Forensic Science International 242 (2014) 210–218

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Rapid detection by direct analysis in real time-mass spectrometry (DART-MS) of psychoactive plant drugs of abuse: The case of *Mitragyna speciosa* aka "Kratom"

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ARTICLE INFO

Article history:
Received 10 May 2014
Received in revised form 28 June 2014
Accepted 1 July 2014
Available online 14 July 2014

Keywords:
DART-MS
Kratom
Legal drug alternatives
Mitragyna speciosa
Mass spectrometry
Forensic analysis

ABSTRACT

Mitragyna speciosa, also known commonly as "Kratom" or "Ketum", is a plant with psychoactive properties that have been attributed to the presence of various indole alkaloids such as mitragynine and 7-hydroxymitragynine. *M. speciosa* use is gaining popularity internationally as a natural and legal alternative to narcotics. As a drug of abuse, its detection and identification are not straightforward, since *M. speciosa* plant material is not particularly distinctive. Here, we show that direct analysis in real time-mass spectrometry (DART-MS) can be used not only to rapidly identify *M. speciosa* plant material and distinguish it from other plants, but also to distinguish between *M. speciosa* plant varieties, based on differences between their chemical profiles. The method is rapid and the analysis expeditious. Plant material such as that found at a crime scene can be analyzed directly with no sample pre-preparation steps. Furthermore, we show that the basis set of principal components that permit characterization of the plant material can be used to positively identify *M. speciosa*.

1. Introduction

In response to the expansion of controlled substances lists and the institution of analog drug laws, the past decade has witnessed the ascendance of "legal" psychoactive substances, so labeled because although purported to have mind altering characteristics, their manufacture, possession, and use remain unscheduled in many countries. Trade in whole plant legal psychotropics presents numerous perceived advantages to the drug manufacturer and user, not the least of which are the ease of sale and distribution through the internet, and the fact that the evidentiary value of such plants or plant products is limited due to the challenge of identifying them. Thus, unlike the cannabis plant that has characteristic well-recognized foliage and easily identified scheduled alkaloids, numerous other plants with physical characteristics that are not widely known and which contain unscheduled psychoactive substances, are available and exploited. This allows both the user and manufacturer to enjoy freedom from prosecution, while at the same time, exposing both to the potentially life-threatening consequences of unregulated exposure to dangerous and toxic active ingredients. The challenges this imposes on law enforcement agencies and crime laboratories are obvious, as the availability of rapid and facile testing protocols for identification of such substances or the plants from which they are derived can be limited or non-existent.

An example of one such plant is *Mitragyna speciosa*. Known colloquially as "Kratom", it is endemic to tropical and sub-tropical regions of Southeast Asia and Africa. It has been used in traditional medicine to treat intestinal disorders, muscle pain, coughing, and diarrhea, as well as for its psychotropic effects [1–5]. Preparations of the plant's aerial parts have been shown to have analgesic, euphoric, and anti-depressant effects [4]. Thai and Malaysian laborers and farmers have been reported to use Kratom as a stimulant in order to provide stamina and relief from sore muscles [6]. Although planting *M. speciosa* in Thailand has been illegal since 1943 and ingestion of the plant was outlawed in 1979, Kratom remains a popular drug in Thailand [4].

Even in those countries where it is currently unscheduled, Kratom is of particular interest to the forensics community because multiple poisonings and fatalities have been associated with its use [7–9]. In these cases, testing for the presence of Kratom was prompted by either self-reporting by the user [7], residual Kratom found in the surroundings of the deceased, or a history of

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http://dx.doi.org/10.1016/j.forsciint.2014.07.005
0379-0738/

past opioid use [8]. As there are no published toxicity thresholds for the psychoactive components of Kratom, notably mitragynine, it is often difficult to ascertain the extent to which ingested Kratom contributes to poisonings or fatalities [8].

Although the plant is often ingested alone, Kratom has also been found to be a component of herbal smoking blends that have become popular in the past decade. In Germany and Sweden, products sold under the name "Krypton" were actually enhanced Kratom preparations that also contained both caffeine and O-desmethyltramadol [9]. In 2009 alone, nine fatal overdoses attributed to the use of Krypton were reported [4,10].

Even though Kratom is unscheduled in the United States (US), the US Drug Enforcement Administration has classified it as a "Drug of Concern" and issued a bulletin on Kratom in January 2013 [11]. Kratom use in the US has increased in recent years. Whereas only one case was reported in 2010, that number increased to 44 in 2011, and then it further increased to 81 cases during the first six months of 2012 [1]. Mitragynine and other *M. speciosa* alkaloids, as well as their metabolites, are often not part of routine drug screens. Although testing protocols such as an ELISA assay [12], as well as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFL)-based identification systems [13,14] have been developed, the adoption of such protocols by forensics labs may be slow due to the significant escalation in costs that adoption of these methods may impose. Nevertheless, other testing methods specific for Kratom have recently been reported, reflecting the increasing importance of the emerging problem of Kratom abuse. These methods include HPLC– or LC–MS/MS analysis of Kratom extracts [6,15–20].

The psychotropic activity of Kratom has been attributed to various indole alkaloids whose presence and concentration vary as a function of where the plant is grown [11]. Mitragynine and its stereoisomers mitraciliatine, speciogynine, and speciociliatine, as well as the related compound paynantheine, are abundant alkaloids present in the leaves of *M. speciosa* [21] and these species are reported to be agonists of κ and μ-subtype opioid receptors (Fig. 1) [2,3,5,11,15,20,22,23]. In terms of opioid receptor agonism, mitragynine is comparable with morphine. However, 7-hydroxymitragynine has been reported to exhibit 13–17 times the agonism of morphine toward opioid receptors [24–26]. These studies have also shown that 7-hydroxymitragynine induces morphine-like tolerance and withdrawal in rats [25,26].

Anecdotal reports describe symptoms of withdrawal in humans that include intense craving, chronic fatigue, insomnia and sudden nerve pain [27,28]. As mitragynine and 7-hydroxymitragynine have not been found in any other plant [2,5], including those in the Rubiaceae family of which *M. speciosa* is a member, both compounds could serve as possible chemical markers and/or chemotaxonomic identifiers of Kratom.

