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Method Title: Identification and Characterization of Indole and Oxindole Alkaloids from Leaves of *Mitragyna speciosa* Korth Using Liquid Chromatography–Accurate QToF Mass Spectrometry

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DIETARY SUPPLEMENTS

Identification and Characterization of Indole and Oxindole Alkaloids from Leaves of *Mitragyna speciosa* Korth Using Liquid Chromatography–Accurate QToF Mass Spectrometry

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Alkaloids have been reported to be the major physiologically active constituents in *Mitragyna*. An analytical method was developed to provide an alternative, fast method for characterization of alkaloids from various *M. speciosa* samples. The separation was achieved using an RP octylsilyl (C8) column, MS detection, and a water–acetonitrile with formic acid gradient as the mobile phase. Ultra-HPLC/quadrupole time-of-flight MS analysis and characterization were performed on 12 corynanthe-type indole/oxindole alkaloids obtained from the leaves of *M. speciosa* Korth. The indoles and oxindoles had an open E ring with or without substitution occurring at the C9 position. The full single mass spectrum of alkaloids showed a strong signal for the protonated molecule $[M+H]^+$. The product ion spectrum of mitragynine type of alkaloids showed strong response at *m/z* = 174.0901 suggestive of an ion containing an odd number of nitrogen atoms corresponding to formula $C_{11}H_{12}NO$, which is characteristic of indole alkaloids. A multivariate statistical analysis technique, principal component analysis, was used to show discrimination between the *M. speciosa* samples. The results indicated that the analytical method is suitable for QC testing of various *Mitragyna* commercial samples and can be used to evaluate market products purported to contain *M. speciosa*.

Extracts of the leaves of *Mitragyna speciosa* Korth (family Rubiaceae) have been used traditionally as a substitute for opium in Thailand and Malaysia (1–4). Many pharmacological studies have been conducted on the alkaloidal extracts of *M. speciosa* leaves (1–5). Over 20 indole or oxindole alkaloids have been isolated from this plant, which has been a good source of alkaloids. The alkaloidal content varied based on locality and time of collection. Mitragynine, a major constituent of *M. speciosa*, has an opioid agonistic activity and was one of the first 9-methoxy corynanthe-type alkaloids isolated from young leaves of *M. speciosa*. A minor constituent, 7-hydroxymitragynine, is much more potent than mitragynine or morphine (1, 2). Compounds like isocorynantheidine, isopaynantheine, and mitraciliatine with 3*R* configuration are the dominant ones, although speciogynine, an indole alkaloid with 3*S* configuration, also occurs as one of the main alkaloids in 2-year-old plants (6).

Several analytical methods have been reported for the quantitation of mitragynine alone or with a few other alkaloids (3, 4, 7–15). Based on the literature, mitragynine was found to be the major constituent followed by other compounds, i.e., speciogynine, speciociliatine, and paynantheine (3). Parthasarathy et al. (7) developed an analytical method for qualitative and quantitative analysis of mitragynine using HPLC-diode array detector for plant samples and commercial kratom drinks. Another group developed methods using LC/electrospray ionization (ESI)-MS (8) and GC/MS (7) for the analysis of mitragynine. Profiling of diastereoisomer alkaloids (mitragynine, mitraciliatine, speciogynine, and speciociliatine) from plant samples of kratom was developed using nonaqueous capillary electrophoresis/MS (10). The major alkaloids of *M. speciosa* (mitragynine, paynantheine, speciogynine, and speciociliatine) and several metabolites have been detected in the urine of rats and humans following ingestion of plant extract or its individual alkaloids (11–17). Recently, our group isolated a series of alkaloids from *M. speciosa* (18) that have been used to develop the separation method and characterization described in this article.

The objective of this described work was to develop a single qualitative LC/quadrupole time of flight (QToF)-MS/MS method for the separation, characterization, and chemical profiling of alkaloids in association with chemometric analysis not only for assessing quality but also for the study of the variations in active constituents among samples of *M. speciosa*. Usually all alkaloids occur in multicomponent mixtures, and separation of these from other groups of compounds is the first requirement

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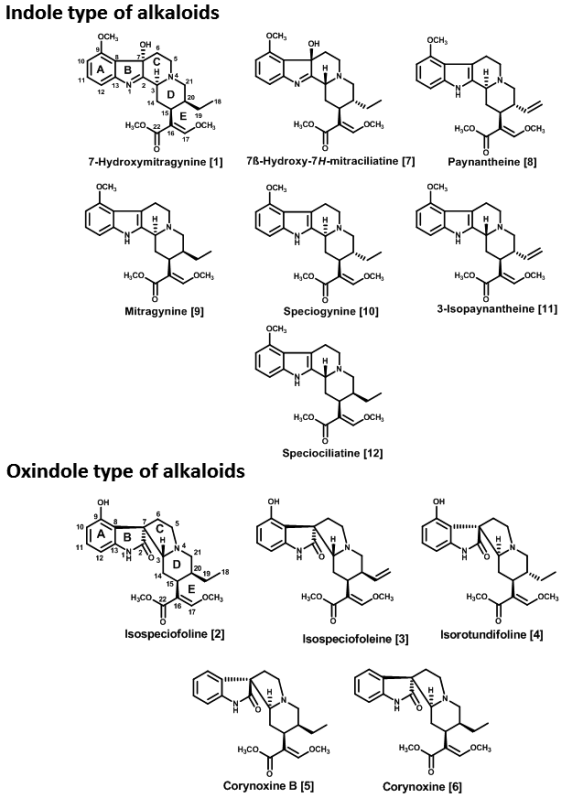

