition of its degradation allowing it to reach the brain.[35] Also, MAO-A and SERT inhibition by β-carbolines allows for an increase in the extracellular levels of neurotransmitter monoamines in different brain regions, which may also contribute to the psychoactive effect of the beverage. A recent review highlights that there could be more synergistic mechanisms between DMT and β-carbolines than it is currently understood, and may explain the complex pharmacology of ayahuasca.[38]

Regarding the chemical analysis of the beverage, several methods have been developed for the detection and quantification of psychoactive alkaloids in ayahuasca samples involving high-performance liquid chromatography-ultraviolet (HPLC-UV),[35,39−41] HPLC with a fluorescence detector (HPLC-FL),[42,43] gas chromatography (GC) with nitrogen-phosphorus detection (GC-NPD),[42−44] GC-MS,[45,46] LC-MS/MS,[40,47,48] and UHPLC-MS/MS.[36] Also, a recent report describes a novel methodology for estimation of the DMT content in ayahuasca samples using quantitative nuclear magnetic resonance (qNMR).[49] In these reports, sample preparation for analysis of the whole sample involves dilution and separation of the solids suspended in the beverage by filtration[35,47] or centrifugation.[40,42,43,48,49] Other protocols rely on the analysis of an alkaloid extract obtained by liquid−liquid extraction[42,45,49] or solid-phase extraction.[39,44,46] In other cases, the ayahuasca sample is lyophilized followed by a solid extraction.[35]

It is worth noting that previous studies have focused on the detection and quantification of the alkaloids present in the drink with less attention to other nonalkaloid components or the composition of the solids suspended in the beverage, which are usually removed before analysis, but are ingested by users on some occasions. In addition, there is anecdotal evidence that these solids possess psychoactive properties, something that prompted us to investigate the presence of residual alkaloids in their composition.

In this manuscript, we analyzed whole ayahuasca samples to detect nonalkaloid components and to characterize the composition of the solids suspended in the beverage by NMR spectroscopy. In addition, we analyzed an organic extract of each sample to characterize the alkaloid profiles by NMR and LC-MS/MS. Finally, a novel, easy, and fast method using quantitative NMR for the simultaneous quantification of compounds **1**−**5** in ayahuasca was developed and validated. qNMR offers some important advantages over more traditional chromatographic methods.[50] First, the use of a universal internal or external standard allows the quantification of many components in a single spectrum. Second, it is a noninvasive, nondestructive technique allowing the study of the same sample over an extended period of time. Third, it is a fast technique that presents a linear response over 6 orders of magnitude in concentration, from uM to M range.

RESULTS AND DISCUSSION

For this study, seven ayahuasca samples were donated by users that participate in rituals in Uruguay, where groups that use the decoction have emerged since the early 1990s in different settings.[6,51−54] These include the religious Brazilian churches, neoshamanic practitioners, and holistic centers which use the beverage for psychological healing.[55] Samples were therefore labeled according to the group of reference of the donor as "Religious", where ayahuasca is used for religious purposes (samples R1, R2, and R3), "Therapeutic", where ayahuasca is used in groups for psychological healing and as a therapeutic for SUDs (samples T1, T2), and "Neoshamanic", where ayahuasca is used for psychospiritual purposes (samples N1 and N2).

Analysis of Nonalkaloid and Nonsoluble Components. As mentioned earlier, previous studies have focused on the detection and quantification of the alkaloids present in ayahuasca with less attention to other nonalkaloid components or the composition of the solids suspended in the beverage. To this effect, selected ayahuasca samples were centrifuged, the

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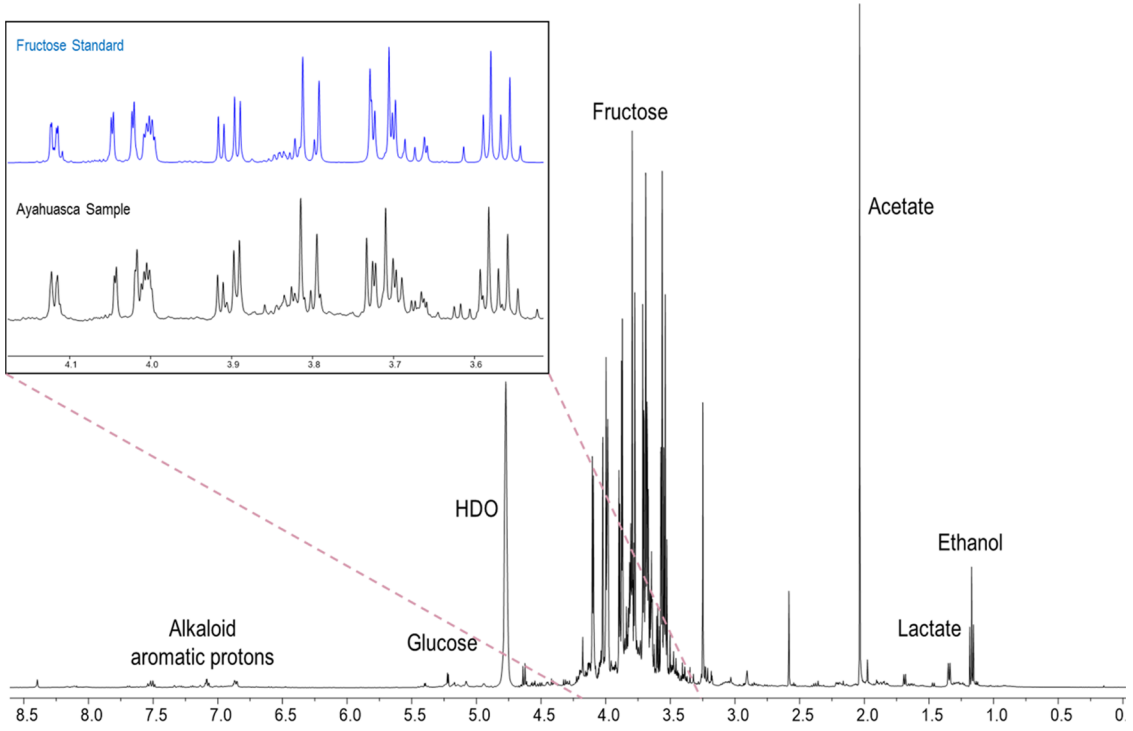