Currently the evidentiary value of Kratom is limited, as plant material found at a crime scene may be difficult to identify. Evidence could be found as live plants, dried leaves, or a powdered leaf

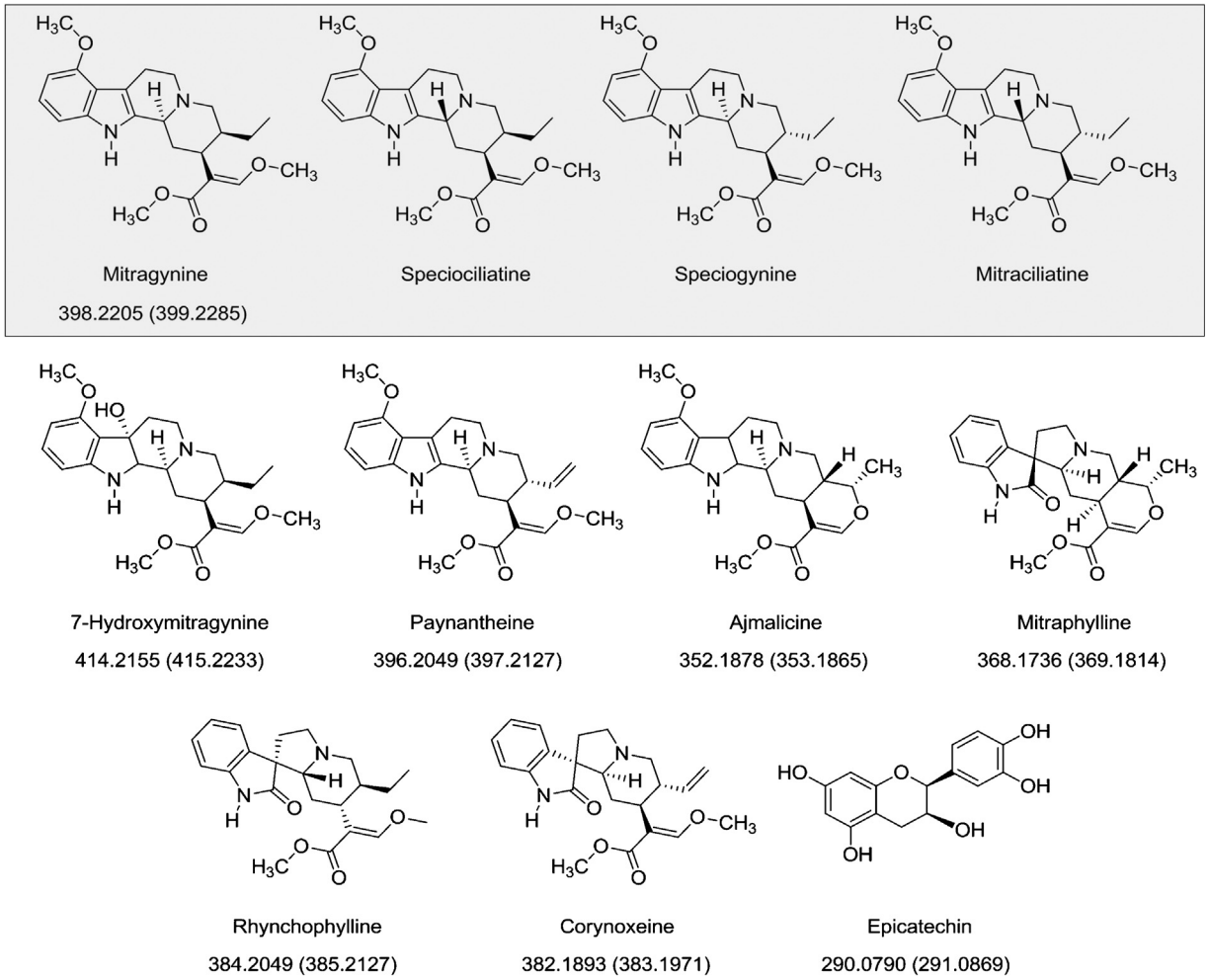

Fig. 1. The names and structures of alkaloids and a flavonoid reported to be found in *M. speciosa*. Their calculated masses as well as the calculated masses of their protonated forms (in parentheses) are shown. Molecules in the shaded area are stereoisomers of one another and therefore have the same molecular weights.

product, and it may be difficult to distinguish true plant-based evidence from innocuous plant-derived food residue. Furthermore, the amount of plant material could be small, making it difficult to perform analyses involving extractions. Although botanic evidence is often characterized by physical features (and *M. speciosa* was recently reported to be characterized through the use of light microscopy [29]), this ceases to be important if the plant material is too well pulverized, which diminishes its importance as evidence [30–32]. DNA analysis has also been used to identify botanic traces, but this method is often time-consuming and yields little useable information in those cases where the plant genome has not been mapped [31,32]. Unfortunately, this is the case for most plants including *M. speciosa*. If a more robust and streamlined method of analysis can be developed, the evidentiary value of Kratom and other plant-based evidence could be dramatically enhanced.

Direct analysis in real time (DART) is an ambient ionization source that permits rapid analysis by mass spectrometry (MS) of gases, liquids and a diversity of solids in various forms, including plant material [33–37]. It has been shown to be an effective tool for characterizing and identifying drug evidence, drug residues from clandestine labs, and synthetic cannabinoid-laced plant material [34,35]. When interfaced with a high resolution (HR) time-of-flight (TOF) mass analyzer, DART provides a powerful means by which instantaneous ionization and MS identification of compounds can be made from very small samples with no extraction or other time and resource consuming sample pre-preparation steps.

In this work, we report how high resolution (HR) DART-MS can be used to instantaneously identify *M. speciosa* based on its distinct chemical signature. Standards were used to confirm the presence in the plant material of the unique psychoactive alkaloids mitragynine and/or its stereoisomers and 7-hydroxymitragynine, as well as a third alkaloid of significant abundance, mitraphylline. Furthermore, linear discriminant analysis (LDA) was successfully used to distinguish between *M. speciosa* and other plants of abuse such as those in the *Datura* genus. The utility of this method in distinguishing between the two most common varieties of *M. speciosa* plants available in the US, Rifat and Bumblebee, was also demonstrated. The statistical analysis system was tested with an "unknown" *M. speciosa* variety and shown to enable correct species classification of the plant from the mass spectral signature of its leaves.