Figure 1. Structures of reference compounds.

for detailed structural analysis of alkaloids. This paper describes a method to resolve and characterize 12 indole and oxindole diastereomer alkaloids. The instrumentation consists of an ultra-HPLC (UHPLC) system coupled with a QToF mass spectrometer that can be used for chemical fingerprinting analysis of *M. speciosa* and is also suitable for the QC of various commercial samples. The fragmentation patterns for 7-hydroxymitragynine [**1**], isospeciofoline [**2**], isospeciofoleine [**3**], isorotundifoline [**4**], corynoxine B [**5**], corynoxine [**6**], 7β-hydroxy-7*H*-mitraciliatine [**7**], paynantheine [**8**], mitragynine [**9**], speciogynine [**10**], 3-isopaynantheine [**11**], and speciociliatine [**12**] were studied with proposed structures (Figure 1) for each significant product ion. With this characterization and chromatographic optimization, alkaloidal mixtures containing a large number of diastereoisomers were separated in extracts of *M. speciosa* leaves. The method offered more information about the chemical constituents of *M. speciosa* with the diastereomeric alkaloids identified and characterized according to retention times (RTs) and mass spectra.

Experimental

UHPLC/QToF-MS Instrumentation and Conditions

The UHPLC system was an Agilent Technologies (Santa Clara, CA) Series 1290 comprising the following modular components: a binary pump, vacuum solvent microdegasser, autosampler with 100-well tray, and thermostatically controlled column compartment. Separation was achieved on an Agilent Zorbax SB-octylsilyl (C8) RRHD column (2.1 × 100 mm, 1.8 µm). The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.23 mL/min, with the gradient elution program as given in Table 1.

Each run was followed by a 5 min wash with 100% B and an equilibration period of 5 min with 85% A/15% B. A 2 µL volume of sample was injected. The column temperature was 35°C. The mass spectrometric analysis was performed with a hybrid QToF mass spectrometer (Model No. G6530A, Agilent Technologies) equipped with an ESI source with Jet Stream technology using the following parameters: drying gas (N_2) flow rate, 9.0 L/min; drying gas temperature, 250°C; nebulizer, 35 psig; sheath gas temperature, 325°C; sheath gas flow, 10 L/min; capillary, 3500 V; skimmer, 65 V; Oct radio frequency (RF) V, 750 V; and fragmentor, 125 V. All operations and acquisition and analysis of data were controlled by Agilent MassHunter Acquisition software version A.05.00 and processed with MassHunter Qualitative Analysis software Version B.06.00. Each sample was analyzed in the positive ion mode over the range of *m/z* = 100–1100 and extended dynamic range (flight time to *m/z* 1700 at 2 GHz acquisition rate). Accurate mass measurements were obtained by means of reference ion correction using reference masses at *m/z* 121.0509 (protonated purine) and 922.0098 [protonated hexakis (1*H*, 1*H*, 3*H*-tetrafluoropropoxy) phosphazine or HP-921] in the positive ion mode, while *m/z* 112.9856 [deprotonated trifluoroacetic acid (TFA)] and 1033.9881 (TFA adducted HP-921) were used in the negative ion mode. The compounds were confirmed in each spectrum. For this purpose, the reference solution was introduced into the ESI source via a T-junction using an Agilent Series 1200 isocratic pump and a 100:1 splitter set at a flow rate of 20 µL/min.

For recording ToF mass spectra, the quadrupole was set to pass all ions (RF only mode), and all ions were transmitted into the pusher region of the ToF analyzer where they were mass analyzed with a 1 s integration time. For the ESI-MS/MS collision induced dissociation (CID) experiments, precursor ions of interest were mass selected by the quadrupole mass filter. The selected ions were then subjected to collision with nitrogen in a high pressure collision cell. The collision energy was optimized to afford good product ion signals, which were subsequently analyzed with the ToF mass spectrometer. Analysis was performed in the reflectron mode with a resolving power of about 10,000 at *m/z* 922. The instrument was set to the extended dynamic range (up to 10^5 with lower resolving power). MS/MS spectra were recorded simultaneously at a rate of 2.0 spectra/s. In order to filter selected precursor ions and their isotopes for MS/MS, an isolation window of 1.3 *m/z* was set for the quadrupole. MS/MS studies were performed by isolating $[M+H]^+$ ions. The fragmentation pattern was obtained

Table 1. Mobile phase gradient elution program

Time, min	A, %	B, %
0.00	85	15
20.00	65	35
30.00	0	100
35.00	0	100

for the molecular ions of the 12 alkaloids by means of CID of the protonated molecule.

