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Application of ambient ionization high resolution mass spectrometry to determination of the botanical provenance of the constituents of psychoactive drug mixtures

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ABSTRACT

A continuing challenge in analytical chemistry is species-level determination of the constituents of mixtures that are made of a combination of plant species. There is an added urgency to identify components in botanical mixtures that have mind altering properties, due to the increasing global abuse of combinations of such plants. Here we demonstrate the proof of principle that ambient ionization mass spectrometry, namely direct analysis in real time-high resolution mass spectrometry (DART-HRMS), and statistical analysis tools can be used to rapidly determine the individual components within a psychoactive brew (Ayahuasca) made from a mixture of botanicals. Five plant species used in Ayahuasca preparations were subjected to DART-HRMS analysis. The chemical fingerprint of each was reproducible but unique, thus enabling discrimination between them. The presence of important biomarkers, including *N*,*N*-dimethyltryptamine, harmaline and harmine, was confirmed using in-source collision-induced dissociation (CID). Six Ayahuasca brews made from combinations of various plant species were shown to possess a high level of similarity, despite having been made from different constituents. Nevertheless, the application of principal component analysis (PCA) was useful in distinguishing between each of the brews based on the botanical species used in the preparations. From a training set based on 900 individual analyses, three principal components covered 86.38% of the variance, and the leave-one-out cross validation was 98.88%. This is the first report of ambient ionization MS being successfully used for determination of the individual components of plant mixtures.

1. Introduction

Despite continuing advances in separations science, the ability to accurately determine the originating constituents of compounded mixtures of complex plant matrices remains a challenge in analytical chemistry. One area that would benefit significantly from progress in this regard is forensics, where knowledge of the component species of psychoactive brews made from multiple plants can be of critical importance to law enforcement, toxicologists and medical practitioners treating patients who have overdosed on psychoactive substances. The United Nations Office on Drugs and Crime (UNODC) has reported an alarming and exponential rise in the use of unscheduled psychoactive plant substances. This increase has been attributed to multiple factors including the desire by users to: (1) experience mind-altering effects without fear of prosecution from violation of current drug laws; (2) intensify the effects of more mainstream prescription and/or illicit drugs; and (3) have "spiritual" or "supernatural" experiences. Many users also have the perception that plant-based psychoactive substances are safer than scheduled substances because they are "natural." These widely held perspectives belie the dangers associated with consumption of these products. The use of these botanical psychotropic products has been implicated in multiple poisonings and fatalities [1–8].

Although inroads are being made into the identification of abused substances derived from single plant species,[9,10] a major challenge that confronts efforts to curtail the use of plant psychoactives is the absence of suitable analytical methods to accurately identify compounded plant-based products. Prescription and illicit drugs, as well as synthetic cathinones and cannabinoid products, are generally comprised of pure or semi-purified components that can be identified by conventional analytical techniques. However, the complex matrices associated with whole plant materials or concoctions made from them, make

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definitive identification of the constituents very difficult. Since scheduling of mind-altering products requires the ability to identify the outlawed substance, and since methods for identification are by and large non-existent for plant-based psychotropics (except in the cases of cannabis, peyote, opium and a few others), law enforcement has been rendered powerless to intervene. Furthermore, because it is often impossible to determine the identity of ingested mind-altering plants in cases of drug overdoses, appropriate life-saving protocols cannot be administered.

One well-known hallucinogenic beverage of South American origin is Ayahuasca, also commonly known as yage, hoasca or caapi [11–16]. Its consumption is an important part of some South American religious ceremonies and it is purported to cause users to experience visions and clairvoyance. Although its use among some groups in ceremonial religious contexts is legal, the appeal of Ayahuasca as a recreational drug is increasing in the United States and Europe. However, despite its legal status, the indiscriminate consumption of Ayahuasca and the plants used to make the brew has been implicated in poisonings [2,6,17–19]. It has also been found in clandestine laboratories where more mainstream drugs of abuse are manufactured [20–22]. When observed in the context of crime scene investigations, psychoactive plant substances or brews are not easily identifiable, particularly in cases where varying combinations of plant materials are used to make the concoctions, as is the case with Ayahuasca. For this reason, it is important to be able to determine the plant species from which the brews are made so that the identity of the mind-altering compounds can be known and appropriate treatment protocols for overdoses can be applied.

The hallucinogenic effects of Ayahuasca are derived from *N*,*N*-dimethyltryptamine (DMT) which is extracted from botanical material and concentrated during the brewing process. DMT-containing plants commonly used in preparation of the brew include *Psychotria viridis* (Chacruna), *Mimosa hostilis* (Jurema) and *Diplopterys cabrerana* (Chaliponga) [12,23–25]. The effects of oral administration of DMT are minimal because of the action of monoamine oxidase (MAO) enzymes which de-aminate the compound to form non-psychoactive derivatives. For this reason, the psychoactive effects of orally ingested DMT require concurrent consumption of an MAO inhibitor (MAOI). Plant-derived β-carboline alkaloids such as harmine, harmaline and tetrahydroharmine (Fig. 1) [16,18,24,26–28] serve this purpose. Thus, Ayahuasca brews contain plant combinations that contribute both DMT and β-carbolines [23,25,28–30]. *Banisteriopsis caapi* (yage) and *Peganum harmala* (Syrian rue) are the two most common β-carboline-containing plants used in these preparations. With inhibition of the MAOs by the harmala alkaloids, the effects of DMT, which include intense visual hallucinations, auditory distortions and an altered sense of being, occur. These experiences are attributed to DMT-promoted 5-HT_{2A}, 5-HT_{1A}, and 5-HT_{2c} serotonergic receptor agonism [28,31]. Peak effects occur rapidly and diminish within 30 min, without the extended period of mind-altering effects that occur with other hallucinogens [32].

While DMT is a Schedule I substance in the United States and under international law, Ayahuasca brews and the plant materials from which the psychoactive compounds are derived remain in a legal grey area. Plants containing scheduled compounds or infusions made from them are not included in the 1971 Convention of Psychotropic Substances, and in the United States, only marijuana, peyote and psilocybin mushrooms are specifically scheduled plant and fungal materials [33,34]. Because the presence of identification protocols is a necessary prerequisite to the legislation of mind-altering materials, the development of methods to accurately confirm the identity of plant-based drugs of abuse is of high concern, as highlighted in the 2009 National Academy of Science report on the status of forensic sciences in the United States [35]. The characterization of Ayahuasca or its plant constituents in a forensic context is mainly limited to the detection of DMT in the brew or botanical material. Conventional time intensive protocols utilizing chromatographic or hyphenated methods such as HPLC-fluorescence,[36] gas chromatography (GC)– or liquid chromatography–mass spectrometry (LC–MS) have been used to isolate and identify DMT and harmala alkaloids in Ayahuasca [30,37–41]. Typically, plant samples or Ayahuasca brews are subjected to liquid-liquid or Soxhlet extraction, which consume large amounts of solvent prior to analysis by HPLC or LC [41]. Sample preparation for GC methods can be even more cumbersome because aqueous samples cannot be analyzed directly. Furthermore, there are no standard operating protocols for the analysis of the plant materials used for Ayahuasca, including *P. viridis*, *M. hostilis*, *D. cabrerana*, *B. caapi* and *P. harmala*. Intensive method development is also needed to optimize chromatographic separation of the components in the brew, which adds to the overall analysis time. For unscheduled substances such as Ayahuasca brews and the psychoactive plant materials used for the concoction, it is often not worth the investment of time or resources to conduct the analysis, especially in light of the casework backlogs faced by forensic laboratories [42].