2. Materials and methods

2.1. Plant materials

Four *M. speciosa* (Kratom) plants were purchased from an online vendor (Kratom Collection-World Seed Supply, Mastic Beach, NY, USA): two plants of the Rifat variety and two plants of the Bumblebee variety, both 6–7 months in age. The live plant material was collected and analyzed in November, 2013. A 4–6 month old *M. speciosa* plant that served as an "unknown" variety used to assess the accuracy of the linear discriminate analysis was purchased from Mazatec Gardens (Houston, TX, USA). The "unknown" plant material was collected and sampled in February, 2014. *Datura ferox*, *D. inoxia*, and *D. wrightii* seeds were purchased from an online vendor (Georgia Vines, Claxton, GA, USA) and analyzed in May 2014.

2.2. Chemical standards

Mitragynine was purchased from Cayman Chemical (Ann Arbor, MI, USA). Mitraphylline and 7-hydroxymitragynine were purchased from Cerilliant Corporation (Round Rock, TX, USA). Epicatechin was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.3. 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 time-of-flight mass spectrometer (JEOL USA, Peabody, MA, USA) in positive ion mode. The DART ion source parameters 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 peak voltage 600 V. Spectra were obtained over the mass range of m/z 50–800 at 1 spectrum per second. The helium flow rate for the DART source was 2.0 L s^{-1}. The resolving power of the mass spectrometer was 6000 FWHM.

In-source collision induced dissociation (CID) was performed on plant material and standards by the application of "function switching", which is a set of instrument parameter settings that allow for the simultaneous acquisition of both low cone voltage and high cone voltage mass spectra. To do this, the orifice 1 voltage was varied from 20 V, 30 V, 60 V and 90 V, with the extent of fragmentation increasing with increasing voltage [34,38]. All other DART-SVP and AccuTOF parameters remained the same. Mitragynine and mitraphylline standards were tested directly by dipping the closed end of a melting point capillary tube into the pure standard and suspending the coated surface of the tube between the DART ion source and the mass spectrometer inlet. Solubilized 7-hydroxymitragynine standard (100 μg/mL in acetonitrile) was sampled in the same manner.

Clippings (approximately 1.0 cm × 0.5 cm) of *M. speciosa* leaves were sampled directly by gripping each sample with tweezers and suspending it between the ion source and the mass spectrometer inlet. Leaf extracts prepared by suspending each leaf clipping into 50 μL of absolute ethanol, sonicating for 20 min, and then letting the sample stand for 2 h, were also analyzed. Four microliter aliquots of each ethanol extract were then applied, one aliquot per sampling window, to a 12-sample QuickStrip™ sample card (Ionsense, Saugus, MA, USA). The card was mounted on a linear rail system (IonSense, Saugus, MA, USA) that moved laterally from left to right through the open air space between the ion source and the mass spectrometer inlet (Supplementary Fig. 1) at a rate of 1.0 mm/s. This arrangement permitted high throughput analysis and enabled optimal and consistent positioning of samples in the helium gas stream. The extracts of the Rifat variety were subjected to function switching conditions for comparison to standards. *Datura* seeds were sliced and were sampled directly by holding a seed-half between the ion source and the mass spectrometer inlet with a vacuum tweezer apparatus.

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.forsciint.2014.07.005.

Calibration, spectral averaging, background subtraction, and peak centroiding were performed using TSSPro3 (Shrader Analytical Labs, Detroit, MI, USA) data processing software. Mass Mountaineer software (ChemSW, Fairfield, CA, USA) was used for mass spectrum analysis, spectral elemental composition and isotope analysis, as well as for Linear Discriminant Analysis (LDA)-facilitated classification and discrimination [39]. Calibration was performed using a polyethylene glycol mixture (PEG 200, 400, 600, and 1000).

3. Results

Fig. 2 shows representative examples of the Bumblebee and Rifat strains of Kratom and illustrates that by visual inspection alone, the plants are difficult to distinguish. *M. speciosa* has been reported to contain a number of alkaloid compounds in the aerial parts [4,11,40]. As a preliminary step, we sought to ascertain which if any of these compounds could be detected in the leaf samples. A

Fig. 2. Whole plant Bumblebee (left) and Rifat (right) varieties of *M. speciosa*. Comparison of the whole plants and leaves (insets) illustrates the difficulties inherent in differentiating between the two plants based on morphological features.

head to tail plot with the top and bottom panels being the average of 6 HR-DART-MS spectra of the Bumblebee and Rifat *M. speciosa* varieties respectively, is shown in Fig. 3. The figure illustrates that although the two varieties share prominent chemical features, there are numerous peaks that appear to be unique to one variety versus the other (discussed below). Additionally, relative abundances of common peaks also vary between varieties. In each case, the spectra were reproducible, in that the analysis of different plants of the same variety, as well as leaf clippings from the same or different plants, yielded similar spectra. The spectra were collected in positive ion mode under soft ionization conditions (i.e. an orifice 1 voltage of 20 V) so that no fragmentation of leaf constituents was observed. Therefore, the peaks in each spectrum generally represent the protonated molecular ions of unique individual compounds. The spectra show that in each case, greater than 141 leaf compounds are detectable by mass spectrometry (estimated using a 2% relative abundance threshold).

The observed high resolution *m*/*z* values were compared to the expected masses of compounds reported to have been detected in

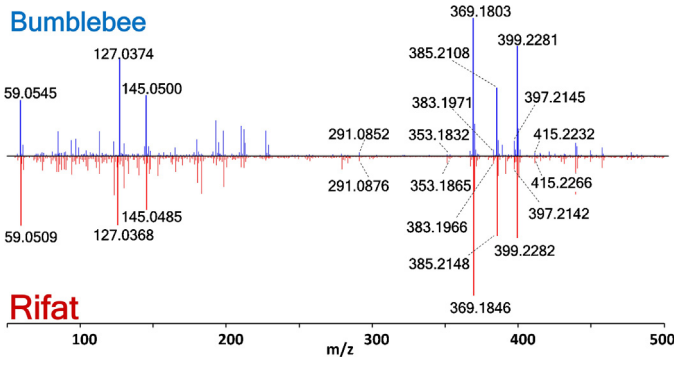

Fig. 3. Positive ion mode HR DART-TOF-MS spectra of leaf clippings of Bumblebee and Rifat varieties of *M. speciosa*, illustrated as a head-to-tail plot. While the spectra are remarkably similar, there are not only differences in the relative abundances of common compounds, but there are also peaks that appear in Bumblebee and not Rifat, and vice versa. The statistics associated with both spectra are shown in Table 1.