Chemicals and Plant Materials

(**a**) *Chemicals.*—Eleven reference indole alkaloids, isospeciofoline [**2**], isospeciofoleine [**3**], isorotundifoline [**4**], corynoxine B [**5**], corynoxine [**6**], 7β-hydroxy-7*H*-mitraciliatine [**7**], paynantheine [**8**], mitragynine [**9**], speciogynine [**10**], 3-isopaynantheine [**11**], and speciociliatine [**12**], were isolated at the National Center for Natural Products Research (NCNPR, University of Mississippi, University, MS); their identity and purity were confirmed by chromatographic (TLC, HPLC) methods, by the analysis of the spectral data [IR, one dimensional (1D)- and two dimensional (2D)-NMR, and ESI-high resolution (HR)MS], and comparison with published spectral data (18–21). The purity of these isolated compounds was greater than 90%. The alkaloid 7-hydroxymitragynine [**1**] was obtained from Chromadex (Santa Ana, CA). Acetonitrile and formic acid were of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ). Water for the HPLC mobile phase was purified using a Milli-Q system (EMD Millipore Corp., Billerica, MA).

(**b**) *Plant materials.*—Dried whole leaves of *M. speciosa* (No. 12433) were purchased online, and the leaf samples were authenticated morphologically by Vijayasankar Raman, botanist at the NCNPR. Dried leaf powder of Nos. 2796, 10852-10862, and 10869–10873 were also purchased online. Samples No. 10869-10873 were obtained from the website address www.ethnobotanicals.com, and samples No. 12433 and 10852–10862 were obtained from the website address www.bouncingbearbotanicals.com. All these samples were labeled as *M. speciosa*. Specimens of all samples are deposited at the NCNPR's botanical repository at the University of Mississippi.

Standards

All standards were prepared in a concentration range from 0.5 to 2 μg/mL in methanol.

Sample Preparation

(**a**) *Methanol extraction.*—A 50 mg amount of dry plant samples was sonicated (Mechanical Ultrasonic Cleaner, Fisher Scientific) in 2.5 mL methanol for 30 min followed by centrifugation (Centrific Centrifuge, Fisher Scientific) for 15 min at 959 × *g*. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated three times, and the supernatants were combined. The final volume was adjusted to 10 mL with methanol and mixed thoroughly. Prior to injection, the solution was passed through a 0.45 μm PTFE membrane filter (Millex Samplicity, EMD Millipore Corp.). The first 1.0 mL was discarded, and the remaining volume was collected in an LC sample vial. For plant samples in which a few constituents were highly concentrated, the sample solutions were further diluted by a factor of 10.

(**b**) *Acid-base extraction.*—The separation of the alkaloids from the methanolic extract of *M. speciosa* (No. 12433) was carried out by mixing with 0.1 N hydrochloric acid solution. Subsequently, the acidic solution was rendered alkaline (pH 9–10) with 10% dilute NH_4OH solution to liberate the alkaloids from other components. The basified solution was then extracted with ethyl acetate. The solvent was removed under reduced pressure, and the traces of moisture were removed with anhydrous sodium sulfate. The dried samples were dissolved in methanol prior to injection.

Results and Discussion

The QToF mass analyzer provided identification of compounds using accurate masses of full spectra in MS and MS/MS modes. Accurate mass determination leads to chemical formula identification that can provide structural information when forming product ions using MS/MS. The large amount of data was mined using the Agilent Molecular Features Extractor, which resolved coeluting interferences and grouped the isotopic clusters, all adducts, dimers, and trimers if present.

The total ion chromatograms of alkaloids from the *Mitragyna* extracts were obtained using UHPLC/QToF-MS in the positive ESI mode. In Table 2, the MS and MS/MS data for compounds **1**–**12** detected in the positive ESI mode are listed, including the proposed molecular formulas and possible compounds on the basis of RT, calculated mass, and information from reference standards. The mass spectra of reference compounds were investigated in the positive ESI mode (*see* Supplemental Material Figure 1S on *J. AOAC Int*. website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

Data Processing

MassHunter Workstation software, including Qualitative Analysis (version B.06.00), was used for processing both raw MS and MS/MS data, including molecular feature extraction, background subtraction, data filtering, and molecular formula estimation. To perform subtraction of molecular features (MFs) originating from the background, analysis of a blank sample (methanol) was carried out under identical instrument settings, and background MFs were removed. MassHunter was used to generate molecular formulas, searched in an in-house generated library. Ions with identical elution profiles and related *m/z* values were extracted as a single MF within the algorithm used for full MS data. MFs were characterized by RT, intensity at the apex of the chromatographic peak, and accurate mass. Various intensity thresholds, i.e., 1000, 5000, and 10 000 counts per second (cps) were tested for MF extraction in the RT range from 5 to 25 min. MS profiles of the blank sample (methanol) were subtracted from the samples to correct for the background. Background subtracted data were converted into compound exchange format files for further use in Mass Profiler Professional (MPP). MPP (Agilent, version 12.6) was used for statistical evaluation of technical reproducibility and comparison of leaf samples of *M. speciosa*. In MPP, the RT and *m/z* alignment across the sample sets was performed using a tolerance window of 0.2 min and 20 mD.