Figure 2. ^{1}H NMR spectra of the ayahuasca sample N1 (that was centrifuged and diluted with water and D_2O) recorded using a water suppression scheme. Fructose is found as the main component of the sample as well as minor amounts of glucose. Ethanol, acetate, and lactate are also detected in the beverage, as well as signals corresponding to the aromatic protons of the psychoactive alkaloids.

Table 1. Estimated Content of Fructose, Glucose, Ethanol, Lactate, and Acetate for the Ayahuasca Samples T1, N1, N2, R1, R2, and R3[a]

sample	fructose		glucose		ethanol		acetate		lactate	
	g/50 mL	RSD	g/50 mL	RSD	g/50 mL	RSD	g/50 mL	RSD	g/50 mL	RSD
T1	33.67	1.99	5.27	1.50	0.90	0.72	0.09	4.78	nd	nd
N1	6.38	0.64	1.46	1.83	1.96	1.90	0.05	6.48	nd	nd
N2	9.29	2.53	0.90	2.11	0.39	2.22	0.04	4.23	0.16	2.63
R1	14.90	0.72	1.67	1.54	0.25	1.44	0.08	0.40	0.04	3.29
R2	3.07	2.78	0.37	0.92	nd	nd	0.01	1.36	0.95	2.47
R3	8.10	4.06	1.73	1.32	0.02	8.38	0.03	4.0	nd	nd

[a]Values were obtained by the PULCON method and are expressed in grams per 50 mL of ayahuasca. nd = not detected. Three independent dilutions of each sample were prepared to estimate the RSD in each case.

resulting supernatants were diluted using distilled water containing 10% D_2O, and the corresponding solids were washed with water, dried, and finally dissolved in DMSO-d_6. ^{1}H NMR spectra of the two solutions were recorded.

Figure 2 shows the water-suppressed ^{1}H NMR spectra of the diluted supernatant for sample N1. Surprisingly, fructose was found as the major component of the beverage. This was corroborated by a comparison of the complex patterns of signals corresponding to the different keto, pyranose, and furanose tautomers of the monosaccharide with the spectrum of an authentic sample.[56] Glucose is also present as a minor component as noticed by the resonances for the anomeric protons of the α- and β-pyranosic forms, at 5.22 and 4.63 ppm respectively. Also minor signals corresponding to the aromatic protons of the psychoactive alkaloids were detected in the aromatic region of the NMR spectra. The intensity of the latter signals with respect to those of fructose reflects a minor alkaloid content relative to carbohydrate content. As indicated in Figure 2, signals for ethanol, lactate, and acetate were also detected in the N1 sample. Other samples such as R1, R2, R3, N2, and T1, which are used in different ritual settings, showed a similar chemical profile in the ^{1}H NMR spectra (see Figures S1 and S2), indicating that similar compositions can be found in samples from different origins.