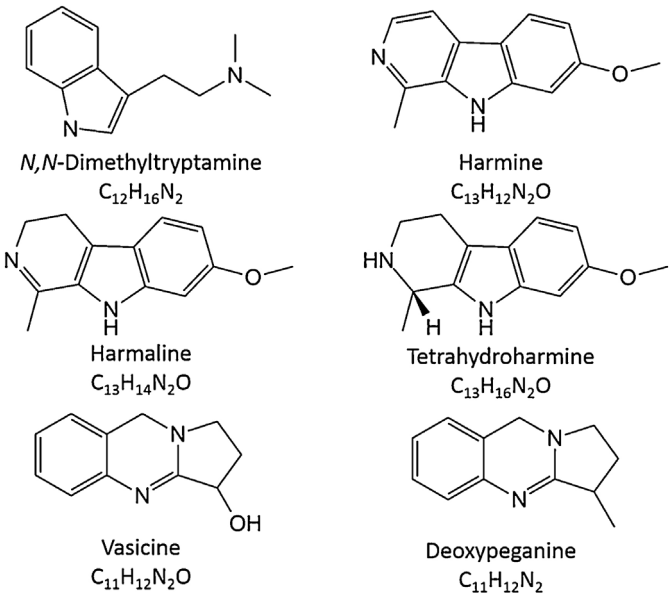

Fig. 1. Psychoactive alkaloid *N*,*N*-dimethyltryptamine (DMT) and β-carboline MAOIs reported to be found in Ayahuasca brews.

The advent of ambient ionization mass spectrometry, and direct analysis in real time-high resolution mass spectrometry (DART-HRMS) in particular, has allowed analysts to circumvent some of the aforementioned difficulties in identifying forensic material including botanical or plant-based substances. Solids, liquids and gases can be easily analyzed using DART-HRMS without the need for lengthy sample preparation steps such as extractions or derivatization [43,44]. It has been demonstrated previously that forensically relevant plant material, including seeds, leaves, and wood, can be analyzed for detection of biomarkers, and that the chemical fingerprint produced can provide species-level discrimination and identification of mind-altering plant substances using statistical analysis tools [9,10,45]. However, this method has not been successfully applied to compounded mixtures comprised of complex matrices. We surmised that DART-HRMS might be an ideal method for the characterization and identification of Ayahuasca brew components. Unlike the case with GC–MS, it can be used for direct analysis of aqueous samples and no

extraction steps, filtrations, pH adjustments, derivatizations or other sample processing steps are required. Furthermore, the individual botanical species of which the brew is comprised can be sampled in the same manner, which permits utilization of the chemical fingerprint of each for identification of the plant species within the brew. The coupling of the DART-HRMS data with statistical analysis processing might then furnish accurate mixture component information, a determination that is impractical, if not impossible, using chromatographic or hyphenated methods, due to the time investment required to obtain the large datasets needed for validation.

To test the proof of principle that chemometric processing of DART-HRMS derived chemical profiles of mixtures of plant products might enable plant constituent determination, we applied the method to Ayahuasca concoctions. In-source collision-induced dissociation was used to confirm the presence of DMT in *P. viridis*, *M. hostilis* and *D. cabrerana*, and the two harmala alkaloids harmine and harmaline were confirmed in *B. caapi* and *P. harmala.* Principal component analysis (PCA) was successfully applied to the DART-HRMS spectra of Ayahuasca brews made following 6 different recipes, and the plant species of which each brew was comprised were rapidly and accurately determined with >98% confidence.

2. Experimental

2.1. Botanical materials

Banisteriopsis caapi shredded vine and *Peganum harmala* seeds were purchased from World Seed Supply (Mastic Beach, NY, USA). *Mimosa hostilis* root bark and *Psychotria viridis* dried leaves were purchased from Mr. Botanicals (mrbotanicals.com, Netherlands). *Diplopterys cabrerana* leaves were purchased from Herbal Flame (Hollywood, FL, USA).

2.2. Chemical standards

N,*N*-Dimethyltryptamine was purchased from Cerilliant Corporation (Round Rock, TX, USA). Harmaline, harmine, and vasicine standards were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.3. Ayahuasca brew preparation

Ayahuasca brews were made according to published protocols [24] that were scaled down to make smaller batches (using approximately 30 g of plant material). The first Ayahuasca brew was made using 15 g of *B. caapi* vine and 15 g of *P. viridis* leaves that were pulverized using a Hamilton Beach coffee grinder (Glen Allen, VA, USA). The mixture was refluxed in 250 mL of water with 5 mL of lemon juice (Sicilia brand, Clifton, NJ, USA) for 3 h. The liquid was passed through a coffee filter and the filtrate was analyzed by DART-HRMS. The remaining residue was refluxed in another 250 mL aliquot of water with 5 mL of lemon juice for 3 h. The suspension was again filtered and the filtrate analyzed by DART-HRMS. The two filtrate fractions were then pooled and again analyzed by DART-HRMS. All other Ayahuasca brews were made following the protocol described above, but using the following plant material combinations: Ayahuasca brew 2 was prepared with 24 g of *M. hostilis* root bark and 6 g of *P. harmala* seeds that were ground; Ayahuasca brew 3 was made using 24 g of *P. viridis* leaves and 4 g of *P. harmala* seeds; Ayahuasca brew 4 consisted of 25 g of *B. caapi* vine and 7.5 g of *M. hostilis* root bark; the fifth and sixth Ayahuasca brews were made using 7 g of *D. cabrerana* leaves and 20 g of *B. caapi* vine, and 24 g of *D. cabrerana* leaves and 4 g of *P. harmala* seeds, respectively. For ease of reference, the brew compositions are listed in Table 1.

Table 1
Botanical species composition of six Ayahuasca brews.