Method Development

Targeted analysis of reference compounds and auto-MS/MS analysis of *M. speciosa* methanol extracts of leaves were performed using an RP chromatographic system without considering any specific group of compounds. UHPLC/QToF-MS conditions

Table 2. Accurate mass measurements of the compounds observed in the ESI-MS and ESI-MS/MS spectra with RT

No.	RT, min	Calculated *m/z* $[M+H]^+$	Precursor ion experimental *m/z* $[M+H]^+$	Error, mDa	Elemental composition	Major ions in MS/MS spectra (key fragment ions)	Identification
1	7.94	415.2227	415.2217	–1.0	$C_{23}H_{29}N_2O_5$	397.2132 (24%) 190.0851 (100%) 110.0964 (16%)	7-Hydroxymitragynine
2	8.66	401.2071	401.2066	–0.5	$C_{22}H_{27}N_2O_5$	369.1792 (12%) 257.1269 (100%) 203.0799 (33%) 176.0696 (45%)	Isospeciofoline
3	8.69	399.1944	399.1913	–3.1	$C_{22}H_{26}N_2O_5$	283.1418 (33%) 217.0963 (30%) 203.0803 (28%) 176.0694 (100%) 148.0744 (27%) 108.0800 (30%)	Isospeciofoleine
4	10.61	401.2071	401.2066	–0.5	$C_{22}H_{28}N_2O_5$	257.1266 (30%) 176.0695 (100%) 148.0737 (33 %) 110.0960 (51%)	Isorotundifoline
5	10.82	385.2122	385.2119	–0.3	$C_{22}H_{28}N_2O_4$	353.1836 (5%) 241.1322 (92%) 187.0852 (51%) 160.0746 (100%) 110.0959 (29%)	Corynoxine B
6	11.54	385.2122	385.2116	–0.6	$C_{22}H_{28}N_2O_4$	353.1845 (5%) 241.1321 (74%) 187.0850 (39%) 160.0742 (100%) 110.0958 (27%)	Corynoxine
7	12.89	415.2227	415.2224	–0.3	$C_{23}H_{30}N_2O_5$	397.2101 (72%) 281.1630 (30%) 227.1164 (100%) 190.0853 (15%)	7β-Hydroxy-7*H*-Mitraciliatine
8	17.43	397.2122	397.2124	0.2	$C_{23}H_{28}N_2O_4$	236.1266 (12%) 224.1268 (14%) 174.0907 (100%) 159.0668 (9%) 108.0804 (9%)	Paynantheine
9	17.69	399.2278	399.2276	–0.2	$C_{23}H_{30}N_2O_4$	238.1424 (34%) 226.1428 (33%) 174.0901 (100%) 110.0958 (21%)	Mitragynine
10	18.84	399.2278	399.2278	0	$C_{23}H_{30}N_2O_4$	238.1427 (26%) 226.1425 (19%) 174.0906 (100%) 110.0962 (14%)	Speciogynine
11	19.66	397.2122	397.2123	0.1	$C_{23}H_{28}N_2O_4$	236.1275 (12%) 224.1256 (13%) 200.1053 (19%) 174.0904 (100%)	3-Isopaynantheine
12	19.91	399.2278	399.2278	0	$C_{23}H_{30}N_2O_4$	238.1423 (26%) 226.1427 (26%) 174.0903 (100%) 110.0957 (18%)	Speciociliatine

were optimized to detect the maximum number of compounds, including the 12 reference standards. The concentration of sample solutions was optimized to 0.5 mg/mL. QToF provides accurate MS/MS spectra using internal mass calibration during acquisition and mass drift compensation (collected in the single MS mode and applied to the MS/MS as a rolling average). Mass accuracy better than 5 ppm was obtained. Figure 2 represents the base peak chromatogram of an alkaloidal methanolic extract of *M. speciosa.* The mobile phase gradient was optimized to elute all the detectable compounds within 30 min. The elution was continued for an additional 5 min to ensure complete removal of the sample from the column. RT variability across the samples was found to be 3–4 s with an RSD of less than 1–1.5%.

Extraction

The plant extract from *M. speciosa* was a complex mixture of alkaloids with a wide range of polarities. Two extraction techniques were used. First, the plant material (No. 12433) was

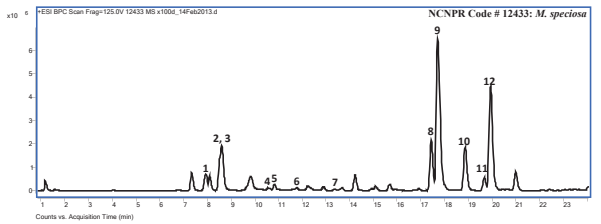

Figure 2. Base peak chromatogram of *M. speciosa* methanolic extract analyzed using UHPLC/QToF-MS in positive ESI mode. Compound numbers are defined in the text.

exhaustively treated with methanol as previously described. Many alkaloids also could be directly extracted from the alcoholic extracts. Second, the methanolic extract (No. 12433) was further purified using acid-base extraction. The extraction procedure for alkaloids from an aqueous acidic medium is based on their general basic properties. The alkaloids form salts in aqueous acidic media that may show improved solubility and stability at low pH values. In addition, protons in the aqueous acidic media may assist in breaking the sample matrix to release the analytes more easily. In comparison, both methods showed the presence of all 12 compounds.

Characterization of Alkaloids

The use of LC/ESI-MS was investigated for the characterization of corynanthe-type indole alkaloids from leaves of *M. speciosa*. The corynanthe-type indole alkaloids have a conjugated pentacyclic skeleton; however, in the alkaloids from *M. speciosa*, the E ring is opened. MS/MS fragmentation of reference standards was carried out and compared with that of alkaloids from plant samples. The structures of compounds **1**–**12** were elucidated by interpretation of spectral data. The use of LC/ESI-MS fragmentation demonstrated the ability to distinguish related compounds based on RTs and product ions. The *M. speciosa* extracts were found to contain many isomeric or isobaric compounds; thus, the availability of reference standards was critical.

