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A Fully Validated LC-MS Quantitation Method for Psychoactive Compounds Found in Native South American Plant Species

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Abstract: Psychoactive drugs are compounds that alter the function of the central nervous system, resulting in changes in perception, mood, cognition, and behavior. A subclass of psychoactive drugs, psychedelics, are hallucinogenic drugs that can trigger psychedelic experiences and possible changes in mental perception. The potential use of psychedelics as a therapeutic has led to an increase in clinical research focusing on the treatment of mental disorders including anxiety and depression. There are numerous species belonging to *Psychotria* and *Banisteriopsis* which have been reported to contain psychedelic and psychoactive compounds; however, there is a lack of validated analytical methods for raw plant material, which is crucial if these plants are to be commercially cultivated for medicines. This study provides a fully validated method using ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS) for the following six compounds: tryptamine, N,N-dimethyltryptamine (DMT), 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT), tetrahydroharmine (THH), harmaline, and harmine. The validated method was used to determine the psychoactive concentrations in *Psychotria viridis*, *Psychotria carthagenensis*, *Banisteriopsis caapi*, and *Alicia anisopetala*. Validation parameters were established; linearity (R^2 = 0.988–0.999), limit of detection (LOD) (0.06–0.11 ng/mL), limit of quantitation (LOQ) (0.18–0.34 ng/mL), accuracy, precision, extraction efficiency (>98%), recovery (74.1–111.6%), and matrix effect (70.6–109%) were all evaluated. All six compounds eluted within nine minutes, with a total analysis time of 20 min including column equilibration. This method establishes a high-throughput method for the robust analysis of psychedelics which may see future use in agricultural research and industry.

Keywords: DMT; harmine; harmaline; tetrahydroharmine; psychedelics; LCMS; *Psychotria*; *Banisteriopsis*

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1. Introduction

The use of alternative medicines for various human health issues, including psychological disorders, is a growing trend, with many people turning to natural and holistic remedies. The interest in investigating psychedelic drugs such as N,*N*-dimethyltryptamine (DMT) as an alternative medicine is increasing. Historically, these drugs have been used in spiritual and cultural rituals and investigated for their therapeutic potential in the mid-20th century [1–4]. However, the prohibition of psychedelic drugs in the late 1960s to 1970s has restricted research into their potential therapeutic benefits. Recently, there has been evidence to suggest that these compounds may have therapeutic potential for various mental health conditions such as depression, anxiety, and post-traumatic stress disorder (PTSD) [5,6]. Psychedelic compounds are classed as hallucinogenic drugs that, when ingested, influence psychological, visual, and auditory perception, often resulting in an altered state of mind [7]. These psychedelic hallucinogens are a subclass of psychoactive drugs, broadly defined as substances that affect the mind or behaviour. Psychoactive substances generally change or alter the function of the central nervous system, resulting

in changes in perception, mood, cognition, and behaviour. In many countries, psychoactive compounds are prohibited, with the exception of alcohol, caffeine, and nicotine, making the research of these drugs difficult. Consequently, there is a knowledge gap on the health effects of these compounds. Psychedelics are classified as prohibited drugs and the potential health benefits of psychedelics require more studies. Therefore, it is important to continue research on psychedelic and psychoactive compounds so that their therapeutic potential can be evaluated such that society may benefit from them.

N,*N*-dimethyltryptamine (DMT), 5-methoxy-N,*N*-dimethyltryptamine (5-MeO-DMT), as well as the harmala alkaloids, tetrahydroharmine (THH), harmaline, and harmine, are psychedelic and psychoactive compounds of interest. This is due to their ability to induce psychological, visual, and auditory changes, resulting in an altered state of consciousness. They are currently being evaluated in clinical trials for the therapeutic effects on post-traumatic stress disorder (PTSD) and major depressive disorder (MDD) [7,8]. DMT occurs naturally in plants and animals; however, it can also be produced synthetically. DMT levels in *Psychotria viridis* fluctuate depending on the time of the day, with the morning and late evening having the highest levels of DMT [9]. DMT, obtained from natural sources or produced synthetically, can be administered via inhalation, injection, and oral ingestion; however, the psychedelic effects are shortened from ingesting DMT without a monoamine oxidase inhibitor (MAOI) [10]. This combinatory approach prevents DMT deamination by the enzyme monoamine oxidase, which is found in the central nervous system (CNS), rendering it inactive [11]. The psychoactive effects of DMT are known to last up to 45 min in the human body [12] where DMT acts as an agonist for 5-HT (serotonin) receptors, G protein-coupled receptors (GPCRs), which bind to 5-HT, a natural neurotransmitter, which then triggers various physiological effects [13]. There are several classes of 5-HT receptors, 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7, each with different biological roles [13]. DMT acts upon 5-HT_{2A} receptors, where guanine nucleotide-binding proteins stimulate phospholipase C activity and release diacylglyerceol and inositol triphosphates, which causes the activation of protein kinase C and Ca^{2+} release. This results in neuronal excitability, synaptic plasticity and neurotransmitter release, resulting in psychoactivity [14].