To our knowledge, this is the first time fructose is reported as a major component of ayahuasca samples and we postulate that this is due to sugars present in the vegetal materials used for the preparation of the beverage. In fact, previous studies have identified the fructose-based disaccharide β-D-fructofuranosyl-(2 → 5)-fructopyranose and other glycosides containing glucose units, such as Banistenosides A and B, in *B. caapi*,[57] which may hydrolyze after intensive boiling in water and would account for the sugars present in the beverage. Also, some glycosylated steroids have been detected in the leaves of *P. viridis*.[58]

The presence of a high fructose content in ayahuasca agrees with some aspects related to the drink and its preparation. A recent report highlights the different preparations made by the *Santo Daime* church in Brazil,[36] where the most concentrated forms of the sacrament are named as "miel" (honey) and "gel",

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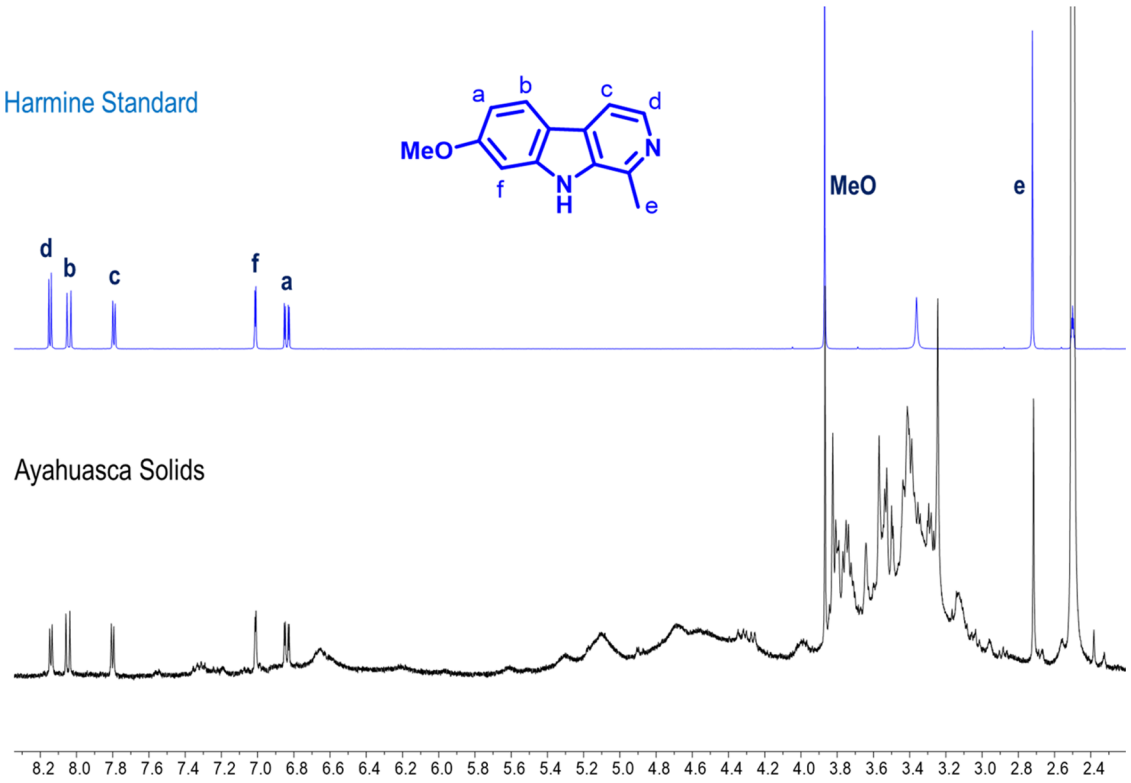

Figure 3. ^{1}H NMR spectra of the solids suspended in the ayahuasca sample N1 compared with a standard of harmine. Solids were separated by centrifugation, washed with water, dried *in vacuo* at 40 °C, and dissolved in DMSO-d_6.

referring to their visual aspect and taste and consistent with high sugar content. Also, the usual dark brownish color of ayahuasca is likely related to the presence of melanoidins, polymers produced by the Maillard reaction of fructose with proteins that could be present in the decoction, a process that is favored by the extensive boiling in water required for some preparations of the brew.[59]

To obtain quantitative data, the content of fructose, glucose, ethanol, acetate, and lactate for samples T1, N1, N2, R1, R2, and R3 was estimated using the PULse Length-based CONcentration determination (PULCON)[60] qNMR approach, selecting individual peaks for each component in the ^{1}H NMR spectra (see Table S1). As mentioned before, qNMR presents some advantages in comparison with traditional chromatographic methods for quantitative analysis. It allows the simultaneous identification and quantification of several components in a complex mixture. Furthermore, no calibration curves are needed for each analyte since only an internal or external standard is required. Finally, the method is fast and nondestructive.[61]

Results shown in Table 1 are expressed in grams per 50 mL of ayahuasca, to estimate their presence in a standard volume ingested of the brew. Porcentual relative standard deviation (RSD) was calculated as: standard deviation of three independent dilutions of the ayahuasca sample × 100/concentration average.