Protonation is believed to take place on the amine nitrogen atom. Compounds **1**–**12** showed abundant $[M+H]^+$ ions in the positive ion spectra, which were selected as precursor ions for CID experiments. These compounds were grouped into indole type and oxindole type alkaloids (connected between C3–C7).

Mitragynine type indole alkaloids (7-hydroxymitragynine [1], 7β-hydroxy-7H-mitraciliatine [7], paynantheine [8], mitragynine [9], speciogynine [10], 3-isopaynantheine [11], and speciociliatine [12]).—Mitragynine was the most abundant compound present in the plant (*M. speciosa*) and was isolated as a major compound. Chemically, mitragynine is the 9-methoxy corynantheidine, a molecule structurally related to yohimbine. Mass spectrometric analysis suggested the molecular formula $C_{23}H_{30}N_2O_4$ from the positive HR-ESI-MS data (*m/z* 399.2278 $[M+H]^+$; Table 2). The MS/MS key product ions were *m/z* 238.1424, 226.1428, 174.0901, and 110.0958 (Table 2). The most abundant MS/MS product ion, $[M+H-225]^+$, corresponds to the loss of piperidine derivative ($C_{12}H_{19}NO_3$) to form methyl substituted fragment ion at *m/z* 174.0901 as shown on the suggested fragmentation pathway of this compound (Figure 3). The presence of the even mass fragment ion at *m/z* 174.09 is suggestive of an odd number of nitrogens corresponding to the formula $C_{11}H_{12}NO$ characteristic of indole alkaloids. It also showed a less abundant peak at *m/z* 367.2018 $[M+H-32]^+$, which was expected to arise from a molecule with the proton on the oxygen of the methoxyl moiety present in acrylate group of the molecule. Product ions with *m/z* 367.2018 and 174.0901 contain the indole fragment, while *m/z* 238.1427, 226.1428, and 110.0958 contain only the nonaromatic portion of the molecule. The CID spectrum showed peaks at *m/z* 238.1427, 226.1428, and 110.0958. The peak at *m/z* 238.1427 resulted from the cleavage at the C5 position in the C ring to form the fragment ion $[M+H-161]^+$, the dihydropyridine derivative with loss of methoxy indole moiety. The peak at *m/z* 226.1428 was the ion $[M+H-173]^+$ formed due to the neutral loss of the methoxy indole group; dissociation of the bond takes place between C5 and nitrogen in piperidine in the C ring. The peak at *m/z* 110.0958 was a fragment ion of $[M+H-289]^+$, due to the loss of the methoxy methylacrylate moiety and resulting in formation of a piperidine derivative.

A similar pattern was followed for the compounds speciogynine and speciociliatine ($C_{23}H_{30}N_2O_4$, *m/z* 399.2278 $[M+H]^+$), which are diastereoisomers of mitragynine. Although experimentally, as shown in Table 2, there are differences in the abundances of the product ions of these diastereoisomers, they cannot, within experimental error, be used to distinguish these compounds. However, they were separated chromatographically and could be identified by RT comparison to standards. Paynantheine and isopaynantheine gave protonated molecular ions $[M+H]^+$ at *m/z* 397.2124 that corresponded to the molecular formula of $C_{23}H_{28}N_2O_4$. The alkaloid paynantheine, which is of the 9-methoxycorynantheine type, and isopaynantheine, the dehydro analog of mitraciliatine, showed major fragment product ions at *m/z* 174.09 (Table 2).

Understanding this fragmentation pattern can be helpful to resolve unknown alkaloids in complex mixtures. The major alkaloids *m/z* 399.2276 (calculated *m/z* 399.2278) were identified in Figure 2 to be mitragynine (RT = 17.69 min), speciogynine (18.84 min), speciociliatine (19.91 min), and unknown mitraciliatine (21.00 min) by comparison with reference standards and the literature (11).

In the plant, 7-hydroxymitragynine was a minor compound. HR-ESI-MS gave protonated molecule $[M+H]^+$ at *m/z* 415.2217

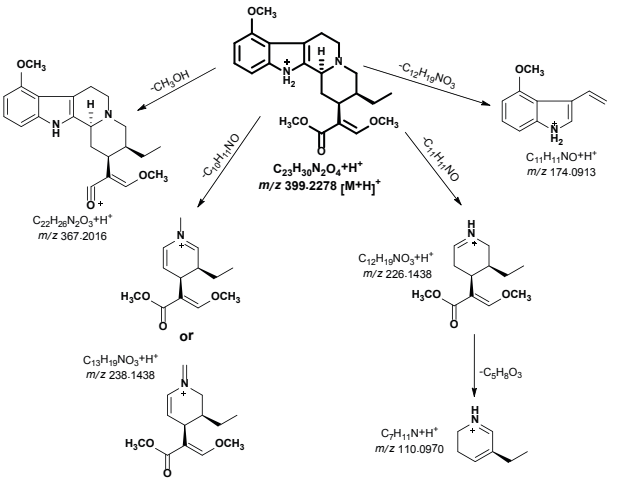

Figure 3. Proposed fragmentation pathway of mitragynine.