	DMT-contributing species	Harmala alkaloid-contributing species
Brew 1	*P. viridis*	*B. caapi*
Brew 2	*M. hostilis*	*P. harmala*
Brew 3	*P. viridis*	*P. harmala*
Brew 4	*M. hostilis*	*B. caapi*
Brew 5	*D. cabrerana*	*B. caapi*
Brew 6	*D. cabrerana*	*P. harmala*

2.4. Mass spectral data collection and analysis

Mass spectra were acquired using a DART-SVP ion source (IonSense, Saugus, MA, USA) coupled to a JEOL AccuTOF high resolution time-of-flight mass spectrometer (JEOL USA, Peabody, MA, USA) in positive-ion mode. The parameters of the DART ion source were: grid voltage, 250 V; and gas heater temperature, 350 °C. The mass spectrometer settings were: ring lens voltage, 5 V; orifice 1 voltage, 20 V; orifice 2 voltage, 5 V; and RF ion guide ("peaks") voltage, 600 V. Mass spectra were acquired over the m/z range 60–800 at a spectral acquisition rate of 1 spectrum per sec. The helium flow rate for the DART ion source was 2.0 L s^{-1}. The resolving power of the mass spectrometer was 6000 FWHM.

In-source collision-induced dissociation (CID) can be used for structural confirmation of compounds in the plant materials through comparison of in-source CID spectra of authentic standards to those of the plant material obtained under identical conditions. For structural confirmation of DMT, harmaline and harmine, in-source CID was performed on plant material and standards using "function switching," whereby the orifice 1 voltage of the mass spectrometer was varied from 20 V to 30, 60, and 90 V to induce fragmentation of the compounds within a single analysis. The RF ion guide ("peaks") voltage was set to 400 V for CID analysis and the mass range was set to m/z 40–800. All other ion source and mass spectrometer parameters were as described above.

The *P. viridis* and *D. cabrerana* leaves were tested directly by grasping the material with tweezers and suspending it between the ion source and mass spectrometer inlet. The *M. hostilis* root bark and *B. caapi* vine were sampled in the same manner. The *P. harmala* seeds were sliced in half transversely and then held with tweezers between the ion source and inlet of the mass spectrometer. Five analyses were averaged in each spectrum and five replicates were obtained for each species. DMT, harmine and harmaline were sampled by dipping the closed end of a melting point capillary tube into the standard and presenting the coated surface of the tube to the space between the DART ion source and mass spectrometer inlet.

The Ayahuasca brews were analyzed by inserting 10 DipIt® tip capillary tubes (IonSense, Saugus, MA, USA) into the liquid and then mounting the tips into the 12-unit sample holder on a linear rail system (IonSense, Saugus, MA, USA) that moved laterally from left to right between the ion source and the mass spectrometer inlet at a rate of 1.0 mm/s. This sampling process provided reproducible, high-throughput analysis of the Ayahuasca brews. Five analyses were averaged for each spectrum and thirty spectra were collected for each of the Ayahuasca brews. In each case, ten spectra were collected from the first fraction, ten from the second fraction and ten from the combined fraction, resulting in 900 individual analyses.

2.5. Mass spectral data processing

Calibration of all spectra was performed using polyethylene glycol (PEG) 600. Spectral averaging, calibration, background

subtraction and peak centroiding were performed using TSSPro3 (Shrader Analytical Labs, Detroit, MI, USA) data processing software. Mass Mountaineer software (RBC Software, mass-spec-software.com) was used for mass spectral analysis, elemental composition determination, isotopic analysis and principal component analysis.

3. Results

3.1. DART-HRMS analysis of Ayahuasca botanical components

The botanical species used in the making of Ayahuasca brews contain compounds that are known to be either psychoactive (e.g. DMT) or serve as MAOIs (e.g. harmaline and harmine). To determine the presence of these compounds in the plant materials, five different botanical species were purchased from various internet vendors and the plant materials were subjected to DART-HRMS analysis. The results of the analyses are shown in Fig. 2, with the corresponding mass measurement data presented in Table 2. In each case, an average of five spectra is shown and each peak in the spectrum represents a unique protonated compound.

Panels a, c and e are representative spectra of *P. viridis*, *M. hostilis* and *D. cabrerana*, respectively, plants that are reported to contain the psychoactive alkaloid DMT. In each case, a peak at nominal mass *m*/*z* 189 was detected, consistent with the presence of protonated DMT ($C_{12}H_{16}N_2 + H^+$). *N*-Methyltryptamine ($C_{11}H_{14}N_2 + H^+$) at nominal *m*/*z* 175 was also detected in the *P. viridis*, *M. hostilis* and *D. cabrerana* botanical samples. In each case, the peak at nominal *m*/*z* 189 was the most abundant in the spectrum at 100% relative intensity, and the relative intensity of *N*-methyltryptamine varied between 0.4 and 9.8%. In the analysis of the harmala alkaloid-containing plants *B. caapi* and *P. harmala*, masses consistent with previously reported compounds were detected [25,26,29,30,40,46]. In the *B. caapi* shredded vine, peaks at nominal *m*/*z* 199, 213, 215 and 217 corresponding to harmol ($C_{12}H_{10}N_2O + H^+$), harmine ($C_{13}H_{12}N_2O + H^+$), harmaline ($C_{13}H_{14}N_2O + H^+$) and tetrahydroharmine ($C_{13}H_{16}N_2O + H^+$), respectively were identified, with *m*/*z* 213 presenting as the base peak. Masses consistent with harmine ($C_{13}H_{12}N_2O + H^+$, *m*/*z*