5-MeO-DMT is a methoxylated derivative of DMT found in the plant *Anadenanthera peregrina* and various toad species [15]. It binds to 5-HT_2 and 5-HT_{1A} and inhibits monoamine reuptake, resulting in a prolonged psychedelic effect [16,17]. Studies have shown a potential benefit of using this derivative in the treatment of patients with anxiety, depression, post-traumatic stress disorder (PTSD), as well as growth in neural plasticity [15,18].

The harmala alkaloids are a class of indole alkaloids found in high concentrations within *Banisteriopsis caapi* plants and include harmine, harmaline, and THH. These psychoactive alkaloids function as monoamine oxidase inhibitors (MAOI), inhibiting monoamine oxidase A (MAO-A); however, they are not hallucinogenic. MAO-A is found in the CNS on the outer membrane of neuron mitochondria and breaks down monoamines including DMT and 5-MeO-DMT. MAOI inhibits monoamine breakdown thus prolonging psychoactivity [11]. South American indigenous peoples would combine *B. caapi* and *P. viridis* to create an ayahuasca brew which would induce psychedelic effects when consumed [9,19]. While there are some indicators of the therapeutic effects of harmine and harmaline individually, they are predominately used for their MAOI ability which enables DMT to be orally active. Clinical research on THH is sparse, but there is evidence that it acts as a serotonin reuptake inhibitor which may contribute to psychoactivity whilst its role as a MAOI is a weaker, secondary action [20]. Harmine has been shown to exhibit anti-cancer properties; however, more research is needed to fully understand its effects [21]. Meanwhile, harmaline has been reported to have antiviral effects, for example, against herpes [20].

The literature reports a range of techniques, such as High Performance Liquid Chromatography (HPLC), Direct Analysis in Real Time–High Resolution Mass Spectrometry (DART-HRMS), and Gas Chromatography Mass Spectroscopy (GCMS), to quantify DMT, harmine, harmaline, and THH concentration in *P. viridis*, *B. caapi*, and ayahuasca

brews [9,11,19,22–24]. Additionally, Liquid Chromatography Mass Spectroscopy (LCMS) has been used for the analysis of DMT in human plasma and urine samples for the purpose of drug testing [25–27]. Most reported analyses have focused on the ayahuasca brew or human plasma and urine [11,19,22,23]. There are studies that have investigated the plant matrices of the ayahuasca (starting material), as we do here; however, they do not provide a fully validated method which is evaluated for extraction efficiency, matrix effect, and recovery [9,24]. *Psychotria carthagenensis* was studied by Rivier and Lindgren (1972) [19], and they found that DMT was contained at 0.66% dry weight. *Alicia anisopetala* is a vine plant like *B. caapi* and is marketed as the "Black" ayahuasca according to online vendors; however, there is no literature on the analysis of any psychedelic compounds in this species.

Previous research on the quantitation of these psychoactive compounds in these plants used various analytical methods, but these were not fully validated [9,11,19,22–24]. For a method to be robust and reproducible, it should include the evaluation of extraction efficiency to ensure extraction methods are exhaustive, evaluate any potential matrix effects which may enhance or supress signals due to plant matrices, and evaluate compound recovery, which determines how much analyte can be recovered by the extraction process. In this paper, we have developed and fully validated a single LCMS method to quantify six psychedelics, DMT, 5-MeO-DMT, THH, harmine, harmaline, and tryptamine, in four plant species, *P. viridis*, *B. caapi.*, *P. carthagenensis,* and *A. anisopetala* leaf samples. Research is scarce in this area and given the increased interest in psychedelic compounds for therapeutic benefit, it is important to identify, quantitate, and chemotype plant species that produce psychoactive and psychedelic compounds. This will lead to a greater understanding of which species of South American native plants are the ideal sources of psychedelic compounds that are suitable for use in clinical trials to treat psychological disorders.

2. Materials and Methods

2.1. Reagents and Standards

All reagents, water in 0.1 formic acid (mobile phase A), acetonitrile 0.1% formic acid (mobile phase B), and methanol were of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). All standards were commercially purchased from Novachem Pty Ltd. (Heidelberg West, Melbourne, Victoria, Australia) as a distributor. N,*N*-dimethyltryptamine (DMT), N,*N*-dimethyltryptamine-D4 (DMT-D_4), 5-methoxy-N,*N*-dimethyltryptamine (5-MeO-DMT), and harmine-D_3 were supplied by Cerillant Corporation (Round Rock, TX, USA); tryptamine hydrochloride was supplied by HPC Standards GmbH (Am Wieseneck, Cunnesdorf, Germany); THH was supplied by LoGiCal GmbH (Im Biotechnologiepark Park, Luckenwalde, Germany); harmine was supplied by the National Measurement Institute (North Ryde, NSW, Australia), and harmaline was supplied by Cayman Chemical (Ann Arbor, MI, USA). Purity of standards were >98%, according to the individual certificates of analysis.