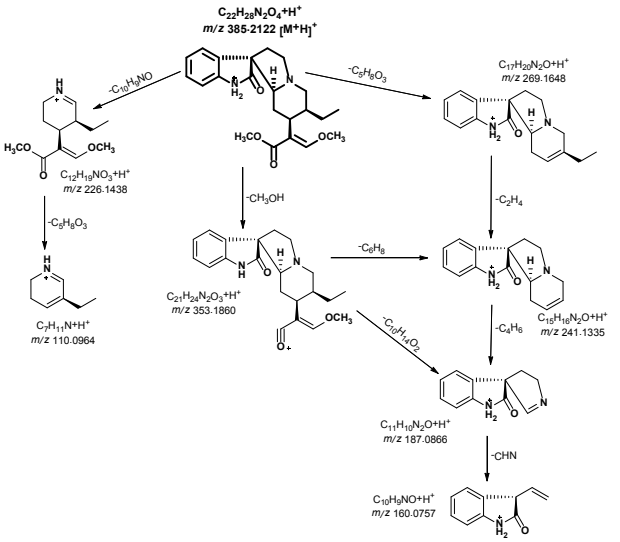

Figure 4. Proposed fragmentation pathway of corynoxine B.

that corresponded to the molecular formula of $C_{23}H_{30}N_2O_5$. The most abundant MS/MS product ion was $[M+H-225]^+$ at *m/z* 190.0891, corresponding to the loss of $C_{12}H_{18}NO_3$. The hydroxyl and methoxyl groups have been confirmed by their fragmentation pattern. The methoxyl group at the C9 position from the published literature (5) and C7 substitution of the hydroxyl group gave *m/z* 190.0891. The key fragments detected were *m/z* 397.2132 $[M+H-18]^+$, 190.0851 $[M+H-225]^+$, and 110.0964 $[M+H-305]^+$ (Table 1). The peak at *m/z* 397.2132 $[M+H-18]^+$ resulted from the loss of H_2O from the molecular ion via the hydroxyl group at the C7 position, which acquired hydrogen ion from the C6 position and resulted in the formation of double bond at positions C6 and C7. The most abundant ion was $[M+H-225]^+$ at *m/z* 190.0891, which is similar to mitragynine except for the presence of the hydroxyl group at position C7.

A similar fragmentation pathway was observed for 7β-hydroxy-7*H*-mitraciliatine, which gave a protonated molecule $[M+H]^+$ at *m/z* 415.2224 that corresponded to the molecular formula of $C_{23}H_{30}N_2O_5$. These two compounds were differentiated based on RTs (Table 2).

Oxindole type alkaloids (isospeciofoline [2], isospeciofoleine [3], isorotundifoline [4], corynoxine B [5], and corynoxine [6]).—In this group of compounds the E ring is open and the C ring is five membered where the connection between C3–C7 positions is established. The fragmentation pattern was slightly different from mitragynine type compounds. These are further divided into two groups with or without hydroxylation substitution at the C9 position.

For corynoxine and corynoxine B, HR-ESI-MS gave protonated molecule $[M+H]^+$ at *m/z* 385.2119 and 385.2116, respectively, that corresponded to the molecular formula of $C_{22}H_{28}N_2O_4$. The key fragments detected were *m/z* 353.1836 $[M+H-32]^+$, 241.1322 $[M+H-144]^+$, 187.0852 $[M+H-198]^+$, 160.0746 $[M+H-225]^+$, and 110.0959 $[M+H-275]^+$ (Table 2). These compounds do not have hydroxylation substitution at the C9 position and are 16 Da units less compared to other groups of compounds (Figure 4). The ions *m/z* 353.1836 $[M+H-32]^+$ and *m/z* 110.0959 $[M+H-275]^+$ have the same fragmentation pattern or pathway as that of mitragynine. The presence of a carbonyl group adjacent to the nitrogen in the indole group makes the fragmentation pattern different from mitragynine. The peak at *m/z* 241.1322 $[M+H-144]^+$ resulted from the loss of both the methoxy methylacrylate moiety and the ethyl group at the C20 position from the molecular ion. The ion $[M+H-198]^+$ at *m/z* 187.0852 was observed due to cleavage of C-C bonds at C3–C14 and C4–C21 positions. The peak at *m/z* 160.0746 was formed due to loss of hydrogen cyanide from the ion $[M+H-198]^+$, which was the major fragment ion.

For isospeciofoline, HR-ESI-MS gave protonated molecule $[M+H]^+$ at *m/z* 401.2066 that corresponded to the molecular formula of $C_{22}H_{28}N_2O_5$. The hydroxyl group was characterized at the C9 position, same as 7-OH-mitragynine according to the reported literature (5). The key fragments detected were *m/z* 369.1792 $[M+H-32]^+$, 257.1269 $[M+H-144]^+$, 203.0799 $[M+H-198]^+$, and 176.0696 $[M+H-225]^+$ (Table 2). It is an isomer of isorotundifoline, which gives the same molecular formula and similar fragments. Isospeciofoleine gives protonated molecule $[M+H]^+$ at *m/z* 399.1913 that corresponds to the molecular formula of $C_{22}H_{26}N_2O_5$. These compounds may result with an additional double bond at position C20. The key fragments detected were *m/z* 283.1418 $[M+H-116]^+$, 217.0963 $[M+H-182]^+$, 203.0803 $[M+H-196]^+$, 176.0694 $[M+H-143]^+$, 148.0744 $[M+H-251]^+$, and 108.0800 $[M+H-291]^+$ (Table 2). All four compounds show the presence of a hydroxyl group based in their fragments.