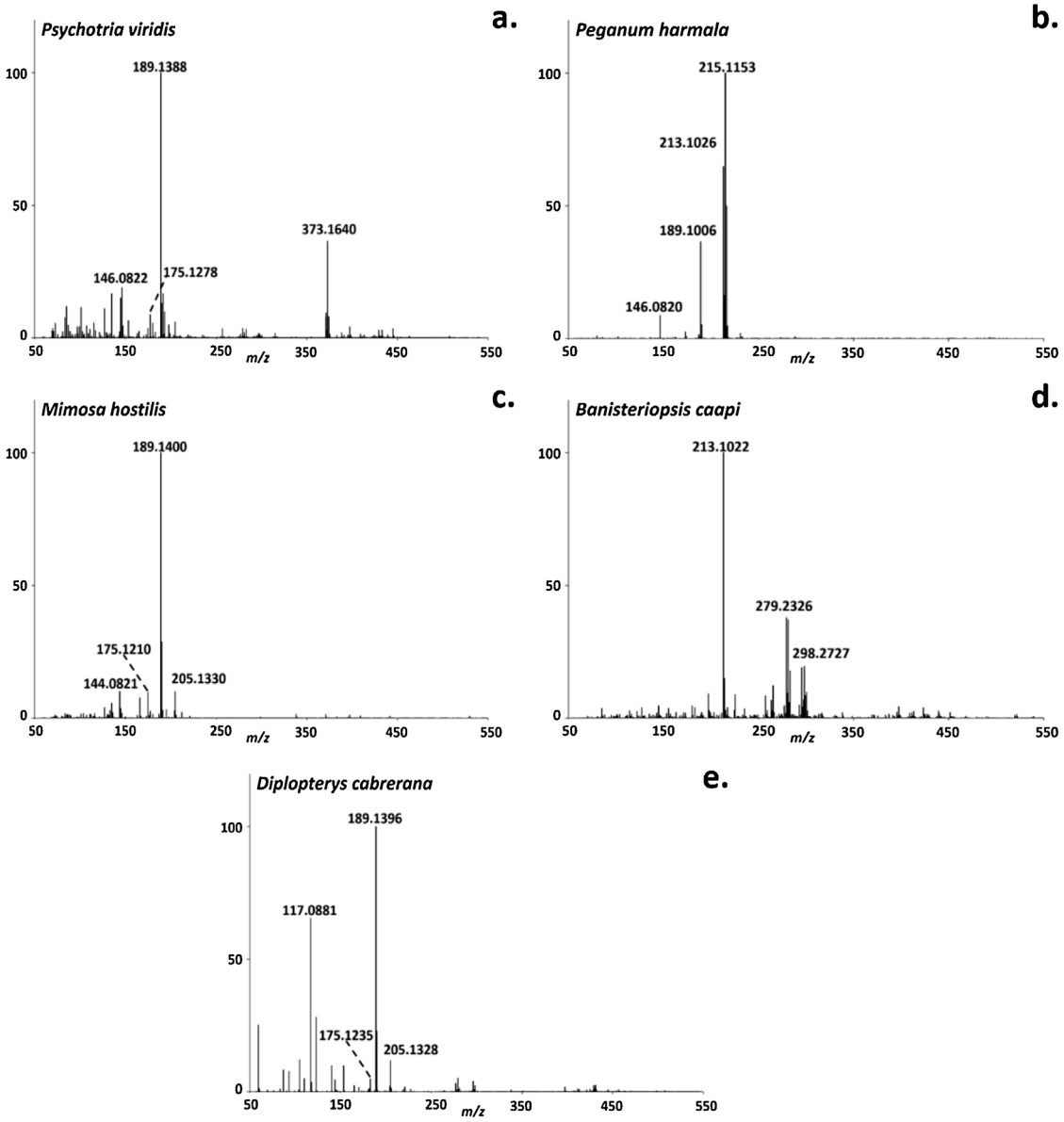

Fig. 2. DART-HRMS soft ionization spectra of plant materials commonly used in Ayahuasca preparations. Panel a: *P. viridis;* Panel b: *P. harmala*; Panel c: *M. hostilis*; Panel d: *B. caapi*; Panel e: *D. cabrerana*. The mass measurement data associated with these spectra are shown in Table 2.

Table 2
Mass measurement data of Ayahuasca brew botanical components at 20 V. The corresponding spectra are shown in Fig. 2.

Product	Compound	Formula	Measured	Calculated	Diff.[†]	Rel. Int.[‡]
Psychotria viridis	*N*-Methyltryptamine[a]	$C_{11}H_{14}N_2+H^+$	175.1278	175.1235	−4.3	1.6
	Dimethyltryptamine[b]	$C_{12}H_{16}N_2+H^+$	189.1388	189.1392	0.4	100.0
Mimosa hostilis	*N*-Methyltryptamine[a]	$C_{11}H_{14}N_2+H^+$	175.1210	175.1235	2.5	9.8
	Dimethyltryptamine[b]	$C_{12}H_{16}N_2+H^+$	189.1400	189.1392	−0.8	100.0
Banisteriopsis caapi	Harmol[a]	$C_{12}H_{10}N_2O+H^+$	199.0875	199.0871	−0.4	1.6
	Harmine[b]	$C_{13}H_{12}N_2O+H^+$	213.1022	213.1028	0.6	100.0
	Harmaline[b]	$C_{13}H_{14}N_2O+H^+$	215.1153	215.1184	3.1	3.0
	Tetrahydroharmine[a]	$C_{13}H_{16}N_2O+H^+$	217.1353	217.1341	−1.2	4.1
Peganum harmala	Deoxypeganine[a]	$C_{11}H_{12}N_2+H^+$	173.1069	173.1079	1.0	2.5
	Vasicine[b]	$C_{11}H_{12}N_2O+H^+$	189.1006	189.1028	2.2	36.6
	Harmine[b]	$C_{13}H_{12}N_2O+H^+$	213.1026	213.1028	0.2	64.9
	Harmaline[b]	$C_{13}H_{14}N_2O+H^+$	215.1153	215.1184	3.1	100.0
Diplopterys cabrerana	*N*-Methyltryptamine[a]	$C_{11}H_{14}N_2+H^+$	175.1235	175.1235	−1.5	0.4
	Dimethyltryptamine[b]	$C_{12}H_{16}N_2+H^+$	189.1392	189.1392	−0.4	100.0

[a] The corresponding mass was consistent with the formula of the indicated compound which has previously been isolated from the species listed.
[b] The presence of this compound was confirmed through comparison of the in-source collision-induced dissociation (CID) spectrum of the plant material with the in-source CID spectrum of an authentic standard.
[†] Differences are reported in millimass units (mmu). Measured masses fell within 5 mmu of the calculated masses.
[‡] Relative intensities are reported in percent.

213.1026), and harmaline ($C_{13}H_{14}N_2O+H^+$, *m/z* 215.1153) were also present in the *P. harmala* seeds, in addition to peaks at *m/z* 173.1069 and 189.1006, corresponding to deoxypeganine ($C_{11}H_{12}N_2+H^+$) and vasicine ($C_{11}H_{12}N_2O+H^+$) respectively. In the case of *P. harmala*, the peak at *m/z* 213 corresponding to harmaline was the base peak. The peak at *m/z* 215, consistent with the presence of harmine, had a relative intensity of 64.9%.

3.2. In-source collision-induced dissociation experiments

DART-HRMS analysis under soft-ionization conditions provides chemical formula information that allows tentative assignment of compounds based on those previously reported to be present in the analyzed species. This is done through comparison of in-source CID spectra of authentic standards to those of plant material obtained under identical conditions [9,10]. The AccuTOF "function switching" feature enables the simultaneous collection of mass spectra under soft ionization conditions (i.e. low orifice 1 voltages), and in-source CID conditions (using higher orifice 1 voltages) which promote fragmentations characteristic of the molecule being analyzed. To confirm the presence of DMT in *P. viridis*, *M. hostilis* and *D. cabrerana*, in-source CID analysis using function switching was performed. For DMT, an orifice 1 voltage of 60 V yielded the best results in that the protonated precursor molecule was retained while the fragment ions were still detected. Representative spectra are illustrated in Fig. 3 as head-to-tail plots. In each of