One mixed stock standard was prepared at 100 µg/mL DMT, DMT-D_4, 5-MeO-DMT, tryptamine, THH, harmine, harmine-D_3, and harmaline in 80:20 (volume/volume) methanol/water solution. The working standard concentrations were 0.001, 0.01, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 µg/mL, prepared as serial dilutions from the 100 µg/mL stock standard. All standards were stored at −80 °C in amber vials to prevent degradation, until they were required for analysis.

2.2. Plant Acquisition and Growth Conditions

All plant species used for method development and validation were commercially sourced. *Psychotria carthagenensis* variety (var.) Sameruca, *Banisteriopsis caapi* var. caupuri, and *Alicia anisopetala* (common name, Black Ayahuasca) were purchased from Herbalistics Pty Ltd. (Sunshine Coast, QLD, Australia). Plants were maintained in a Victoria government cultivation facility in controlled-environment rooms (CER). *P. carthagenensis*, *B. caapi*, and *A. anisopetala* were maintained in a CER with a relative humidity of 80%, temperature of 25 °C, and a 20:4 day-and-night cycle. Fresh leaves from *P. viridis* var. DW08, a backcross

between the *P. viridis* 'Nexus' and 'Shipbo' varieties, were purchased from Herbalisitics Pty Ltd. (Sunshine Coast, QLD, Australia).

2.3. Sample Preparation

A total of 2–3 g of fresh whole leaves (3–6 per plant) were sampled directly from the plant and flash frozen in liquid nitrogen. All leaf tissues were then then freeze dried for at least 48 h (Martin Christ Gefriertrocknungsanlagen GmbH Alpha 1–4 LD Plus (Osterode am Harz, Germany)) at −54 °C and to a pressure of 0.011 mbar, then weighed again to measure moisture loss and subsequently stored at −80 °C until processing.

2.4. Extraction Method

Samples were placed in liquid nitrogen for one minute and ground into a fine powder using a SPEX SamplePrep 2010 Geno Grinder (Metuchen, NJ, USA) for a total of three minutes at 1300 rpm in one-minute intervals. After grinding, 10.0 ± 0.2 mg of each sample was weighed into a 2 mL Axygen microtube and extracted with 1 mL of 80% methanol (*v*/*v*), vortexed for 5 min, sonicated for 5 min, and then centrifuged at 13,000 rpm (15.7× *g*) for 5 min. The supernatant was transferred to a 2 mL Axygen microtube, and the extraction process repeated once more. Supernatants from the first extraction and second extraction were combined and vortexed for 5 min. A total volume of 1 mL of the combined supernatant was transferred into a 1.5 mL amber HPLC vial and diluted 1 in 100 to ensure responses were within the quantitative range of the instrument. All plant extracts were replicated seven times to ensure repeatability. Both undiluted and diluted samples were used for the method validation.

2.5. Extraction Efficiency Method

Extraction efficiency was evaluated by weighing 10 ± 0.2 mg of plant material and following the extraction procedure outlined in Section 2.4. The aim was to repeat the extraction process (addition and removal of 1 mL 80:20 methanol), re-extracting the sample until all target psychedelic compounds were extracted. The extraction process was repeated four times in total and performed in triplicate, with each re-extraction analysed separately.

2.6. Pre-Extraction Spike Preparation

To determine analyte recovery (RE), samples (10.0 ± 0.2 mg) were extracted as detailed in Section 2.4; however, for the initial extraction step, the sample was spiked with of 50 µL of 10 µg/mL standard, and 950 µL of 80% methanol was added to make a final volume of 1 mL. The final concentration was 0.25 µg/mL for the low spike (LS). This method was repeated for the high spike (HS) using 20 µL of 100 µg/mL, giving a final concentration of 1 µg/mL. RE was calculated using the following formula [28–30], where the peak area response of the pre-extraction spiked sample is subtracted by the peak area response of the non-spiked sample, which is then compared with the response of the neat standard at the same concentration used for the spike:

$$\%\text{RE} = \frac{\text{spiked sample} - \text{no spike sample}}{\text{spike level}} \times 100 \quad (1)$$