M. speciosa (Fig. 1 and Table 1). Of these reported compounds, observed accurate masses representing the protonated forms of seven known alkaloid molecular formulas were seen in the Bumblebee and Rifat spectra. These formulas were $C_{23}H_{30}N_2O_4$ (corresponding to mitragynine and/or its stereoisomers speciociliatine, speciogynine and mitraciliatine with a mass of 399.2284), $C_{21}H_{24}N_2O_4$ (corresponding to mitraphylline and/or its stereoisomers with a mass of 369.1814), $C_{23}H_{28}N_2O_4$ (corresponding to paynantheine and/or its diastereomer with a mass of 397.2127), $C_{23}H_{30}N_2O_5$ (corresponding to 7-hydroxymitragynine with a mass of 415.2233), $C_{22}H_{28}N_2O_4$ (corresponding to ±rhynchophylline with a mass of 385.2127), $C_{21}H_{24}N_2O_3$ (corresponding to ajmalicine with a mass of 353.1865), and $C_{22}H_{26}N_2O_4$ (corresponding to ±corynoxeine with a mass of 383.1971). Since the stereoisomers have very similar fragmentation patterns, it is usually not possible to differentiate between them using mass spectral techniques alone. However, the alkaloids mitragynine and 7-hydroxymitragynine are unique to *M. speciosa* and can therefore be used to identify the plant even if the stereoisomer identity is unknown. Although it can be seen that both the spectra of the Bumblebee and Rifat varieties have these two and several other peaks in common, the spectra are nevertheless distinct and contain components unique to each variety (see later). Other prominent peaks such as those at *m*/*z* 127 and 145 have formulas that are consistent with the presence of pyrolysis fragments of sugars commonly found in plants (i.e. $C_6H_7O_3$ and $C_6H_9O_4$, respectively) [41]. Other minor peaks whose corresponding molecular formulas are also consistent with compounds previously detected in Kratom, include those at *m*/*z* 383 and 291. The accurate masses associated with these peaks are consistent with the presence of corynoxeine and epicatechin, respectively. The statistics associated with the spectra in Fig. 3 are listed in Table 1.

The lack of availability of authentic standards for some of these tentatively assigned compounds precluded the possibility of confirming all of the assignments. However, through comparisons with the spectra of authentic standards of mitraphylline, mitragynine and 7-hydroxymitragynine determined under collision induced dissociation (CID) conditions, we were able to confirm that the peaks at *m*/*z* 415, 399, and 369 in the CID spectra of the Bumblebee and Rifat varieties corresponded to compounds

Table 1
Statistics for the HR-DART-MS spectra of the Bumblebee and Rifat leaf clippings shown in Fig. 3.

	Compound[a]	Formula	Measured $[M+H]^+$	Calculated $[M+H]^+$	Difference (mmu)[b]	Abund.
Bumblebee	Mitragynine (and/or its stereoisomers)	$C_{23}H_{30}N_2O_4$	399.2281	399.2284	0.3	79.7
	Mitraphylline	$C_{21}H_{24}N_2O_4$	369.1803	369.1814	1.1	100.0
	Paynantheine	$C_{23}H_{28}N_2O_4$	397.2145	397.2127	1.8	11.4
	7-Hydroxymitragynine	$C_{23}H_{30}N_2O_5$	415.2232	415.2233	0.1	2.8
	Rhynchophylline	$C_{22}H_{28}N_2O_4$	385.2108	385.2127	1.9	49.5
	Epicatechin	$C_{15}H_{14}O_6$	291.0852	291.0869	1.7	2.9
	Ajmalicine	$C_{21}H_{24}N_2O_3$	353.1832	353.1865	3.3	1.7
	Corynoxeine	$C_{22}H_{26}N_2O_4$	383.1970	383.1971	0.1	5.0
	Acetone	C_3H_7O	59.0545	59.0497	4.8	53.3
Rifat	Mitragynine (and/or its stereoisomers)	$C_{23}H_{30}N_2O_4$	399.2282	399.2284	0.2	55.3
	Mitraphylline	$C_{21}H_{24}N_2O_4$	369.1846	369.1814	-3.1	100.0
	Paynantheine	$C_{23}H_{28}N_2O_4$	397.2142	397.2127	1.5	8.8
	7-Hydroxymitragynine	$C_{23}H_{30}N_2O_5$	415.2266	415.2233	-3.3	3.2
	Rhynchophylline	$C_{22}H_{28}N_2O_4$	385.2148	385.2127	-2.1	58.8
	Epicatechin	$C_{15}H_{14}O_6$	291.0876	291.0869	-0.7	3.6
	Ajmalicine	$C_{21}H_{24}N_2O_3$	353.1865	353.1865	0.0	1.9
	Corynoxeine	$C_{22}H_{26}N_2O_4$	383.1971	383.1971	0.5	5.1
	Acetone	C_3H_7O	59.0509	59.0497	-1.2	54.3

[a] Compound names are tentatively assigned based on the match between the mass spectrometrically determined elemental compositions and compounds that have been identified in *M. speciosa* (shown in Fig. 1).
[b] Measured masses fell within the 5 mmu tolerance of the mass spectrometer.

7-hydroxymitragynine, mitragynine and/or its stereoisomers, and mitraphylline, respectively (Fig. 4a–c). For example, in the case of 7-hydroxymitragynine (Fig. 4a), the protonated molecular ion peak was apparent at *m*/*z* 415 for both the standard and Rifat variety. The CID conditions (90 V) resulted in the formation of fragments at *m*/*z* 397, 238 and 226 in the spectrum of the standard. These same fragments appeared in the CID spectrum of the Rifat variety and thus the presence of 7-hydroxymitragynine was confirmed. Similarly, the CID spectra of the mitragynine standard and Rifat leaf (Fig. 4b) both showed the molecular ion peak at *m*/*z* 399, with fragments at *m*/*z* 397, 238 and 226. Fig. 4c of the mitraphylline and Rifat leaf spectra under CID conditions showed a molecular ion peak at *m*/*z* 369 with fragment peaks at *m*/*z* 337, 309 and 160, confirming the presence of mitraphylline in the plant.