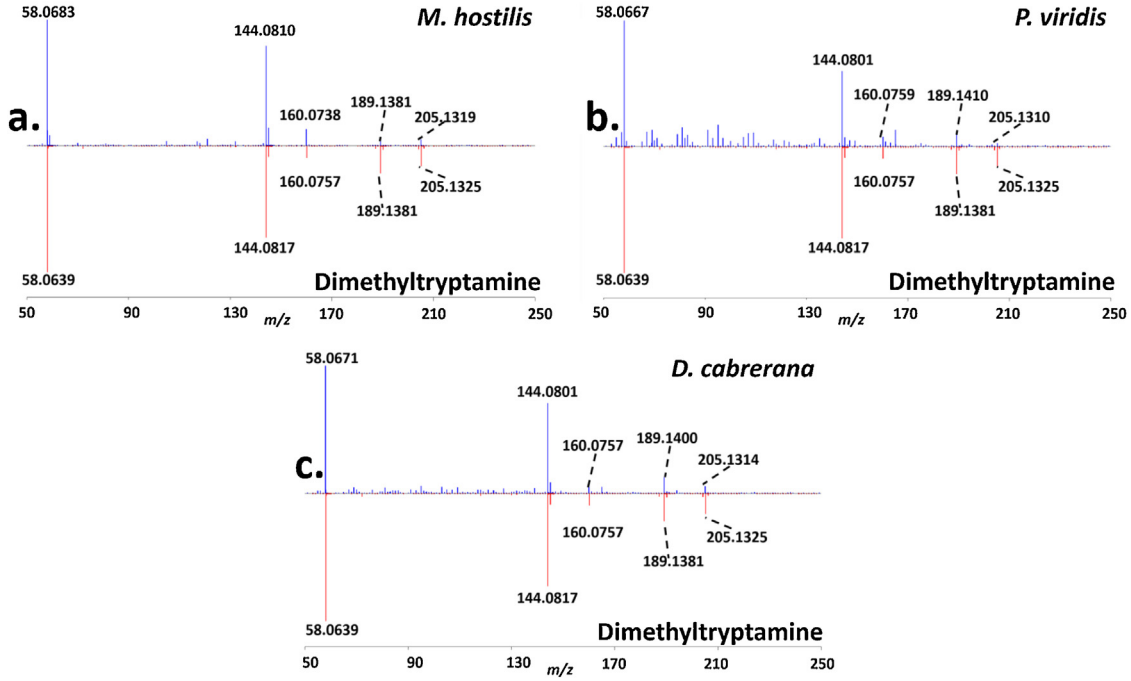

Fig. 3. Comparison of positive-ion mode DART-HRMS spectra under in-source CID conditions of *M. hostilis*, *P. viridis* and *D. cabrerana* vs. a dimethyltryptamine (DMT) standard, rendered as head-to-tail plots. In each case, the top spectrum is that of the indicated plant material and the bottom spectrum that of the DMT standard. The protonated precursor (nominal *m/z* 189) and fragment ions (nominal *m/z* 160, 144, 58) are observed in the DMT standard and in the plant products. Panel a: compar-ison of *M. hostilis* to DMT; Panel b: comparison of *P. viridis* to DMT; Panel c: comparison of *D. cabrerana* to DMT. The mass measurement data associated with these spectra are shown in Tables S-1–S-3.

the Panels a through c, the in-source CID spectrum of the plant material is on top and that of DMT appears on the bottom. The mass measurement data associated with the spectra are presented in Supplementary Material Tables S-1–S-3. The precursor peak for DMT at nominal *m*/*z* 189 was detected in the plant material as well as in the standard in each case. Fragment ions at nominal *m*/*z* 160, 144, and 58 corresponding to $C_{10}H_{10}NO$, $C_{10}H_{10}N$, and C_3H_8N, respectively, were detected in *P. viridis*, *M. hostilis*, *D. cabrerana* and the standard, confirming the presence of DMT in each of the plant materials.

In-source CID analysis was also performed on *B. caapi* and *P. harmala* to confirm the presence of harmine and harmaline, the bioactive β-carbolines reported [16,18,24,26–28] in these plants. Fig. 4 shows the in-source CID spectra at 90 V of *B. caapi* and *P. harmala* rendered as head-to-tail plots, with mass measurement data presented in Supplementary Material Tables S-4–S-7. In Panels a and b, the in-source CID spectrum of the plant material is on top and that of harmaline appears on the bottom, while in Panels c and d, harmine appears on the bottom of the head-to-tail plots. The in-source CID spectrum of the harmaline standard shown in Panels a and b exhibited a precursor peak at nominal *m*/*z* 215 with fragment ions at nominal *m*/*z* 200, 174, 172, 159 and 68. The precursor peak and corresponding fragment ions were also found in the *B. caapi* and *P. harmala* in-source CID spectra. However, the abundances in the *B. caapi* spectrum were much lower, due to the overall lower intensity of the harmaline peak in the soft ionization spectrum of the plant material (Fig. 2d). Harmine was confirmed to be present in both *B. caapi* and *P. harmala* based on the comparison of the plant material 90 V spectra to that of the harmine standard. Panels c and d exhibited a precursor peak at nominal *m*/*z* 213 for the harmine standard, as well as fragment ions at nominal *m*/*z* 199, 198, 171 and 170. The 90 V spectra of *B. caapi* and *P. harmala* both exhibited a precursor peak at nominal *m*/*z* 213 and fragments at nominal *m*/*z* 199, 198, 171 and 170, confirming the presence of harmine in the botanical material. In-source CID also confirmed the presence of vasicine in the *P. harmala* seeds (Fig. 4e). The 90 V spectra of the seed and vasicine standard have a precursor peak at nominal *m*/*z* 189, with fragments at nominal *m*/*z* 171, 144, 143, 118, 91 and 68 (Supplementary Material Table S-8).

3.3. Classification of Ayahuasca brew DART-HRMS data by principal component analysis

Upon determination of the unique chemical fingerprints associated with each of the botanical materials typically used in Ayahuasca, brews were concocted to ascertain whether the identity of the plants used in the extraction could be elucidated. Six brews were made following readily available recipes:[24] brew 1: *B. caapi* and *P. viridis*; brew 2: *M. hostilis* and *P. harmala*; brew 3:

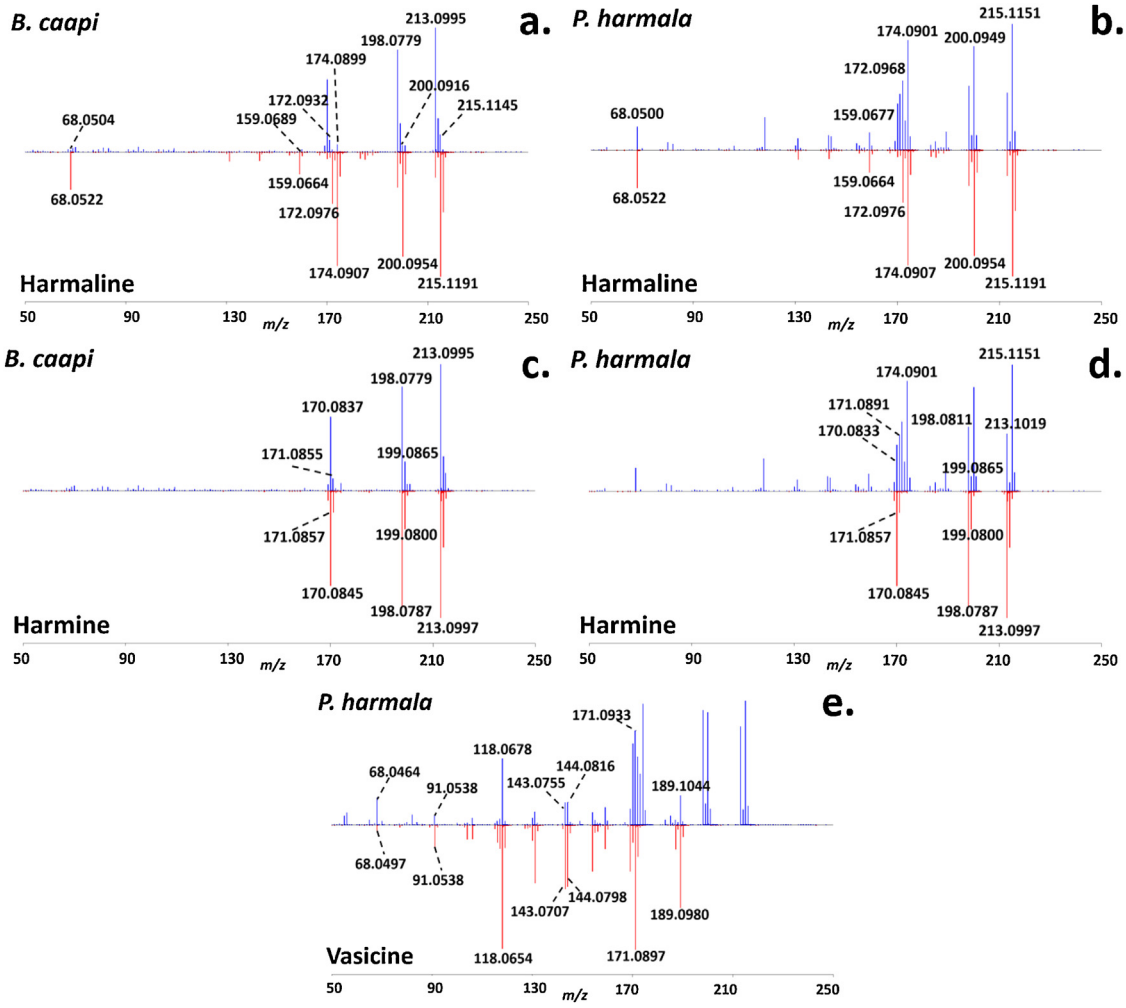

Fig. 4. Comparison of positive-ion mode DART-HRMS spectra under in-source CID conditions of *B. caapi*, and *P. harmala* vs. harmala alkaloid standards, rendered as head-to-tail plots. In each case, the top spectrum is that of the indicated plant material and the bottom spectrum that of the noted authentic standard. Panel a: comparison of *B. caapi* to harmaline; Panel b: comparison of *P. harmala* to harmaline; Panel c: comparison of *B. caapi* to harmine; Panel d: comparison of *P. harmala* to harmine; Panel e: comparison of *P. harmala* to vasicine. The mass measurement data associated with these spectra are shown in Tables S-4–S-8.

P. viridis and *P. harmala*; brew 4: *M. hostilis* and *B. caapi*; brew 5: *D. cabrerana* and *B. caapi*; and brew 6: *D. cabrerana* and *P. harmala*. The DART-HRMS analysis of the six brews conducted under soft ionization conditions (i.e. orifice 1 voltage of 20 V) are illustrated in Fig. 5, with corresponding mass measurement data listed in Supplementary Information Table S-9. Each spectrum is an average of five spectra, with each peak representing a protonated molecule. A total of 30 averaged spectra were obtained for each brew, resulting in 900 individual analyses. The inset in each Panel is an image of the indicated brew, and illustrates the subtle differences in the appearances of the brews made by the different methods. DMT (nominal *m*/*z* 189), *N*-methyltryptamine (nominal *m*/*z* 175), harmine (nominal *m*/*z* 213) and harmaline (nominal *m*/*z* 215) were detected in all of the brews, with intensities ranging from 77.1% to 100%, 1.1% to 5.1%, 36.8% to 77.1% and 2.1% to 73.7%, respectively. Harmane (nominal *m*/*z* 183) was present in brews made with *B. caapi* (Panels a, d, and e) and deoxypeganine (nominal *m*/*z* 173) was detected in the brews containing *P. harmala* seeds (b, c and f).

In previous studies, multivariate statistical analysis methods were applied to DART-HRMS data to differentiate species and cultivars of plant-derived samples [9,10,45]. In these cases, the DART-HRMS spectra were visually very different, making it clear that multivariate statistical analysis approaches would be successful in distinguishing species. However, despite being comprised of different constituents, the spectra of the various Ayahuasca brews were remarkably similar. This is illustrated by the heat map renderings of the data, which represent a total of 900 individual analyses (Fig. 6). High intensity peaks are dark red in color and lower intensity peaks appear in lighter shades. Overall, comparisons of heat maps representing replicates within a single class, or spectra of different classes, were remarkably very similar, with the highest intensity peaks appearing at *m*/*z* 189, 213, and 215, representing DMT, harmine and harmaline, respectively.

Therefore, principal component analysis (PCA) was applied to the dataset to determine if the botanical species of origin from which each brew was comprised could be determined. Using five feature masses (Table 3), a PCA plot was generated using three principal components (Fig. 7). Each brew is represented by thirty data points: brew 1 by blue circles, brew 2 by red squares, brew 3 by green triangles, brew 4 by turquoise circles, brew 5 by pink