Regarding fructose, the concentrations in the different samples ranged from 3.07 to 33.67 g/50 mL of ayahuasca. This may reflect different amounts of plant material used in the preparation of the brew, as well as different preparation procedures since different degrees of cooking and concentration of the resulting beverage have been described.[34,35]

Some samples exhibited a very high fructose content (e.g., T1 and R1), which may also contribute with some of the known gastrointestinal effects described in some individuals after drinking the brew. These effects, such as vomiting, nausea, and diarrhea, are traditionally interpreted as purification effects and are considered an important part of the ayahuasca experience in some rituals. Although these effects have been attributed to an increase of serotonin levels related to the action of β-carbolines at the central nervous system level (i.e., vomiting induced by vagus nerve stimulation) and/or peripherally (i.e., excessive intestinal stimulation),[62] the possibility of causing diarrhea or intestinal symptoms in some users by an osmotic effect generated by the high fructose concentration in the distal small intestine and colon cannot be ruled out. In fact, it is known that a high percentage of the population presents fructose malabsorption,[63] a disease characterized by colicky abdominal pain, flatulence, and diarrhea as a consequence of an accumulation of unabsorbed fructose in the gut.[64,65] Although merely speculative, this hypothesis agrees with observations which indicate that *pharmahuasca* preparations, where only pure alkaloids are consumed, do not produce intense gastrointestinal symptoms as ayahuasca preparations do.[33] Therefore, precautions should be taken before individuals presenting hereditary fructose intolerance, a rare inborn disease characterized by a deficiency of the enzyme aldolase B, ingest ayahuasca since other conditions such as hypoglycemia, liver, and kidney failure could be developed.[66] Finally, the presence of fructose in the brew may also alter the bioavailability of the alkaloids, which could be another factor explaining the difference between the traditional preparations and pharmahuasca's formulations.

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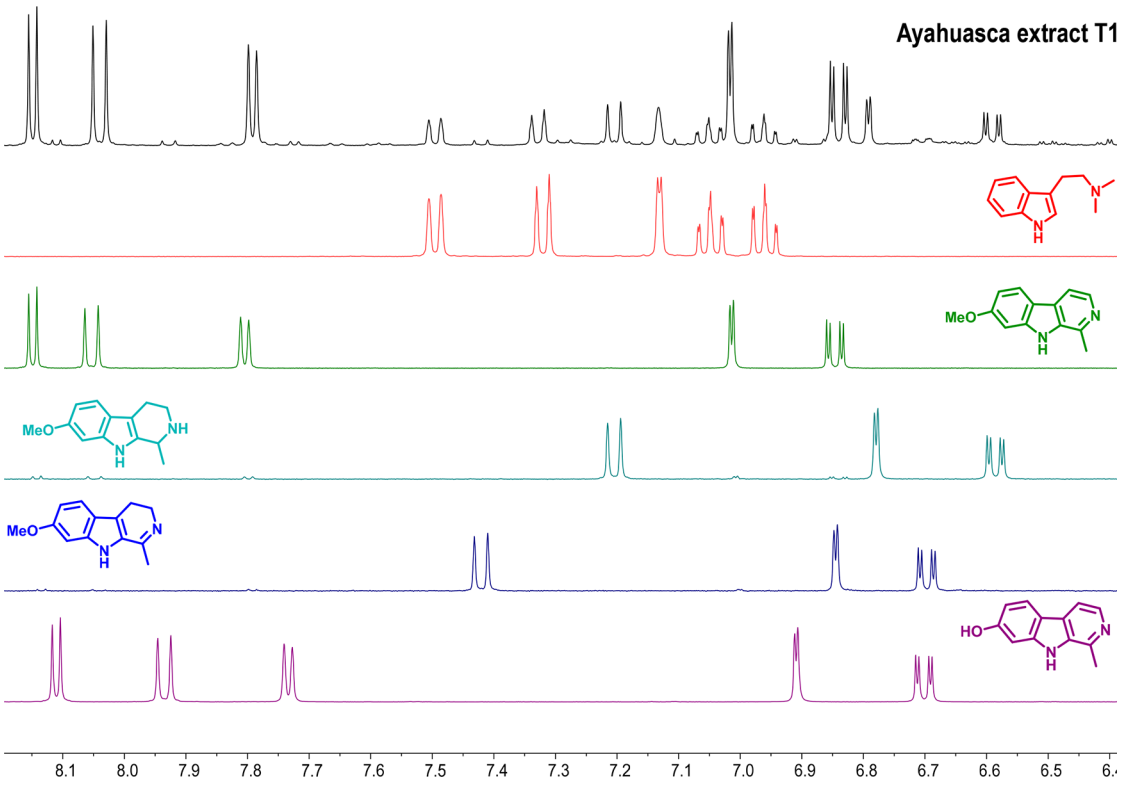

Figure 4. Representative ^{1}H NMR spectra of the ayahuasca extract T1 (aromatic region), and comparison with the authentic standard of **1**, **2**, **3**, **4**, and **5**.

Ethanol, lactate, and acetate were also present in the analyzed ayahuasca samples and could be attributed to fermentation products of fructose and glucose. Their content is highly variable within the samples, which may be due to the high variability in brewing conditions, procedures, and storage.