The mass spectra for both Kratom varieties showed a peak at *m*/*z* 291, a mass which corresponds to protonated epicatechin, a compound previously identified in Kratom. In order to determine whether this peak represented epicatechin, the fragmentation pattern obtained from the CID spectrum of an authentic standard of this compound was compared to the CID spectrum of the Rifat leaf samples. Fig. 5 shows the head to tail plot of the CID spectrum of epicatechin (bottom panel) and the Rifat plant (top panel). Although the fragmentation of pure epicatechin is not extensive, comparison of the two spectra shows that there are no epicatechin fragments in the representative Kratom CID spectrum. This implies that epicatechin is not present in the leaf samples we analyzed. However, the possibility that the plant samples analyzed did contain epicatechin but in amounts too low to detect CID fragments cannot be ruled out.

The prevalence of *M. speciosa* identification protocols that involve detection of alkaloids in plant extracts (as opposed to detection of compounds by direct analysis of plant parts), prompted us to analyze extracts by HR-DART-MS. A typical positive ion mode HR-DART-MS of an ethanol leaf extract is shown in Fig. 6. This head-to-tail plot illustrates that although the major alkaloids (i.e. mitragynine and/or its stereoisomers, 7-hydroxymitragynine, mitraphylline, paynantheine, ±rhynchophylline, ajmalicine and/or its stereoisomers, ±corynoxeine – Fig. 1) as well as the tentatively identified flavonoid epicatechin can be detected by direct analysis of both the fresh leaf and the leaf extract, a significant number of the chemical substances observed in the leaf are not carried up into the ethanol extract. This is exemplified by the fact that the whole leaf analysis showed the presence of approximately 150 compounds, whereas analysis of the extract showed only about 62 compounds.

It has previously been shown that data generated by HR-DART-MS can be used with linear discriminant analysis (LDA) to differentiate species [42–44]. Since the ability to not only identify but also to distinguish between plant species can be particularly important in a forensics context, we sought to determine whether the mass spectral data acquired here could be used as a basis for identifying Kratom and distinguishing between it and other plants of abuse. Toward that end, we used a training set comprised of 29 *M. speciosa* samples (12 Bumblebee, 12 Rifat and 5 samples of unspecified variety). From this dataset, 10 *m*/*z* values representing the most abundant and/or characteristic ions were selected for LDA. As a comparison species, we used mass spectral data of *D. ferox*, *D. inoxia*, and *D. wrightii*. The seeds of these plants which are colloquially known as "devil's trumpets" have reportedly been used for millennia for the psychotropic effects of their tropane alkaloids. Representative mass spectra of an average in each case of five *D. ferox*, *D. inoxia*, and *D. wrightii* seeds are shown in Supplementary Fig. 2. Fig. 7 shows the LDA results and illustrates not only the clustering of the *M. speciosa* and *Datura* data, but also that the two taxa are clearly differentiated.

Supplementary Fig. 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.forsciint.2014.07.005.

Our observation of *m*/*z* values both common and unique to Bumblebee and Rifat mass spectra raised the possibility that the two varieties could be distinguished through LDA of the mass spectral data. LDA was employed using a combination of masses common and unique to both varieties, with these masses being the same as those used in the LDA that compared Kratom to *Datura*. These included the accurate masses for peaks at *m*/*z* values 415, 399, 385, 369, 291, 145, 127, 59 (Table 1), as well as *m*/*z* values 316 and 152. The results, shown in Fig. 8, indicate that the two Kratom varieties can be differentiated by LDA based on differences in their chemical signatures. Thus, the Bumblebee strain, represented by blue circles, is clustered and well resolved from the Rifat strain (represented by red squares).

In order to determine whether LDA could be used to discriminate between *M. speciosa* varieties, a plant whose variety was unknown to the analyst was purchased from an online vendor. Plant leaves were analyzed in a manner similar to that described

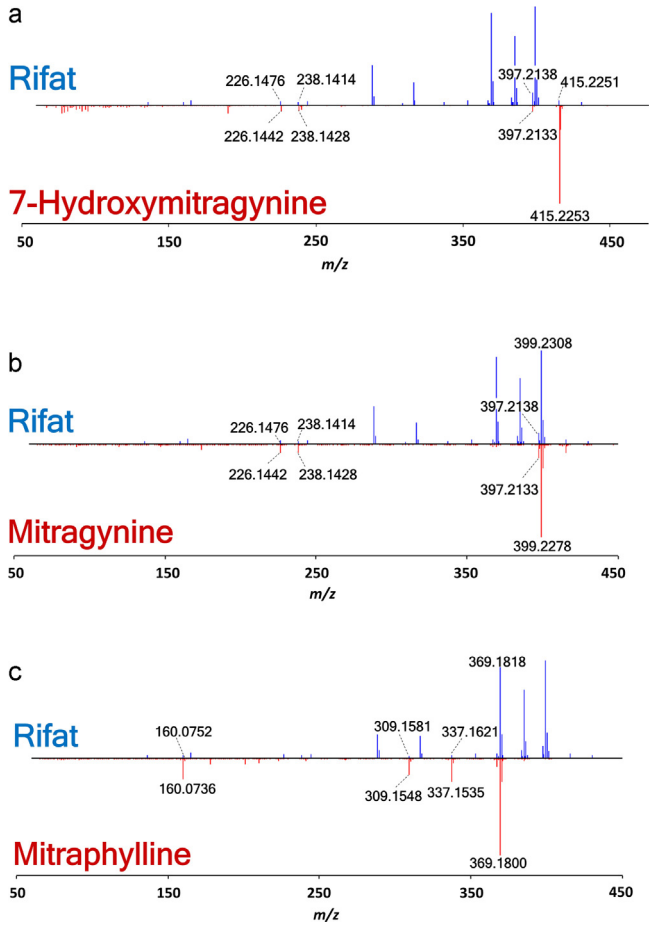

Fig. 4. Positive ion mode HR DART-TOF-MS under collision induced dissociation (CID) conditions of *M. speciosa* leaf extracts (in ethanol) and alkaloid standards. Panel a: head-to-tail plot of CID spectra of Rifat extract and 7-hydroxymitragyinine standard. The protonated molecular ion (*m*/*z* 415) as well as the fragment peaks at *m*/*z* 397, 238 and 226 are present in the spectra of both the extract and the standard. Panel b: head-to-tail plot of CID spectra of Rifat extract and mitragynine standard. The protonated molecular ion (*m*/*z* 399) as well as the fragment peaks at *m*/*z* 397, 238 and 226 are present in the spectra of both the extract and the standard. Panel c: head-to-tail plot of CID spectra of Rifat extract and mitraphylline standard. The protonated molecular ion (*m*/*z* 369) as well as the fragment peaks at *m*/*z* 337, 309 and 160 are present in the spectra of both the extract and the standard. The statistics associated with the mass spectra presented here are outlined in Table 2.

earlier, and the results were imported into the previously compiled data set used for LDA. The results are shown in Fig. 8. The unknown, depicted by black circles, was well clustered alongside the red squares, indicating correctly that the plant was of the Rifat variety.