Spectral Library

An MS/MS spectral library was created by analyzing the 12 reference standards using the described chromatographic method. The data were obtained in positive ESI mode at collision energies of 0, 10, 20, 30, and 40 eV. Multiple collision energies were necessary as fragmentation behavior was different for all 12 compounds. Only singly charged positive $[M+H]^+$ ions were used to produce targeted MS/MS spectra. Chromatographic peaks were found and spectra generated by averaging across the chromatographic peak, and the results were presented to the library building tool (MassHunter PCDL Manager, Version B.04.00). The utility of the MS/MS library was tested on 18 commercial samples of *M. speciosa*. Spectral matching was performed by comparison of the corresponding peaks in the library and unknown spectra within a set mass tolerance. When a corresponding peak was found, a dot product of library peak intensity and unknown peak intensity was calculated. A matching score was then generated by summing the dot products for all the peaks in a given spectra, normalized to produce a score of 0–100 with 100 being a perfect match. Even though accurate mass spectra of the pseudomolecular ion can provide a molecular formula, this alone cannot provide identification of a molecule. The spectral library is a more reliable tool in confirming the identity of compounds, and the software has high search speed. The in-house generated library of compounds specific for *M. speciosa* was used to identify some of these remaining MFs by comparison of the fragmentation in the library spectra.

Chemometric Analysis

This study also demonstrates that metabolomic analysis

using UHPLC/QToF-MS combined with multivariate statistical analysis provides some useful information about *M. speciosa* and can be used as a powerful tool to profile and differentiate phytochemical compositions among different *Mitragyna* samples. Principal component analysis (PCA) was performed using the Agilent MPP version 12.6.1 software. The MFs were further analyzed by PCA in order to determine differences among alkaloids of samples with the same botanical origin but with different geographical origin or varieties. As PCA is an unsupervised method of examining variance in the data, it was used here to show that these samples have significant differences and may indicate different varieties, geographies, or even different growing conditions in the same geography. This is unknown, but the power of discovering differences was shown.

Positive ions with accurate *m/z* values and with a difference corresponding to adduct isotopes or multiply charged species were merged into MFs as a single variable. This single variable, termed an entity, consisted of the MW of the molecule, its RT, and abundance. Entities absent in at least 75% of the samples in a given group were removed to reduce the dimensionality of the data sets prior to PCA. Furthermore, entities were filtered on the basis of *P*-values ($P < 0.02$) calculated for each entity by one-way analysis of variance. This ensured the filtration of MFs which differed in the respective varieties with statistical significance (98% in this particular case). Compounds that satisfied fold change cutoff 2.0 in at least one condition pair were selected for further analysis and differentiation. The extracted entities were mean centered and logarithmically transformed in order to reduce the relatively large differences in the respective abundances.

A two-component PCA score plot of entities mined from the UHPLC/QToF-MS data was utilized to depict general variation of alkaloids among the *M. speciosa* samples (Figure 5). Visual examination of the UHPLC/QToF-MS chromatograms indicated clear difference among three groups of samples. The separation of the three groups of *M. speciosa* samples was observed in the PCA scores plot, where each coordinate represents a sample (Figure 5). PCA was performed to reduce data dimensionality by covariance analysis of 18 samples of *Mitragyna*. The metabolites shown to be significant from the PCA are given in Figure 5. Shown there is variability among entities from leaves of *M. speciosa* samples. For the chemometric analysis, as there was not much difference with other thresholds selected, PCA was used with 5000 cps threshold data because the results with 1000, 5000, and 10 000 cps thresholds were almost equivalent. In other words, there was little difference in the final list of entities determined from setting the above abundance thresholds. Elemental formulae were generated to find plant specific biomarkers. PC1 (gives 52.8% of the variability to the original data set) and PC2 (gives 19.6% of the variability to the original data set) together explain 73% of the total variance of the dataset. The PCA scores plot in Figure 5 is divided into three groups based on the levels and occurrence of alkaloids. Each sample was represented as a point in a scores plot.

A total number of 43 MFs were recorded to be differentially expressed across samples at a threshold of 5000 cps. The PCA tool can be used as analytical model for authentication and showed content or compound variations from the leaves of *M. speciosa*. Hence, it is important to assess the samples to ensure the proper collection of leaves.

Of all samples analyzed, two samples (Nos. 10859 and 2796) were significantly different than the others. This is shown in Table 3 where the detection of the many isomers in each sample is indicated. Four isomers of corynantheidine ($[M+H]^+$ at *m/z* 369.21), one corynoxeine isomer ($[M+H]^+$ at *m/z* 383.19), four isomers of mitragynine ($[M+H]^+$ at *m/z* 399.22), and six isomers at *m/z* 415.22 were found in all samples except samples No. 10859 and 2796 (Figure 5). Similarly five isomers of corynoxine B and/or hydrogenation of corynoxeine ($[M+H]^+$ at *m/z* 385.21) were found in all samples, whereas samples No. 10859 and 2796 showed only two abundant isomers (*m/z* 385.19). Four isomers of isospeciofoline were observed in all the compounds except for two samples (Nos. 10859 and 2796), whereas different isomers were detected in these samples as indicated by their RTs (Table 3). These two samples were labeled as *M. speciosa* but differed in containing the complete profile of *Mitragyna* compounds.