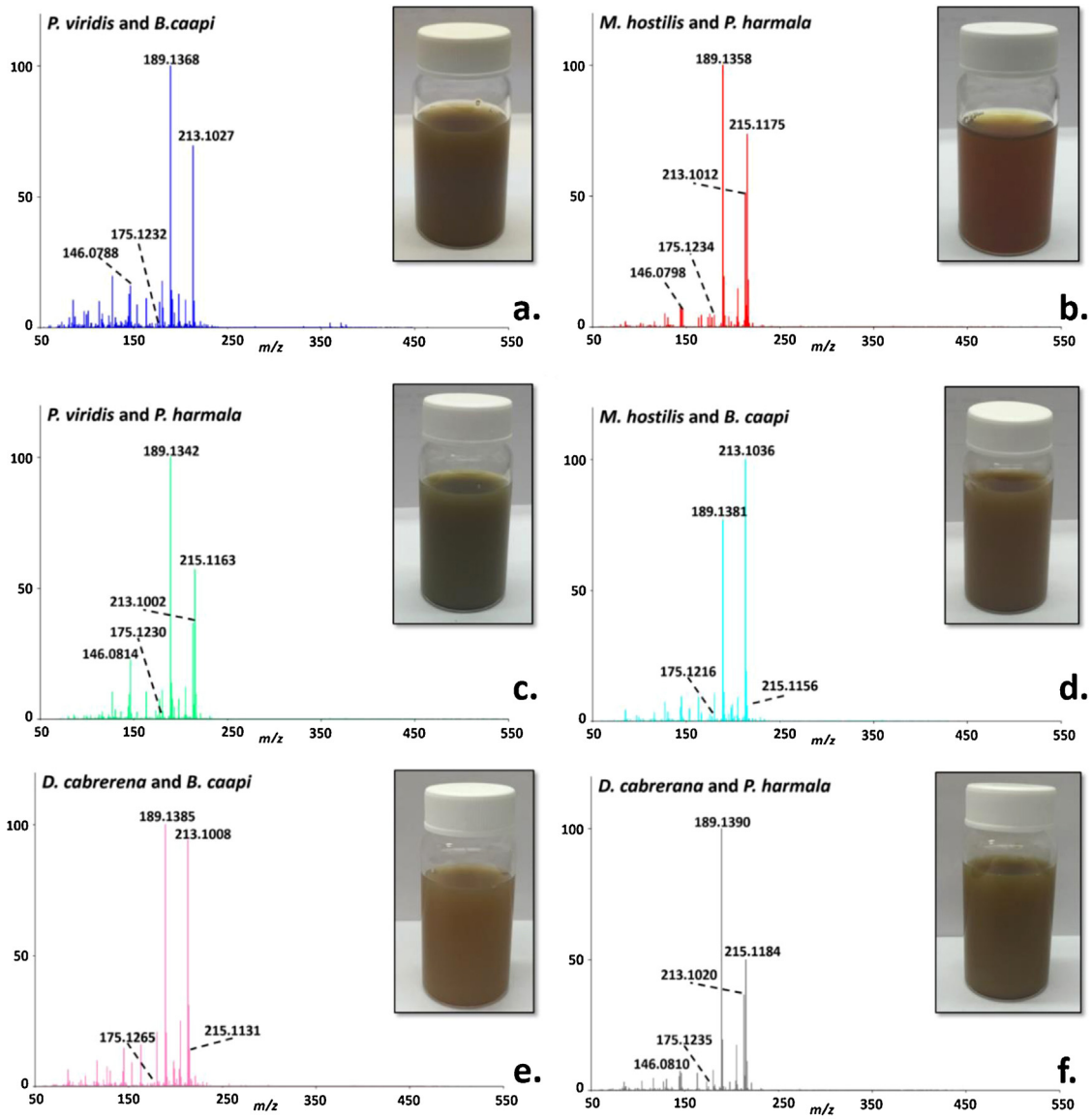

Fig. 5. DART-HRMS soft ionization spectra of Ayahuasca brews. The inset in each Panel is an image of the brew that illustrates the subtle differences in their appearances. Panel a: *P. viridis* and *B. caapi* brew; Panel b: *P. harmala* and *M. hostilis* brew; Panel c: *P. viridis* and *P. harmala* brew; Panel d: *M. hostilis* and *B. caapi* brew; Panel e: *D. cabrerana* and *B. caapi* brew; Panel f: *D. cabrerana* and *P. harmala* brew. The mass measurement data associated with these spectra are shown in Table S-9.

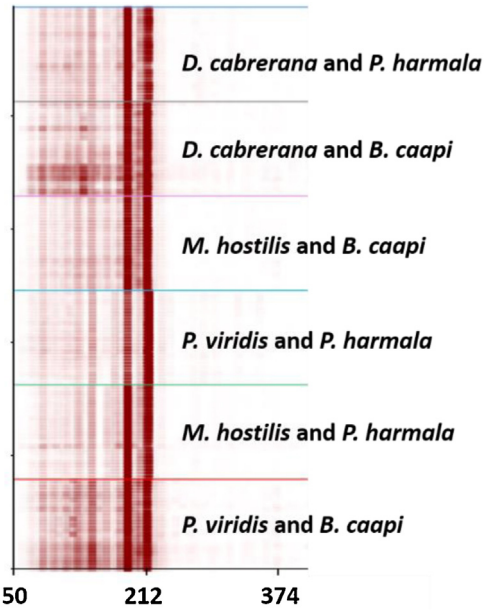

Fig. 6. Heat map renderings of the DART-HRMS spectra of Ayahuasca brews. High intensity peaks are shown in dark red and lower intensity peaks are indicated by lighter shades.

Table 3
Feature masses used for principal component analysis (PCA) of Ayahuasca brews. The PCA plot is shown in Fig. 7.

146.0788	213.1006
189.1351	215.1170
195.0874	–

squares, and brew 6 by grey triangles. Each of the brews clustered independently, clearly reflecting differences between brews that were a function of the botanical components in the mixture. Data points with a low score along PC1 (*x*-axis) contain *P. harmala* as the β-carboline contributing plant, while data with a high PC1 score contain *B. caapi* as the harmala alkaloid contributing plant. With regard to the DMT-containing plant contributions, data points with a high PC2 value (*y*-axis) contain *P. viridis* as the DMT contributor, while data points with low PC2 scores indicate *M. hostilis.* Intermediate PC2 values arise from *D. cabrerana* contributions. Three principal components accounted for 86.38% of the variance, and the leave-one-out cross validation for the training set was 98.88%.

4. Discussion

The ability to identify new psychoactive substances (NPS), especially those in complex matrices, is increasingly important because of the rise in use of alternative drugs of abuse that are marketed and sold to circumvent current drug laws. Plant-based drugs are an NPS subset which, second to ketamine, are the largest group of NPSs emerging on the drug market worldwide [47]. Of the 20 plant-based drugs of concern listed in the UNODC bulletin, four are used in the creation of Ayahuasca brews. Thus, the need to identify these psychoactive materials as well as mixtures or brews made from them will become increasingly necessary, as the sale of the products grows due to their legal status around the world.

There are few published protocols for the identification of the psychoactive components in Ayahuasca brews found as evidence at a crime scene or submitted to forensic drug chemistry laboratories. GC–MS protocols have been developed for the characterization of Ayahuasca brews, [37,39,40] but the sample preparation steps and long chromatographic programs makes routine screening and identification challenging, especially in light of casework backlogs [42]. LC–MS methodology would enable analysis of aqueous Ayahuasca brews to be more straightforward than would be required for GC–MS. Yet, reports of such methods are absent in the literature, particularly regarding identification of Ayahuasca brews and the individual components of psychoactive plant material mixtures. Furthermore, although LC–MS protocols have been developed for identification of DMT and harmala alkaloids in human samples including serum, plasma and blood [2,18,48], there are no reports of identification of Ayahuasca itself in an evidentiary context. Characterization of the specific plant materials used in Ayahuasca preparations (as opposed to the brew itself) has been achieved using LC–MS methodology [49]. However,