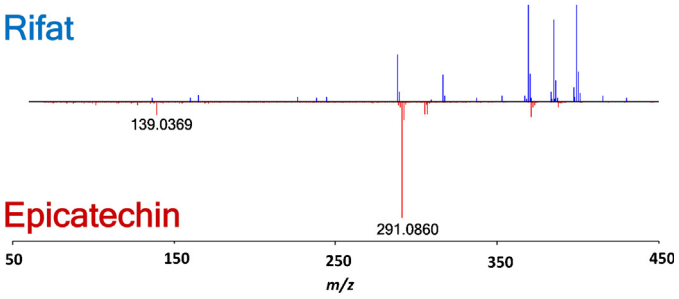

Fig. 5. Head-to-tail plot of the CID spectra of Rifat extract and epicatechin standard. The epicatechin molecular ion (*m*/*z* 291) and the fragment peak at *m*/*z* 139 are observed only in the standard mass spectrum.

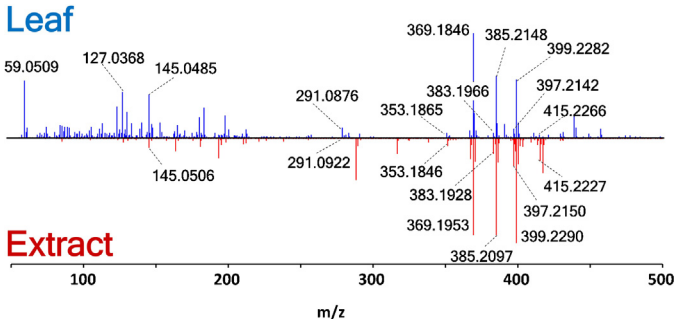

Fig. 6. Positive ion mode HR DART-TOF mass spectra of a leaf clipping and an ethanol extract of the leaf, rendered as a head-to-tail plot. Although the spectra show a large number of peaks in common, including those corresponding to major alkaloids, many more peaks representing individual leaf constituents are observed in the spectrum of the leaf, as opposed to that of the extract.

4. Discussion

The work reported here was prompted by a desire to develop a method that could be used to rapidly triage plant-based forensic evidence to confirm or rule out the presence of *M. speciosa* and distinguish it from other plant material that might be present. The need for such a procedure is increasingly urgent. The advent of the Internet has provided unprecedented access to psychotropic plant products that is unencumbered by the previous constraints of

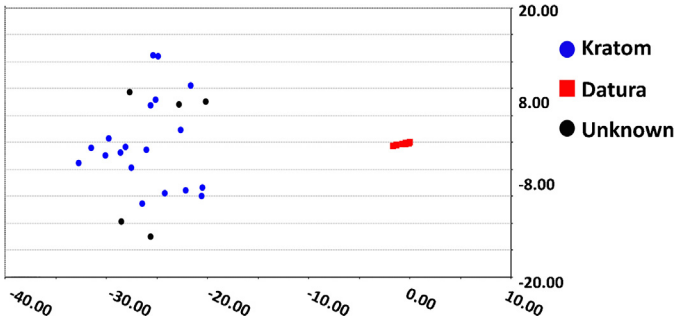

Fig. 7. Linear discriminant analysis (LDA) of mass spectral data derived from HR-DART-MS analysis of Kratom and *Datura* plant material. The plot shows that Kratom and *Datura* plant material can be distinguished based upon 10 principal components. Kratom (blue circles) and *Datura* (red squares) are clustered and well resolved with 100% leave-one-out-cross validation (LOOCV). The unknown plant (black circles) was correctly classified as Kratom with greater than 99% probability in each of the test analyses.

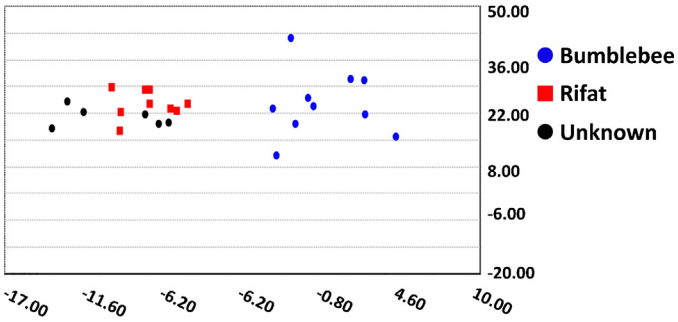

Fig. 8. Linear discriminant analysis (LDA) of Kratom varieties. The LDA plot shows the classification of Bumblebee and Rifat based upon 10 principal components. The two varieties (Bumblebee shown as blue circles and Rifat shown as red squares) are clustered and well resolved. The unknown plant (black circles) was correctly classified as Rifat in 100% of the test analyses.

distance and location. Whereas three decades ago, abuse of *M. speciosa* was restricted primarily to Thailand and Malaysia, its use is now global in scope. Indeed, *M. speciosa* ingestion has now become an increasingly challenging problem for law enforcement agencies in the United States, for example.

Currently, the sheer volume of possible psychotropic plants that can be abused poses challenges for identification efforts because there are in principle a myriad of different protocols that might have to be invoked to ascertain the identity of plant-based evidence. Exploration of all of these is simply not practical, particularly in a crime scene investigation context. Even if it is suspected that a particular plant is being abused, the procedures involved in confirming this can dramatically slow the progress of the investigation. In the case of Kratom, many of the available protocols for its identification are highly specialized and are time consuming enough that it is not practical for crime laboratories to routinely apply them unless investigators receive credible independent information indicating that Kratom was used. Thus, the challenge of identifying plant-based evidence outside of common visually identifiable species like the opium poppy or cannabis, does not lend itself to resolution through the use of methodologies based on well-established hyphenated techniques (e.g. GC–MS or LC–MS), painstaking analysis by microscopy, or time consuming plant DNA analysis simply because for most plants of abuse, such protocols do not exist. However, these methods are excellent when directed toward more definitive confirmatory analyses, particularly in cases where information on stereoisomer identity is desired [18–20,45].