Samples No. 10852, 10855–10858, 10860–10862, 10869–10872, and 12433 (Group 1) showed signals at different RTs for protonated molecules *m/z* 369.21, 383.19, 385.21, 399.22, 401.21, and 415.22. These were grouped together and showed differences in contents, whereas samples No. 10853, 10854, and 10873 (Group 2) showed most of the signals and, hence, had close relationship. For samples No. 2796 and 10859 (Group 3) did not show most of the signals (*m/z* 369.21, 399.22, and 415.22) and were different from other samples (Figure 5 and Table 3). These samples with RT 10–14 min showed a more abundant signal than the one compared to an authenticated sample (No. 12433). The total alkaloidal content substantially varied based on geographic origin and season; however, the presence of major indole alkaloids (mitragynine, speciogynine, and paynantheine) with some variations in the content remained the same irrespective of season or location (22). All of the leaves analyzed were purchased online. Details of these samples were not available, and based on their grouping pattern it was shown that Group 3 samples did not contain major alkaloids. Groups 1 and 2 showed close relationships, and the difference was mainly in the form we received. The Group 1 samples were

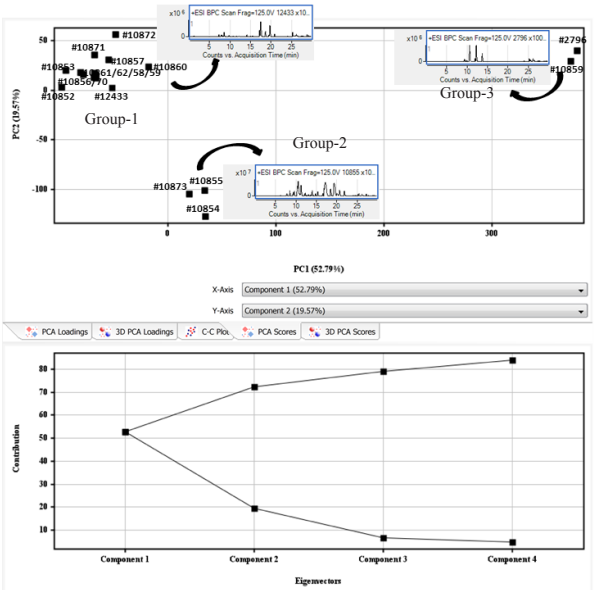

Figure 5. PCA score plots of *Mitragyna* samples.

Table 3. Qualitative analysis of *Mitragyna* samples using UHPLC/QToF-MS

		Sample No.																
$[M+H]^+$, *m/z*	RT, min	10852	10853	10854	10855	10856	10857	10858	10859	10860	10861	10862	10869	10870	10871	10872	10873	2796	12433
369.21	15.5	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	16.5	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	17.8	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
383.19	9.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	10.9	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	11.5								+									+	
385.21	10.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	11.5	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	12.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	13.2	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	13.7	+			+	+	+	+		+	+	+	+	+	+	+			+
399.22	17.7	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	18.8	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	19.9	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	21.0	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
401.21	8.6	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	9.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	9.9	+			+	+	+	+	+	+	+	+	+	+	+	+		+	+
	10.6	+			+	+	+	+		+	+	+	+	+	+	+			+
	10.4		+	+													+		
	11.5		+	+													+		
	12.0								+									+	
	13.8								+									+	
415.22	7.9	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	12.2	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	12.9	+			+	+	+	+		+	+	+	+	+	+	+			+
	13.5	+			+	+	+	+		+	+	+	+	+	+	+			+
	14.2	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	14.9	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
	20.1		+	+													+		
	20.9		+	+													+		

powders and Group 2 samples were extracts. Hence, this work is of great importance for the evaluation of overall quality of leaves of *M. speciosa* samples.

Conclusions

UHPLC/QToF-MS-based metabolite profiling is promising for elucidating the metabolic outcomes as a result of geographical, seasonal, and cultivation method variations. UHPLC/QToF-MS has been applied to the characterization of alkaloids from *M. speciosa* with the advantage of avoiding time-consuming and tedious purification of compounds from the crude extracts. The characteristic fragmentation patterns observed in MS/MS spectra allowed the identification of the indole group and presence of other functional groups. A total of 12 alkaloids were identified by comparing the RT, accurate mass, and MS/MS data with the reference standards. For most compounds, positive ion mode MS gave abundant $[M+H]^+$ ions, and MS/MS in the positive ion mode gave more information for structural elucidation. In addition, UHPLC/ESI-QToF-MS proved to be an effective, sensitive, selective, rapid, and guided method for compound identification, especially for phytochemical research with trace amounts. These results also showed that this method could be used to identify alkaloids from the methanolic extracts and differentiate *Mitragyna* samples using PCA with high sensitivity and reproducibility. Hence, this work is of great importance for the evaluation of the quality of leaves of *M. speciosa* samples, ultimately of great significance in the pharmacological and clinical investigation of dietary supplements of plant origin.

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