Figure 3 shows the ^{1}H NMR spectra of the solids suspended in the sample N1, which were centrifuged, washed, dried, and dissolved in DMSO-d_6. As observed by comparison with an authentic sample of harmine, this alkaloid is present in the insoluble material suspended in the beverage. This finding is important since psychoactivity of the ayahuasca could be influenced by the amount of suspended solids the users ingest, and this depends on the homogenization of the beverage before serving it. Also, from an analytical point of view, removing these solids before analysis may lead to an underestimation of the real amount of harmine present in the whole sample which may be ingested by the users. Figure S3 shows the composition of the solids corresponding to the samples from different origins: R1, R2, R3, and N2. As can be appreciated, all of them contain harmine although in different relative contents, which may reflect differences in filtration or solid loss during the preparation procedures of ayahuasca.

Furthermore, the fact that part of the harmine extracted from *B. caapi* remains undissolved in the beverage could also explain that despite previous reports showing an average ratio of tetrahydroharmine (**2**) to harmine (**1**) near 1:1 in ayahuasca samples, a large survey of source plant material of *B. caapi* used to prepare those samples presented a ratio closer to 1:5.[67] Callaway postulates that this difference in the relative content of these two alkaloids can be explained by a chemical reduction of harmine to tetrahydroharmine during the acidic process of decoction, which would increase the tetrahydroharmine content. However, from a chemical point of view, this process is very unlikely to occur. Harmine is aromatic, and its reduction to harmaline or tetrahydroharmine would require strong reducing agents that are not found under the conditions for the brew preparation. In fact, an example for the synthetic reduction of harmine to tetrahydroharmine requires the quaternization of its nonindolic nitrogen followed by reduction using sodium borohydride.[68] Therefore, we postulate that the relative amount of tetrahydroharmine is higher in the beverage because part of the harmine is lost due to its insolubility during the preparation of the brew (because of sedimentation or filtration of the suspended solids). Also, the harmine content of the brew could be underestimated in some analytical reports since the solids are initially removed during the sample preparation for analysis. A similar observation regarding higher ratios of harmine to tetrahydroharmine in *B. caapi* samples than in the corresponding brew is included in a recent study by Santos et al.[69]

Qualitative Analysis of Alkaloids. For qualitative and quantitative analyses of the alkaloid content, a liquid−liquid extraction of the samples was carried out without previous centrifugation or filtration. In this manner, the suspended harmine would be dissolved in the organic solvent and taken into account in the analysis. Thus, ayahuasca samples were basified with ammonia and extracted five times using ethyl acetate. The combined organic extracts were dried and evaporated *in vacuo* to afford a crude extract, which was further analyzed by TLC, NMR, and LC-MS/MS.

Initial analysis by TLC of the extracts indicated the presence of harmine, tetrahydroharmine, DMT, and traces of harmaline as major alkaloids present in all samples except in T2, where no alkaloids were detected (see Figure S4 in the Supporting Information). The latter sample was thus discarded from further analyses. Next, extracts were analyzed by ^{1}H NMR.

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Table 2. LC-MS Analysis of the Extracts Prepared for Some Ayahuasca's Samples

sample	retention time (min)	molecular weight	MS and MS/MS fragment ion (m/z)	UV/vis (λ max)	identification
T1, N1	1.17	204	205/187/160/132/115	223/271	bufotenine
T1, N2	2.40	174	175/144/132/127/117	278	*N*-methyltryptamine
T1, N1, N2, R1, R2, R3	2.50	188	189/143/127/117/115	278	*N, N*-dimethyltryptamine
T1, N1, N2	3.09	202	203/174/159/131	266/288	tetrahydronorharmine
T1, N1, N2	3.10	200	186/171/158/143/130	251/325	harmalol
T1, N1, N2, R1, R2, R3	3.29	216	217/200/188/173/158/145/130	224/268/292	tetrahydroharmine
T1, N1, N2, R1, R2, R3	3.40	198	183/171/158/140/131/116	249/322	harmol
T1, N1, N2, R1, R2, R3	3.76	214	215/200/185/183/174/172/171/159/143/131	250/374	harmaline
T1, N1, N2, R1, R2, R3	3.91	212	213/198/170/144	246/320	harmine

Table 3. Concentration for DMT, Harmine, Tetrahydroharmine, Harmaline, and Harmol in the Ayahuasca Samples Determined by qNMR (PULCON Approach)[a]

sample	DMT		harmine		tetrahydroharmine		harmaline		harmol	
	mg/g	% RSD	mg/g	% RSD	mg/g	% RSD	mg/g	% RSD	mg/g	% RSD
T1	1.82	3.45	5.61	3.26	2.33	4.43	0.452	2.26	0.290	1.18
N1	0.532	3.66	3.27	4.91	1.10	4.79	0.132	19.8[b]	0.103	6.73
N2	0.607	4.04	2.97	6.93	1.53	9.67	0.234	9.90	0.094	8.20
R1	1.86	1.76	1.65	0.66	1.44	1.95	0.241	5.22	0.073	4.69
R2	1.04	6.99	1.34	2.38	1.11	8.75	0.213	6.80	0.090	5.78
R3	1.32	6.01	1.56	3.65	1.03	2.96	0.161	3.50	0.047	4.33

[a]For each sample, the mean value of three extractions is shown with the corresponding porcentual relative standard deviation. [b]High RSD in this case is explained because of an impurity near the harmaline peak, which affected its integration.