We proposed that one way in which to address this problem is to identify unique and characteristic biomarkers for psychotropic plants of interest, whose presence or absence can be used to inform more definitive determinations of plant identity. Furthermore, we posited that HR-DART-MS can be used to rapidly detect such biomarkers. We used *M. speciosa* to illustrate this principle. The *Mitragyna* family is known to contain ten species of plants, six of which are Asian, and four of which are African. Although *Mitragyna* species contain several characteristic alkaloids, the two which have been established to have central nervous system activity in mammalian systems are mitragynine and 7-hydroxymitragynine. Importantly, of the ten known species in the family, these two alkaloids have only been found to be present in *M. speciosa* [2]. Therefore, *M. speciosa* provided a good test case to investigate the hypothesis that HR-DART-MS could be used to rapidly determine whether fresh leaf material could be identified as *M. speciosa*.

Most of the analytical methods that have been developed to detect the presence or use of *M. speciosa* or its purified active components have focused on detection of mitragynine and/or 7-hydroxymitragynine. However, there exist in *M. speciosa* three other compounds that are known stereoisomers of mitragynine: mitraciliatine, speciogynine, and speciociliatine (Fig. 1). In the majority of published reports, the methods that have been developed to identify or quantify mitragynine do not permit distinction between it and its stereoisomers. These include protocols that utilize TLC, HPLC, GC [29,46] and HPLC with UV diode array detection [47]. On the other hand, a few methods do exist that allow identification and quantitation of *M. speciosa* stereoisomeric alkaloids by the aforementioned methods as well as supercritical fluid extraction with CO_2 [45]. All of these methods involve sample pre-preparation steps such as grinding of the sample, extractions, pH adjustments, or sample derivatization, with the extraction steps alone often requiring upwards of 24 h in some cases. Thus, even if the analysis by TLC, HPLC, GC or MS is relatively short, varying from 10 to 40 min, the sample pre-preparation steps often result in long overall sample analysis times. Indeed, these challenges are contributing to sample testing backlogs in the US [48,49]. It should also be noted that as stated previously, many of the alkaloids found in *M. speciosa* have stereoisomers that are often difficult to differentiate by mass spectrometry alone. However, since the presence of the alkaloids mitragynine (and its stereoisomers) and 7-hydroxymitragynine have only been detected in *M. speciosa*, the masses associated with these alkaloids can still serve as chemotaxonomic identifiers or chemical markers for the species.

We were able to confirm that HR-DART-MS can be used to rapidly identify compounds consistent with the structures of mitragynine and/or its isomers, 7-hydroxymitragynine and mitraphylline in Bumblebee and Rifat varieties of Kratom. Simply suspending a leaf segment in the open air space between the ion source and the mass spectrometer inlet produced mass spectra that were acquired within ~3 s. The soft ionization conditions under which the analyses were conducted resulted in ion chromatograms that showed the protonated forms of >150 compounds in each case. Even so, the HR masses associated with the compounds of interest were clearly discernable and unobscured. Importantly, the plant material could be analyzed directly, and no extractions or other sample pre-preparation steps were necessary. *By this method, 250 leaf samples could be analyzed in 1 h!*

The merits of the use of the 12 sample wire mesh OS card vis-à-vis high throughput sampling deserve comment. Although we observed that analysis of fresh leaf clippings provided data that enabled definitive identification of the plant, we also sampled the leaf using a more traditional extraction method involving analysis of an ethanol leaf extract. This allowed us to compare the results obtained by sampling the leaf directly, to those obtained by using a traditional extraction protocol. Our results demonstrated that MS analysis of 4 μL of ethanol extract that was deposited onto the wire mesh of the OS sampling card, yielded spectra which exhibited the major peaks observed in the fresh sample. Interestingly but perhaps unsurprisingly, a significant amount of information available in the direct leaf analysis was lost in the extract. For example, whereas 141 unique compounds were observed in the HR-DART-MS spectrum of the Rifat variety, only 62 unique peaks were observed in the ethanol extract. This represents a drop in information content of ~56%. Nevertheless, from the point of view of identification of Kratom based on the presence of the distinctive mitragynine isomers and 7-hydroxymitragynine, the extract still serves the purpose, since these prominent alkaloids are still observed. Compared with previously published protocols for the detection of *M. speciosa* alkaloids, analysis of the extract is still extremely rapid, since ~150 individual extracts could be analyzed in 1 h using the wire mesh OS QuickStrip™ sampling cards.

Since the spectra yielded peaks corresponding to protonated unfragmented molecular ions, we sought to confirm our tentative structural assignments by fragmenting authentic standards in those cases where they were available, and comparing the fragmentation patterns in each case to the fragments observed in the CID spectra of the plant samples. A representative example of the results of this type of experiment is shown in Fig. 4a. The head-to-tail plot shows that the fragments formed from 7-hydroxymitragynine under CID conditions are present in the CID spectrum of the leaf sample, and that these fragments are formed at the expense of the molecular ion peak. By this method, we were able to confirm that the peaks corresponding to m/z 399, 415, and 369 in the *M. speciosa* plants corresponded to mitragynine or its stereoisomers, 7-hydroxymitragynine, and mitraphylline, respectively. The mass spectrum of the 7-hydroxymitragynine standard under CID conditions produced characteristic peaks at m/z 415, 397, 238 and 226 which matched fragment peaks of the plant material (Fig. 4a), confirming the presence of 7-hydroxymitragynine. Statistics associated with these spectra are listed in Table 2. The peak at m/z 291 implying the presence of epicatechin was not observed in the CID spectrum. This was not unexpected, as the low

Table 2
Mass spectral statistics associated with CID HR-DART-MS analysis of mitragynine, 7-hydroxymitragynine, and mitraphylline in standards and plant extracts.