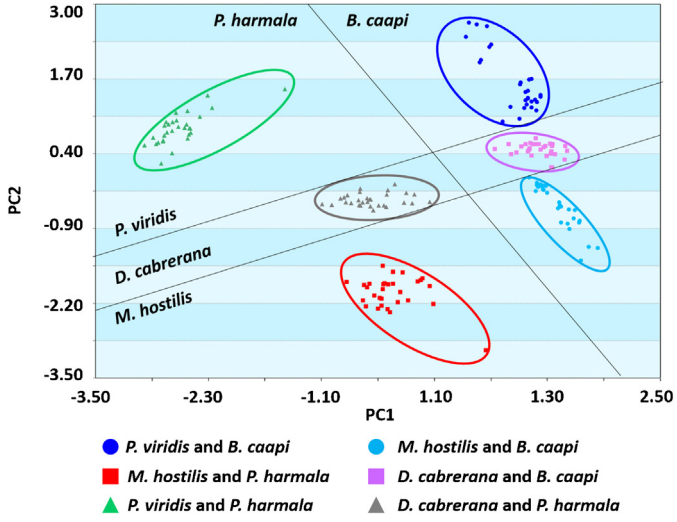

Fig. 7. Principal component analysis (PCA) plot of six Ayahuasca brews constructed using DART-HRMS derived data. Mass spectral data with a low score along PC1 (*x*-axis) contain *P. harmala* as the β-carboline contributing plant, while data with a high PC1 score contain *B. caapi* as the harmala alkaloid contributing plant. Data points with a high PC2 value (*y*-axis) contain *P. viridis* as the DMT contributor, while data points with low PC2 scores indicate *M. hostilis.* Intermediate PC2 values arise from *D. cabrerana* contributions. Three principal components (PCs) accounted for 86.38% of the variation, and the leave-one-out cross validation (LOOCV) was 98.88%. The feature masses used for the PCA are listed in Table 3.

the lengthy sample preparation steps, intensive method development and long chromatographic run times make this an impractical solution for routine analysis.

The approach described here provides a means to rapidly triage forensic sample unknowns and determine whether or not they are Ayahuasca. Specifically, we show that by chemometric processing of DART-HRMS data, it is possible to not only identify the concoctions as Ayahuasca, but also that: (1) the presence of psychoactive constituents could be confirmed without the need for extensive sample preparation steps; and (2) the plant species of which the brews were comprised could be determined. DMT and *N-methyltryptamine* were both detected in *P. viridis*, *M. hostilis*, and *D. cabrerana.* This observation is consistent with the results of previous reports,[23,25,28–30] and supports the use of these plant materials as the main contributors to the hallucinogenic effects of Ayahuasca beverages (Fig. 2). While the most abundant peak in the *P. viridis*, *M. hostilis*, and *D. cabrerana* samples represented DMT, the chemical fingerprint of each of the plant materials was unique between each species. Masses consistent with the harmala alkaloids harmol, harmine, harmaline, and tetrahydroharmine were detected in the *B. caapi* vine material, and deoxypeganine, vasicine, harmine, and harmaline were also tentatively identified in the *P. harmala* seeds. Despite being detected in both species, harmine and harmaline exhibited different relative intensities, with *B. caapi* having a higher abundance of harmine, and *P. harmala* showing a higher relative intensity of harmaline. The use of in-source CID provided confirmation of the tentative assignments of the psychoactive compounds in the plant materials. DMT was confirmed to be present in *P. viridis*, *M. hostilis* and *D. cabrerana* by comparison of the 60 V spectra of the plant material to the 60 V spectrum of the DMT standard. Harmine and harmaline were confirmed to be present in both *B. caapi* and *P. harmala* through comparisons of their 90 V in-source CID spectra with that of the plant material, and vasicine was also identified in the *P. harmala* seeds in the same manner.

Differences in the chemical fingerprints of the plant species in Ayahuasca brews indicated that the use of different plant combinations would slightly alter the overall composition of the brew. Therefore, in principle, the identity of the species of plants used in the beverages could be determined based on the unique chemical fingerprints of the brews. To investigate this hypothesis, six Ayahuasca brews were prepared from various pairings of DMT and harmala alkaloid-containing botanical materials (Fig. 5). In each case, DMT was readily detected at nominal m/z 189, as were harmine and harmaline at m/z 213 and 215 respectively, indicating that the brews were in fact Ayahuasca. Visually, the mass spectral profiles of the brews were remarkably similar (Fig 6). Nevertheless, based on the differences in the chemical compositions of the botanical species used in the brews, statistical analysis processing of the DART-MS data permitted identification of the species in each mixture.

Due to the time required for a single GC– or LC–MS analysis, generation of the voluminous chromatographic data required for the application of statistical analysis methods is not practical. However, the rapidity of DART-HRMS analysis permits the rapid acquisition of the large datasets required for the application of statistical analysis methods to the spectral data. Here, PCA was applied to the Ayahuasca brew fingerprints to determine if the species identity of the plant materials used in the brew could be verified. The PCA plot generated (Fig. 7) showed clear distinctions with no overlap between each of the six brews, as well as tight clustering within each class. Moreover, there were distinctions along PC1 between brews that contained *B. caapi* as the harmala alkaloid contributor to the Ayahuasca beverage, and those that contained *P. harmala* as the source of harmala alkaloids. Similarly, there were distinctions between each of the brews as a function of the DMT-containing plant used in the concoction. Along PC2, data points with higher values were those of *P. viridis*, those with intermediate values aligned with *D. cabrerana*, and those with low PC2 values contained *M. hostilis* as the hallucinogen contributor.

Three principal components (derived from the five selected feature masses) accounted for 86.38% of the observed variance in the PCA plot. The feature masses were associated specifically with the psychoactive compounds present in the plant materials used in making Ayahuasca. The inclusion of additional feature masses was unnecessary for validation of the statistical analysis method, as they did not contribute substantially to the variance. Thus, the leave-one-out cross validation was found to be 98.88%. The use of statistical analysis processing on DART-HRMS data enabled a level of certainty to be associated with the identity of the composition of the brew, an outcome the accomplishment of which has been emphasized as highly desirable by the National Academy of Sciences [35]. Moreover, successful classification by botanical species in this way could provide law enforcement with vendor or manufacturer information that might be essential in sourcing the product.

5. Conclusion

DART-HRMS analysis of Ayahuasca beverages revealed unique chemical fingerprints based on the characteristic signatures of the psychoactive botanical species used in the making of the brews. In-source CID confirmed the presence of the hallucinogen DMT in *P. viridis* leaves, *M. hostilis* root bark, and *D. cabrerana* leaves, and various harmala alkaloids in *B. caapi* and *P. harmala*. The detection of DMT in conjunction with the presence of harmine and harmaline enabled identification of the brews as Ayahuasca. Moreover, PCA revealed that while the spectra of the mixtures were visually similar, the composite of constituents in the six brews were unique enough to enable differentiation of the specific combination of plant species used in making each Ayahuasca beverage. This definitive classification is not possible if currently used conventional methods that simply rely on confirmation of the presence of DMT, are used. The method described herein can be readily applied to a variety of compounded plant-based products, in a manner that circumvents time- and resource-intensive sample preparation steps. Furthermore, it could provide a means for forensic laboratories and emergency medical staff to rapidly and accurately identify abused substances.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.forsciint.2016.06.009.

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