Figure 4 shows the aromatic region of the ^{1}H NMR spectra of the alkaloid extract dissolved in DMSO-d_6 for sample T1, which is representative. Comparison with standards allowed for the identification of harmine, tetrahydroharmine, harmaline, harmol, and DMT as major components detected in all of the studied samples. We were delighted to see that we could identify isolated individual signals of each alkaloid in the mixture, which allowed us to carry out qNMR studies (*vide infra*). Figure S5 shows a comparison of three representative extracts of ayahuasca samples used in different settings (i.e., religious, therapeutic, and neochamanic). Although displaying a different relative content, the same alkaloids profile is detected for each sample, which reflects a standard composition.

Finally, to detect trace amounts of other alkaloids, LC-MS analysis for all of the extracts was carried out (Table 2). In addition to the major alkaloids detected previously by NMR, trace amounts bufotenine (5-hydroxy DMT), *N*-methyltryptamine, tetrahydronorharmine, and harmalol were detected in some samples. The presence of 5-hydroxy DMT (bufotenine) detected in samples T1 and N1 may reflect that *D. cabrerana* was used as a DMT source in this specific preparation since it is reported that this alkaloid is present in the latter and not in *P. viridis*.[35] No appreciable amounts of other alkaloids than tryptamines and β- carbolines were detected in all samples. This is important since it is known that the preparation of ayahuasca may also include variable contents of other admixture plants, which can be a source of another type of alkaloids. In fact, Ott has described almost 100 species of plants as additives to traditional preparations (e.g., Solanaceae species such as *Nicotiana, Brugmansia*, or *Brunfelsia*).[70] Also, adulterants such as the MAO inhibitor moclobemide or other drugs previously reported in ayahuasca preparations from Europe were not found in the samples considered in this study.[36]

Quantitative Analysis of Alkaloids. To quantify the content of DMT, harmine, tetrahydroharmine, harmaline, and harmol present in the extracts, we selected individual peaks for each component in the ^{1}H NMR spectra (see Figure S6) and used the PULCON method.

Three independent extracts were prepared for each ayahuasca sample (each of them prepared by five extractions of the basified sample using EtOAc), dissolved in DMSO-d_6, and the corresponding ^{1}H NMR spectra were recorded. To corroborate that the harmine found in the solids suspended in the beverage was also extracted during this protocol, we carried out a qNMR analysis of the resulting solids of the aqueous phase. We found that 96% of the harmine originally present in the solids of the ayahuasca sample was removed after the ethyl acetate extractions (see Figure S12)

For qNMR analysis, an 86 mM benzoic acid solution in DMSO-d_6 was used as external standard. For each sample, 16 scans were recorded using a 90° pulse width of 13 μs and a relaxation delay of 40−60 s. The method was validated by determining for each analyte the selectivity and specificity, linearity and accuracy, limit of detection (LOD), limit of quantification (LOQ), precision, and their stability in DMSO-d_6 solutions (see the Supporting Information for experimental details and results regarding the validation of the method).

Table 3 shows the results expressed as mg of alkaloids per gram of ayahuasca (mean value) with the respective percentual relative standard deviation (RSD) found for n = 3, calculated as: standard deviation × 100/average. Results were expressed in grams of ayahuasca since the high viscosity of some samples made its proper volumetric measurement difficult (an estimated density for each sample can be found in Table S3). It is important to note that the RSDs presented in Table 3 represent the deviation for the whole extraction method and not only to the analyte measurement.

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Average concentrations (mg/g) and ranges found for each alkaloid in the six samples analyzed were: DMT 1.13 (0.532−1.86); harmine 2.83 (1.34−5.61); tetrahydroharmine 1.48 (1.03−2.33); harmaline 0.221 (0.118−0.452); harmol 0.106 (0.044−0.290). Values for each analyte are in the range described in previous studies that reported analysis of ayahuasca samples (see Table S2 for a detailed comparison between previous analysis and the results found in this study).

Nevertheless, some differences can be found when analyzing the relative content of the alkaloids (see Table S3). While most of the previous studies describe a relative amount for the alkaloids as: harmine ∼tetrahydroharmine > DMT > harmaline, our samples from therapeutic and neoshamanic settings displayed an unusually high harmine/tetrahydroharmine ratio of ∼1.9−3.0 (see Table S2). Religious samples analyzed in this study displayed comparable ratios of harmine and tetrahydroharmine but presented higher amounts of DMT, showing lower β-carbolines:DMT ratios (ca. 1.8−2.7) than samples from therapeutic and neoshamanic settings (ca. 4.8−7.9), see Table S3. This could reflect different amounts of vegetal species used for the preparation of the beverage according to the origin of the sample.