	Compound	Composition	Measured	Calculated	Difference (mmu)
Standards	Mitragynine (and/or its stereoisomers)	$C_{23}H_{30}N_2O_4 + H^+$	399.2278	399.2284	0.6
		$C_{23}H_{28}N_2O_4$	397.2133	397.2127	−0.6
		$C_{13}H_{20}NO_3$	238.1428	238.1443	1.5
		$C_{12}H_{20}NO_3$	226.1442	226.1443	0.1
	7-Hydroxymitragynine	$C_{23}H_{30}N_2O_5 + H^+$	415.2253	415.2233	−2
		$C_{23}H_{28}N_2O_4$	397.2133	397.2127	−0.6
		$C_{13}H_{20}NO_3$	238.1428	238.1443	1.5
		$C_{12}H_{20}NO_3$	226.1442	226.1443	0.1
	Mitraphylline	$C_{21}H_{24}N_2O_4 + H^+$	369.1800	369.1814	1.4
		$C_{20}H_{21}N_2O_3$	337.1535	337.1552	1.7
		$C_{19}H_{21}N_2O_2$	309.1548	309.1603	5.5
		$C_{10}H_{10}NO$	160.0752	160.0762	1.0
Plant extracts	Mitragynine (and/or its stereoisomers)	$C_{23}H_{30}N_2O_4 + H^+$	399.2308	399.2284	−2.4
		$C_{23}H_{28}N_2O_4$	397.2138	397.2127	−1.1
		$C_{13}H_{20}NO_3$	238.1414	238.1443	2.9
		$C_{12}H_{20}NO_3$	226.1476	226.1443	−3.3
	7-Hydroxymitragynine	$C_{23}H_{30}N_2O_5 + H^+$	415.2251	415.2233	−1.8
		$C_{23}H_{28}N_2O_4$	397.2138	397.2127	−1.1
		$C_{13}H_{20}NO_3$	238.1414	238.1443	2.9
		$C_{12}H_{20}NO_3$	226.1476	226.1443	−3.3
	Mitraphylline	$C_{21}H_{24}N_2O_4 + H^+$	369.1818[a]	369.1814	−0.4
		$C_{20}H_{21}N_2O_3$	337.1621	337.1552	−6.9
		$C_{19}H_{21}N_2O_2$	309.1581	309.1603	2.2
		$C_{10}H_{10}NO$	160.0752	160.0762	1.0

[a] Successive spectra showed a higher measured value (369.2042). This increase in mass relative to the value of 369.1818 is attributed to an unresolved interference from a cholesterol fragment that is evident from an observed shoulder peak in the 90 V spectrum.

abundance of this peak in the original spectrum would likely have resulted in any fragment peaks being of low enough abundance to be lost within the baseline noise. Moreover, due to the many hydroxyl groups in its structure, epicatechin is more likely to be seen under negative ion conditions, an experiment that was not performed in this study since the focus was on alkaloids.

The potential utility of the method used here to facilitate identification of *M. speciosa* fresh plant material lies in part with the demonstration that comparison of the mass spectra of leaves of an unknown plant with the mass spectral data generated here, would enable determination of whether or not it is Kratom. A preliminary test of this possibility was conducted using the LDA statistical method. We first developed a minimized profile comprised of 10 characteristic peaks and their corresponding intensities, each of which represented either metabolites that were tentatively identified as known characteristic alkaloids, or common sugar pyrolysis fragments. The alkaloid leaf constituents are distinguishing features of Kratom and to date, every publication that has appeared detailing Kratom secondary metabolite analysis has shown that the characteristic distinguishing alkaloids are present [3,6,11,15,18–22,50,51]. No other plant species has been observed to contain the psychotropic alkaloids identified in *M. speciosa*. The mass spectra of plant material from other plant species that are ingested for their psychotropic effects, namely *D. ferox*, *D. inoxia* and *D. wrightii*, were used to determine if the LDA based on the 10 peak basis set (derived from Kratom mass spectra) would enable differentiation of the two taxa. The resulting LDA scatter plot (Fig. 7) showed that the *Mitragyna* and *Datura* species were well separated, with all the Kratom plants being correctly classified as *M. speciosa* plants, and the *Datura* plants being clustered in a different region. Furthermore, it was observed that of the approximately 150 peaks present within a mass range of *m*/*z* 50–500 in a typical Rifat or Bumblebee spectrum, the 10 that were selected for the LDA were consistently observed to be the most useful biomarkers for differentiation between the two varieties. A head-to-tail plot of the mass spectra of the Bumblebee and Rifat varieties (Fig. 3) showed that these 10 principal components (PCs) could be matched to peaks that were either unique to one plant (e.g. *m*/*z* 152) or were present in both plants but with significant differences in intensity (e.g. *m*/*z* values of 59, 127, 145, 291, 369, 385, 399 and 415). A LDA scatter plot (Fig. 8) showed clustering of the data sets for the Rifat and Bumblebee varieties that was well resolved. Additionally, the Mass Mountaineer software algorithm was used to assess the recognition capability of the LDA dataset for an "unknown" *Mitragyna* variety. The result (Fig. 8) was a recognition capability of 100% (black circles), and thus the plant was correctly classified as being of the Rifat variety, which confirmed the information about the plant's identity that was provided by the vendor. Thus, although the mass spectral peaks observed were very consistent for samples within a specified *M. speciosa* variety, the relative abundance of the peaks varied modestly from sample to sample and this is reflected in the scatter that appears within each of the clusters (i.e. for Rifat and Bumblebee Kratom varieties) in the LDA plot. Nevertheless, the clustering is well enough defined that the two *M. speciosa* varieties are distinguishable by LDA (Fig. 8). This indicates that each variety is unique enough to be distinguished from the other, based on the consistency of their respective chemical profile differences. Importantly, both varieties can correctly be identified as Kratom, and can clearly be distinguished from other plants, such as *Datura* species.

Although the total number of plants analyzed was limited, we propose that the use in the LDA of *m*/*z* values of the distinguishing alkaloids will enable identification of *M. speciosa* plants from many different varieties, including those not included in this study. This hypothesis is being investigated.

5. Conclusions

HR-DART-MS was used to prove the principle that *M. speciosa*, a psychotropic plant of abuse, can be identified solely based on the chemical signature observed through mass spectrometric analysis of its leaves. The method is robust and data acquisition is rapid, with sample analyses requiring only a few seconds, and no sample pre-preparation steps are needed. Furthermore, a classification system to discriminate and distinguish between *M. speciosa* and plants of a different genus, as well as two varieties of *M. speciosa* was developed and tested with an unknown, yielding a correct classification at 100% probability. This facile and rapid method

demonstrates the utility of HR-DART-MS as a means to identify and classify plant material that might be seized as evidence at a crime scene, without the need for conventional extraction or analysis methods.

Acknowledgements

The support of the Research Foundation of the State University of New York, as well as a National Science Foundation grant to RAM and RBC (Grant #1310350) are gratefully acknowledged.

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