2.7. Post-Extraction Spike Preparation

To determine the matrix effect (ME), samples were extracted as outlined in Section 2.4. The first and second extracts were combined and (400 µL) was transferred to a 1.5 mL amber HPLC vial and spiked with 50 µL of 2.5 µg/mL standard to produce a concentration of 0.25 µg/mL, LS. This process was repeated for the HS; 50 µL of 10 µg/mL for a final concentration of 1 µg/mL. Then, 100 µL of DMT-D_4 (5 µg/mL) and harmine-D_3 (2.5 µg/mL) were added as an internal standard (ISTD) to account for matrix effect. A further 1 in 100 dilution was performed to ensure that all compounds fit within the calibration curve. Final spike concentrations were 0.25 µg/mL (LS) and 1 µg/mL (HS). ME was calculated

using the following formula [28–30], where the peak area response of the pre-extraction spiked sample is subtracted by the peak area response of the non-spiked sample, which is then compared with the response of the neat standard at the same concentration used for the spike:

$$\%\text{ME} = \frac{\text{spiked sample} - \text{no spike sample}}{\text{spike level}} \times 100 \quad (2)$$

2.8. Instrumentation Parameters

Analysis was performed on a Vanquish Ultra-High Performance Liquid Chromatography (UHPLC) system (Thermo Fisher Scientific, Bremen, Germany) with a binary pump, autosampler, and temperature-controlled column compartment, coupled with a Q-Exactive (QE) Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) detector. The column compartment temperature was maintained at 30 °C and the autosampler was maintained at 15 °C. The data were acquired in positive ion mode with a mass range of 80–1200 m/z and with the resolution set to 35,000 and the AGC target set to 3×10^6 ions. The spray voltage was set to 3.6 kV. Nitrogen was used as the sheath, with auxiliary and sweep gas flow rates at 28, 15, and 4 L/min, respectively. Prior to data acquisition, a mass calibration was performed with a Pierce ESI Positive Ion Calibration Solution (Thermo Fisher Scientific). Mass spectrometry data were acquired using Thermo Xcalibur V. 3.2 software (Thermo Fisher Scientific Inc., Waltham, MA, USA). The column used was a Thermoscientific Hypersil GOLD C18 150 × 2.1 mm, 1.9 µm column (Thermo Fisher Scientific Inc., Waltham, MA, USA) with an injection volume of 3 µL. The mobile phase consisted of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The following gradient parameters were used: 2% B, 0 min; 35% B, 10 min; 100% B, 11–15 min; 2% B, 15–15.1 min; followed by equilibration to initial conditions at a flow rate of 0.3 mL/min. Blanks (80% methanol) were injected after every full range of prepared standards and after every 10 samples, with a quality control (QC) standard run after every 10 samples.

2.9. Data Processing

Thermofisher Xcalibur LCQuan Quantitative Software version 2.7 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for data processing, and calculation of R^2 values and equations. The calibration curve origin was fit through zero. The data were then exported into Microsoft Excel version 2408 (Microsoft Excel 365 Redmond, WA, USA) to calculate the limit of detection (LOD) and limit of quantitation (LOQ). LOD and LOQ were determined using the LINEST function, where a signal ratio of 3.3:1 from baseline was used for the LOD and a signal ratio of 10:1 from baseline was used for the LOQ.

3. Results and Discussion

3.1. Method Validation

Method validation was performed in accordance with guidelines outlined by Peters et al. (2007) [28], where the following parameters were evaluated: linearity, LOD, LOQ, accuracy, precision, repeatability, matrix effect (ME), and recovery (RE). The compounds eluted within nine minutes and insource fragmentation was observed for the following: tryptamine (144.0803 m/z); DMT-D_4 (148.1051 m/z); DMT (144.0803 m/z); 5-MeO-DMT (174.0909 m/z); and THH (200.1064 m/z and 188.1065 m/z) (Table 1). The mass spectra of each compound have been provided in Supplementary S1 and the elution profile in Supplementary S2. The total method runtime was 20 min, making this a rapid method for the high-throughput analysis of psychoactive compounds in these selected plant species.

Insource fragmentation was present, and the peak responses for each fragment ion and precursor ion for each respective compound were added together to obtain a total response. The reasoning for this was to eliminate any inconsistent precursor ion and fragment ion ratios between samples and standards. To clarify, the MS experiment only requires a precursor ion and retention time to be able to quantify the targeted compounds. Tryptamine and DMT share the same fragment ion (Table 1) but can be distinguished based on retention

time; therefore, both fragment peaks could be used for the integration of each compound separately. Harmine and harmaline coelute, however, can be individually quantitated based on their precursor ions $[M + H]^+$; no insource fragmentation was observed. DMT-D_4 and harmine-D_3 were included as internal standards (ISTD) for DMT, harmaline, and harmine.

Table 1. Compound retention time (RT), precursor ions, and fragment ions caused by insource fragmentation.