Regarding the harmaline/harmine ratio, the recent study by Kaaski et al.[36] showed that the average ratio found for typical ayahuasca preparations was in the range of ∼0.07, which is similar to those found in our study (see Table S3). Ayahuasca samples displaying unusually elevated harmaline:harmine ratios (e.g., ∼0.6) could reflect the addition of *Peganum harmala* seeds (Syrian rue), which are rich in harmaline, during the preparation of the beverage.

As concluding remarks of this study, fructose was detected as a major component of the ayahuasca samples, while harmine was found to be present in the solids suspended in the beverage. To our knowledge, this constitutes the first report to detect these soluble and nonsoluble components in ayahuasca samples. Regarding the alkaloid content, we detected DMT, harmine, tetrahydroharmine, harmaline, and harmol as the major alkaloids present in extracts of all samples while bufotenine, *N*-methyltryptamine, tetrahydronorharmine, and harmalol were detected in trace quantities in some cases. Finally, we reported a novel, easy, fast, and validated qNMR method, which allows for the simultaneous quantification of DMT, harmine, tetrahydroharmine, harmaline, and harmol found in ayahuasca samples.

MATERIALS AND METHODS

Reagents and Chemicals. All solvents were distilled prior to use. Chemicals and reagents including authentic samples of harmine, harmaline, harmane, and 10-methoxyharmalan were purchased from Sigma-Aldrich and used as received. Tetrahydroharmine and *N*,*N*-dimethyltryptamine were synthesized as described below.

Synthesis of (±)-Tetrahydroharmine. Tetrahydroharmine was prepared according to a slight modification of the procedure published by Shulgin et al.[71] Briefly, of PtO_2 (0.1 g) was added to a stirred solution of harmine hydrochloride (1.0 g) in H_2O (25 mL) followed by the dropwise addition of a solution of $NaBH_4$ in water H_2O (0.4 g in 4.0 mL) over the course of 20 min under a nitrogen atmosphere. The pH was monitored periodically, and the reaction mixture was kept acidic by the addition of 1 N HCl as needed. When the starting material was consumed (as checked by TLC analysis), the solution was basified using aqueous NaOH 10%. The aqueous phase was extracted using dichloromethane, and organic extracts were combined, dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield a white solid, which was purified using column chromatography (SiO_2, CH_2Cl_2-MeOH 9:1 + 1% NH_4OH) to afford tetrahydroharmine free base in 81% yield. Also, the crude material can be recrystallized from MeOH to give a reference sample as white crystals (see Figure S7 for the ^{1}H NMR spectra)

Synthesis of *N*,*N*-Dimethyltryptamine Fumarate. DMT fumarate was prepared according to a procedure published by Dunlap and co-workers.[72] Briefly, to an ice-cold solution of tryptamine (1.0 equiv) and glacial acetic acid (5.0 equiv) in MeOH (80 mL/g of tryptamine) was added sodium cyanoborohydride (2.1 equiv) followed by aqueous formaldehyde (37%) (2.6 equiv). The reaction was stirred at room temperature for 5 h, where it was concentrated *in vacuo* to afford a crude mixture, which was dissolved in diethylether and diluted with 1.0 M NaOH (125 mL). The aqueous phase was extracted twice with diethylether, the combined organic extracts were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield a colorless oil (see Figure S7 for the ^{1}H NMR spectra of the obtained DMT as free base). This oil was dissolved in acetone (8 mL) and added dropwise to a boiling solution of fumaric acid (0.50 equiv) in acetone (25 mL). The solution was allowed to cool to room temperature and filtered to render DMT fumarate as a white solid.

Ayahuasca Samples. Samples for analysis were donated by users that participate in rituals in different ayahuasca groups in Uruguay as part of a research project funded by the Junta Nacional de Drogas (Presidencia de la República, Uruguay) and the Espacio Interdisciplinario (Universidad de la República). The project received approval from the División de Sustancias Controladas (Ministerio de Salud Pública, Uruguay).

NMR Sample Preparation. For qualitative analysis of the solids suspended in the samples, 10 mL of ayahuasca was first centrifuged at 2000 rpm for 20 min. The supernatant was discarded, and the solid was washed using 5 mL of distilled water, centrifuged again at 2000 rpm for 10 min, and the supernatant was discarded. The resulting solid was then dried under vacuum drying at 40 °C, and 20 mg was completely dissolved in 800 μL of DMSO-d_6 for NMR analysis.

For qualitative and quantitative analyses of the nonalkaloid components of the ayahuasca samples, 1 mL of ayahuasca was centrifuged at 4500 rpm for 5 min and diluted 50 times with distilled water. This solution (900 μL) was taken and 100 μL of D_2O was added.

For qualitative and quantitative analyses of the alkaloids extracts of the ayahuasca samples, an ayahuasca sample was homogenized for 1 min using a vortex mixer and 2.5 mL was taken and weighted. The sample was basified to pH 12 using NH_4OH and extracted with ethyl acetate (5 × 2.5 mL). After each extraction, the mixture was centrifuged at 2000 rpm for 10 min to facilitate phase separation. The combined organic layers were dried using Na_2SO_4, filtered, and the solvent was evaporated *in vacuo* to afford a dried extract, which was dissolved in DMSO-d_6.