Compound	RT (min)	Precursor Ion (*m*/*z*) $[M + H]^+$	Fragment Ion 1 (*m*/*z*) $[M + H]^+$	Fragment Ion 2 (*m*/*z*) $[M + H]^+$
Tryptamine	5.81	161.1068	144.0803	N/A
DMT-D_4	6.41	193.1625	148.1051	N/A
DMT	6.45	189.1379	144.0803	N/A
5-MeO-DMT	6.64	219.1485	174.0909	N/A
THH	7.60	217.1328	200.1064	188.1065
Harmaline	8.45	215.1172	N/A	N/A
Harmine-D_3	8.48	216.1196	N/A	N/A
Harmine	8.53	213.1011	N/A	N/A

RT—Retention Time, min—minutes, *m*/*z*—mass-to-charge ratio, $[M + H]^+$—positively charged mass, N/A—not applicable, DMT-D_4; dimethyltryptamine-D_4, DMT; dimethyltryptamine, 5-MeO-DMT; 5-methoxy-N,*N*-dimethyltryptamine, THH; tetrahydroharmine.

3.2. Extraction Efficiency

Extraction efficiency was evaluated for all plants and target compounds (Table 2). Extraction efficiency is performed to ensure the extraction method is exhaustive, and hence the need to measure how many extractions are required until all analytes are extracted out of the plant sample and into solution for analysis. At least 87% of all target compounds were extracted in the first step, with two extractions improving the extraction efficiency to at least 98% for all target psychoactive compounds. Subsequent extraction steps did not provide sufficient improvement, and therefore a two-step extraction was adopted. Extractions marked NF were not found to have any compounds present at all during any stage of the extracts.

Table 2. Cumulative percentage of tryptamine, DMT, 5-MeO-DMT, THH, harmaline and harmine extracted via methanol extraction after each subsequent extraction step.

		Psychedelic Compounds (%)					
	Extraction No.	**Tryptamine**	**DMT**	**5-MeO-DMT**	**THH**	**Harmaline**	**Harmine**
Psychotria carthegenensis	1	94	NF	NF	NF	NF	NF
	2	99	NF	NF	NF	NF	NF
	3	100	NF	NF	NF	NF	NF
	4	100	NF	NF	NF	NF	NF
Banisteriopsis caapi	1	NF	NF	NF	99	87	90
	2	NF	NF	NF	100	98	98
	3	NF	NF	NF	100	99	100
	4	NF	NF	NF	100	100	100
Alicia anisopetala	1	NF	NF	NF	NF	NF	NF
	2	NF	NF	NF	NF	NF	NF
	3	NF	NF	NF	NF	NF	NF
	4	NF	NF	NF	NF	NF	NF
Psychotria viridis	1	91	91	NF	NF	NF	NF
	2	98	98	NF	NF	NF	NF
	3	100	99	NF	NF	NF	NF
	4	100	100	NF	NF	NF	NF

(NF) = No compound was detected in extracts. Not found.

3.3. Linearity, LOD, and LOQ

The calibration curves consisted of ten working standards prepared in methanol, with an $R^2 > 0.988$ for all compounds (Table 3). The LOD was determined to be 0.10 ng/mL for tryptamine; 0.08 ng/mL for DMT-D_4; 0.09 ng/mL for DMT; 0.11 ng/mL for 5-MeO-DMT; 0.06 ng/mL for THH; 0.09 ng/mL for harmaline; 0.06 ng/mL harmine-D_3; and 0.11 ng/mL for harmine. The LOQ was determined to be 0.31 ng/mL for tryptamine; 0.27 ng/mL for DMT-D_4; 0.27 ng/mL for DMT; 0.33 ng/mL 5-MeO-DMT; 0.18 ng/mL for THH; 0.29 ng/mL for harmaline; 0.20 ng/mL harmine-D_3; and 0.34 ng/mL for harmine. Chambers et al. (2020) [22] quantified DMT in psychoactive plants with a lower LOQ of 10,000 ng/mL. Santos et al. (2017) [23] used HPLC with LOD values at 7.5, 18.8, 11.6, 6.8, and 17.5 µg/mL for tryptamine, DMT, harmine, harmaline, and tetrahydroharmine, respectively, and LOQ values ranging from 20.6 to 57.1 µg/mL. Our method is several orders of magnitude more sensitive as our LOD values range from 0.06 ng/mL to 0.11 ng/mL and LOQ values range from 0.18 ng/mL to 0.34 ng/mL. Signal-to-noise ratio values have been included in Supplementary S3 for reference.

Table 3. Linearity (concentration range), correlation coefficient (equation), LOD and LOQ for tryptamine, DMT-D_4, DMT, 5-MeO-DMT, THH, harmaline, harmine-D_3, and harmine calibration and internal standards tested.

Compound	Concentration Range (ng/mL)	Equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
Tryptamine	1–10,000	$y = (5.33 \times 10^5)x$	0.999	0.10	0.31
DMT-D_4	1–10,000	$y = (4.84 \times 10^5)x$	0.990	0.08	0.25
DMT	1–10,000	$y = (5.15 \times 10^5)x$	0.991	0.09	0.27
5-MeO-DMT	1–10,000	$y = (6.83 \times 10^5)x$	0.993	0.11	0.33
THH	1–10,000	$y = (6.60 \times 10^5)x$	0.999	0.06	0.18
Harmaline	1–10,000	$y = (1.01 \times 10^6)x$	0.991	0.09	0.29
Harmine-D_3	1–10,000	$y = (9.58 \times 10^5)x$	0.989	0.06	0.20
Harmine	1–10,000	$y = (9.95 \times 10^5)x$	0.988	0.11	0.34

3.4. Accuracy and Precision

Accuracy and precision of the method was determined by calculating the mean and percent relative standard deviation (RSD) of seven repeat injections of the standards and seven independent extracts of each plant. Extracted ion chromatograms of analysed plant samples can be found in Supplementary S4. Repeat injection of the 0.25, 1, and 5 µg/mL standards resulted in an RSD of <2.5 for all analytes across the concentration range (Table 4). Mean concentration for each compound was determined for each species, with the RSD range between 2.86% and 4.83% (Table 5). *P. carthagenensis* contained 0.061 mg/g of tryptamine (RSD 4.1%). In *B. caapi*, THH and harmine were present in high levels at 7.7 mg/g (RSD 4.2%) and 11.4 mg/g (RSD 3.13%), respectively. *B. caapi* also contained 0.69 mg/g (RSD 2.86%) of harmaline, tryptamine, and DMT were below LOD for the method. *P. viridis* contained 0.34 mg/g (RSD 4.83%) tryptamine, and 18.2 mg/g of DMT (RSD 3.47%). 5-MeO-DMT was not detected in any species analysed. The inter-day precision for the standard solution injections was <4% RSD. The inter-day precision for the plant extracted samples was <5% RSD (Supplementary S5), emphasising reproducibility.

Many studies [22,31–33] tend to combine *B. caapi* into an ayahuasca mixture with *P. viridis* or another DMT-containing plant species such as *Mimosa hostilis* or *Diplopterys cabrerana.* The mixture of these plants involves a brewing period of several hours where the plants are exposed to heat. However, the metabolic profile of the combined extract of both plants is only inferred, and an accurate representation of the metabolic profile of *B. caapi* cannot be obtained; *B. caapi* must therefore be analysed on its own. When compared to the previous published data [9,11], DMT levels in *P. viridis* were higher in the current study compared to the mean average found in Callaway et al. (2005) [9]. Callaway et al. (2005) [9] quantified harmala alkaloids in *B. caapi* plants using HPLC and reported mean

concentrations of 4.83 mg/g for harmine, 0.46 mg/g for harmaline, and 1 mg/g for THH. Callaway's method used an extraction solvent mixture consisting of 67% methanol, 11% acetonitrile, and 22% 0.1 M ammonium acetate at pH 8.0, as opposed to the current study using only 80% methanol and 20% water. The current study found higher levels of harmine, harmaline, and THH, possibly because the extraction method illustrated by Callaway was not fully exhaustive, and their method used 100 mg of dried *B. caapi* material in 2 mL of solvent, which is ten times greater in concentration of mass per volume compared that of our study, where we used 10 mg in 2 mL of solvent (two extractions). Furthermore, one extraction was not sufficient in extracting all the compounds found in *B. caapi*, where only 87% of harmaline and 90% of harmine was extracted. Meanwhile, the current method was evaluated for extraction efficiency, with 90 to 100% of the target compounds successfully extracted. It is also possible that the observed differences are due to the nature of the specific plant material used in the different studies.

Table 4. Relative standard deviation (RSD) values (%) for tryptamine, DMT, 5-MeO-DMT, THH, harmaline, and harmine in standard solution at 0.25, 1, and 5 µg/mL calculated from seven replicated injections.

Compounds	0.25 µg/mL	1 µg/mL	5 µg/mL
Tryptamine	2.39	1.39	1.48
DMT-D_4	0.50	0.95	0.69
DMT	1.99	0.98	0.97
5-MeO-DMT	1.09	1.36	1.09
THH	1.46	1.67	1.05
Harmaline	1.99	2.27	2.32
Harmine-D_3	1.08	1.37	1.49
Harmine	0.82	1.58	1.27

Table 5. Concentration of each endogenous compound for four South American plant species.