NMR Experiments. ^{1}H, ^{13}C, and bidimensional NMR spectra used for the characterization of compounds **1**, **2**, **3**, **4**, and **5** and in the qualitative and quantitative analyses of sediments and alkaloid extracts were recorded at 25 °C on a Bruker Neo 400 equipped with a BBO *z*-gradient probe

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operating at ^{1}H and ^{13}C frequencies of 400.13 and 100.62 MHz, respectively. The qualitative and quantitative ^{1}H NMR studies of the nonalkaloid components of the ayahuasca samples were carried out at 25 °C on a Bruker AVANCE III 500 equipped with a TXI *z*-gradient probe and operating at a ^{1}H frequency of 500.13 MHz.

qNMR analyses were carried out using the PUlse Length-based CONcentration (PULCON) method.[60] Concentration measurements with PULCON use the reciprocity principle, which states that the lengths of a 90 or 360° pulse are inversely proportional to the intensity of the NMR signal.[73,74] Therefore, as long as the concentration of a standard is precisely known and the 90° pulse of all of the samples has been calibrated correctly, the concentrations of a problem sample can be determined. The estimation of concentrations of nonalkaloid components in ayahuasca samples employed 8.16 mM ethyl benzoate in $CDCl_3$ as the calibration standard, and experiments were performed using a standard water suppression scheme with 128 scans, presaturation during the relaxation delay of 4 s, and a 90° pulse length of 9.75 μs. Table S1 indicates the individual peaks selected to quantify each component and the method used in each case. It is important to point out that the signals of the anomeric protons from the fructose β-pyranose and glucose α-pyranose mutarotamers at 4.02 and 5.22 ppm, respectively, were employed in the quantification of these two sugars. The areas of these signals are affected by less than 5% by the solvent suppression scheme, and they can be employed to accurately determine the concentrations of all forms of the monosaccharides using ratios reported in the literature.[56,75]

For the quantitation of **1**, **2**, **3**, **4**, and **5** in the alkaloid extracts of ayahuasca samples, 86 mM benzoic acid in DMSO-d_6 was used for calibration, and spectra were recorded with 16 scans, a relaxation delay of 40−60 s, and a 90° pulse length of 13 μs. The validation of the method is described in the Supporting Information.

Thin-Layer Chromatography (TLC) Analysis. Analytical TLC was performed on silica gel 60F-254 plates and visualized with UV light at 254 or 376 nm and/or using stains as *p*-anisaldehyde and $CuSO_4$.[76] To prepare the alkaloid extracts used for TLC analysis, an ayahuasca sample was homogenized for 1 min using a vortex mixer and a 1 mL aliquot was taken. The sample was then basified to pH 12−13 with NH_4OH, and successive extractions were carried out with ethyl acetate (5 × 1 mL). After each extraction, the mixture was centrifuged at 2000 rpm for 10 min to facilitate phase separation. Combined organic layers were dried using Na_2SO_4, filtered, and the solvent was evaporated *in vacuo* to afford a dried extract that was dissolved in ethanol to obtain a 1.4 mg/mL solution.

LC-MS/MS Analyses. The alkaloid profile in the ayahuasca extracts was recorded using an HPLC−DAD-ESI-MS/MS system consisting of a Shimadzu LCMS 8040 equipped with an LC-20AD HPLC pump, a DGU-20A5R solvent degassing module, an SPD-M20A DAD detector, a CTO-20A oven, a SIL-20A injector, and an FCV-32AH high-pressure switching valve with a split connection to the electrospray ionization (ESI)-MS. The data were processed with the LabSolutions LCMS software. For chromatographic separation, a 100 mm × 4.6 mm Phenomenex Kinetex EVO C18 column with a particle size of 5 μm was used, with a flow rate of 1.25 mL/min, and a temperature of 40 °C. Ultraviolet−visible (UV−vis) spectra were recorded in the range of 220−360 nm and detection at nm. Gradient system: Mobile phase (A) 0.1% formic acid and (B) methanol were used. The gradient program was: t0′, 5% B, t3′, 50% B, t5′, 90% B t11′, 90% of B, t11.01′, 5% B. The mass spectrometer was programmed to carry out a full scan over *m*/*z* 100−600 (MS1) in positive-ion detection mode. The heat-block and desolvation line (DL) temperatures were set to 400 and 250 °C, respectively. The nebulizer gas flow rate, drying gas flow rate, CID gas pressure, and ion spray voltage were 3.0, 15.0 L/ min, 230 kPa, and 4.5 kV, respectively. The collision energies for MS/MS experiment were 10, 15, 20, 25, 30, and 35 eV.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00795.

Validation of the qNMR method (PDF)

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Notes

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