	Psychotria carthagenensis		*Banisteriopsis caapi*		*Alicia anisopetala*		*Psychotria viridis*	
Compounds	**Conc. (mg/g)**	**RSD (%)**	**Conc. (mg/g)**	**RSD (%)**	**Conc. (mg/g)**	**RSD (%)**	**Conc. (mg/g)**	**RSD (%)**
Tryptamine	0.061	4.41	NF	N/A	NF	N/A	0.34	4.83
DMT	NF	N/A	NF	N/A	NF	N/A	18.2	3.47
5-MeO-DMT	NF	N/A	NF	N/A	NF	N/A	NF	N/A
THH	NF	N/A	7.7	4.20	NF	N/A	NF	N/A
Harmaline	NF	N/A	0.69	2.86	NF	N/A	NF	N/A
Harmine	NF	N/A	11.4	3.13	NF	N/A	NF	N/A

NF—Not Found. N/A—Not Applicable. RSD (%) was calculated from seven replicated extracts of each respective plant.

The current study analysed fresh leaves and, although they were flash frozen to avoid degradation, it would be of interest to apply this method to the stems as they are traditionally used in the ayahuasca brew. To our knowledge, *A. anisopetala* has no published data regarding its metabolic profile. Despite this, it has been named the 'Black ayahuasca'. However, no psychedelic alkaloids of interest or tryptamine were detected in our samples. Despite Rivier and Lindgren's (1973) [19] findings of 0.66% *w*/*w* of DMT in *P. carthagenensis* leaves, the current study found no traces of DMT or any other psychedelics in the leaves; perhaps the samples used in Rivier and Lindgren's study were collected from a more mature *P. carthagenensis* plant. It was speculated by the authors that 5-MeO-DMT may be present in either of the two *Psychotria* species, similar to *A. peregrina* which contained both DMT and 5-MeO-DMT [34], suggesting a shared metabolic pathway; however, 5-MeO-DMT was not found in any of the four targeted plant species included in this study. To the best of our knowledge, the current work is the only method that provides quantitative

data for tryptamine, DMT, 5-MeO-DMT, THH, harmaline, and harmine in the four selected plant species whilst providing robust and accurate RSD values below 5%. The current work may be applied to other plant tissues such as stems and roots, considering that a further validation method may be needed as these are different plant tissues.

3.5. Matrix Effect

Working standards were used for post-extraction spikes and performed to evaluate the potential matrix effect at concentrations of 0.25 (LS) and 1 µg/mL (HS). Matrix effect is defined as any ion suppression or enhancement of the target compounds in the presence of other compounds in the sample matrix. Spike concentrations were chosen at 0.25 and 1 µg/mL, similar to the concentration levels of the psychoactive compounds found endogenously in these selected plants. Additionally, the medicinal agriculture industry is unlikely to be interested in lower-level concentrations, hence a lower spike concentration was not selected.

Generally, ion suppression was observed (Table 6). Matrix effect values ranged from 72.1% to 111.4% across all compounds and plant species. Post-extraction spikes were performed in triplicate, resulting in RSD values less than 3% (Supplementary S6). Post-extraction spikes were performed on samples which were diluted 1 in 100 *B. caapi* and *P. viridis,* as denoted by an asterisk (*). The species with the highest amount of ion suppression overall was *P. carthagenensis*.

Table 6. Post-extraction spikes for matrix effect values (%) in four South American plant species.

	Psychotria carthagenensis		***Banisteriopsis caapi* (*)**		***Alicia anisopetala***		***Psychotria viridis* (*)**	
Compounds	**LS**	**HS**	**LS**	**HS**	**LS**	**HS**	**LS**	**HS**
Tryptamine	87.4	85.4	74.8	81.6	109.6	95.8	77.2	82.9
DMT-D_4	87.2	96.8	99.8	102.1	95.1	104.6	104.5	111.4
DMT	81.9	91.0	93.8	95.9	89.4	98.3	98.2	104.7
5-MeO-DMT	94.0	92.0	84.6	94.0	101.2	99.6	88.2	95.2
THH	88.8	85.8	95.9	98.1	94.3	93.5	90.6	95.1
Harmaline	85.1	84.9	95.5	99.3	72.1	76.8	98.5	101.6
Harmine-D_3	80.2	84.2	91.6	102.4	92.2	96.9	106.6	110.5
Harmine	77.2	81.0	88.2	98.6	88.7	93.3	102.6	106.4

LS—Low Spike (0.25 µg/mL); HS—High Spike (1 µg/mL). (*)—post-extraction spike samples were performed on samples diluted 1 in 100.

Callaway et al. (2005) [9] and McKenna et al. (1984) [11] quantified DMT in *P. viridis* leaves but did not report any matrix effect which may have influenced the results. The method reported here has resolved this and has utilised ISTD in the form of their isotopically labelled deuterated counterparts to account for this matrix effect. Ion suppression was observed in most plant species, likely due to the presence of non-targeted endogenous compounds co-eluting in the sample. Deuterated ISTDs were used to account for any observed matrix effects. DMT-D_4, served as an ISTD for DMT, where little to no ion suppression was found in *P. viridis*, a plant which contains large endogenous levels of DMT. Harmine-D_3 was used as an ISTD for harmine and harmaline. Using the response factor from DMT-D_4 and DMT, we can recalculate the endogenous concentration of DMT in Table 5 and adjust for the matrix effect, resulting in a concentration of 19.4 mg/g. Using the same method, we will use harmine-D_3 response factors for harmine and harmaline, where the endogenous concentrations are 11.8 mg/g and 0.73 mg/g. *B. caapi* is only plant in this study which contains endogenous levels of harmine and harmaline, where the LS had an ion suppression of 88.2% but the HS had almost no ion suppression with a value of 98.6% for harmine. Harmaline had an ion suppression of 95.5% for the LS but no ion suppression with a value of 99.3% for the HS. THH was found in significant amounts in *B. caapi* where more than 95% of the spike was detected at the LS and HS. 5-MeO-DMT

was not found endogenously in any analysed samples. Recent studies [22–24,31–33] have not investigated or provided information on any matrix effect in plant matrix samples or accounted for any of these effects. It is important to include an ISTD for any quantitative analysis, as the plant matrix can influence results [35,36].

3.6. Recovery

Pre-extraction standard spikes were performed to evaluate recovery at the final concentrations of 0.25 (LS) and 1 µg/mL (HS). Recovery values are calculated using pre-extraction spikes, where a predetermined amount of analyte is added before the extraction step, and any fluctuation in response is recorded. Recovery values were adjusted for any matrix effect (Table 7) and ranged from 74.1 to 111.6% across all the compounds and psychedelic plants. Non-adjusted recovery values can be found in Supplementary S7. Pre-extraction spikes were performed in triplicate with RSD values of less than 5% (Supplementary S8). Pre-extraction spikes of THH and harmine on *B. caapi* and DMT on *P. viridis* were above the linear concentration range due to high endogenous concentrations in each species and have been defined as such.

Table 7. Pre-extraction spikes for the recovery values (%) in four South American plant species adjusted for matrix effect.

	Psychotria carthagenensis		***Banisteriopsis caapi***		***Alica ansiopetala***		***Psychotria viridis***	
Compounds	**LS**	**HS**	**LS**	**HS**	**LS**	**HS**	**LS**	**HS**
Tryptamine	96.9	96.5	91.3	93.4	84.1	89.1	111.6	89.6
DMT	108.8	96.7	109.1	92.5	100.4	81.6	ND	ND
5-MeO-DMT	107.8	100.9	110.2	98.4	91.8	85.9	85.3	84.5
THH	105.9	101.2	ND	ND	77.6	74.1	86.7	86.0
Harmaline	108.0	98.1	94.8	86.1	96.7	83.3	95.4	87.9
Harmine	110.6	99.6	ND	ND	90.4	88.7	97.4	91.2

LS—Low Spike (0.25 µg/mL); HS—High Spike (1 µg/mL); ND—Not determined; above the linear concentration range. Original recovery values can be found in Supplementary S7.

Recent studies [22–24,31–33] have not provided information on the recovery of the extraction methods used. Although this criterion is out of the scope of the studies, it is required to provide a full validation of the analytical method. Although recovery values vary, most values are between 80 and 100% (Table 7); the extraction efficiency studies (Table 2) indicate that >98% of each compound was captured.

4. Conclusions

This study reports on the first fully validated method for quantifying six psychoactive compounds, tryptamine, DMT, 5-MeO-DMT, THH, harmine and harmaline, in *P. carthagenensis*, *P. viridis*, *B. caapi* and *A. anisopetala*, using LC--MS. There are no previous studies describing a fully validated method which determines extraction efficiency, matrix effect, and analyte recoveries. Evaluating the extraction efficiency, matrix effect and analyte recoveries are essential for accurate quantitation and has often been overlooked in studies to date. Additionally, the use of deuterated internal standards accounts for any matrix effect, resulting in a highly accurate, reliable, and high-throughput method that can be applied to other plant tissues containing psychoactive compounds. Having a fully validated method for quantifying compounds to ensure accurate, reliable, and credible results in both research and industry settings underpins the scientific integrity and product quality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/psychoactives3040032/s1, Figure S1: Mass spectra of analyzed compounds; Figure S2: The extracted ion chromatogram LCMS elution profile of 1 µg/mL standard concentration; Table S3: Signal-to-noise ratio values of analyzed compounds at concentration; Figure S4: Chro-

matograms of four analyzed plant samples; Table S5: The relative standard deviation values (%) for targeted standard compounds in neat and plant samples over an inter-period of seven days; Table S6: The percentage relative standard deviation of the post-extraction spike samples in four South American plant species for eight compounds; Table S7: Pre-extraction spikes for the recovery values (%) in four South American plant species; Table S8: The percentage relative standard deviation of the pre-extraction spike samples in four South American plant species for six compounds.

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