Kratom (*Mitragyna speciosa*) validation: quantitative analysis of indole and oxindole alkaloids reveals chemotypes of plants and products

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Abstract:

Many consumers are turning to kratom (*Mitragyna speciosa*) to self-manage pain and opioid addiction. In the United States, an array of capsules, powders, and loose-leaf kratom products are readily available. Additionally, several online sites supply live kratom plants. A prerequisite to establishing quality control and quality assurance standards for the kratom industry, or understanding how alkaloid levels effect clinical outcomes, is the identification and quantitation of major and minor alkaloid constituents within available products and preparations. To this end, an ultra-high performance liquid chromatography-high resolution mass spectrometry method was developed for the analysis of 8 indole alkaloids (7-hydroxymitragynine, ajmalicine, paynantheine, mitragynine, speciogynine, isopaynantheine, speciociliatine, and mitraciliatine) and 6 oxindole alkaloids (isomitraphylline, isospeciofoleine, speciofoline, corynoxine A, corynoxeine, and rhynchophylline) in US-grown kratom plants and commercial products. These commercial products shared a qualitatively similar alkaloid profile, with $12-13$ detected alkaloids and high levels of the indole alkaloid mitragynine ($13.9 \pm 1.1 - 270 \pm 24$ mg/g). The levels of the other major alkaloids (paynantheine, speciociliatine, speciogynine, mitraciliatine, and isopaynantheine) and the minor alkaloids varied in concentration from product to product. The alkaloid profile of US-grown *M. speciosa* "Rifat" showed high levels of the indole alkaloid speciogynine ($7.94 \pm 0.83 - 11.55 \pm 0.18$ mg/g) and quantifiable levels of isomitraphylline ($0.943 \pm 0.033 - 1.47 \pm 0.18$ mg/g). Notably, the alkaloid profile of a US-grown *M. speciosa* seedling was comparable to the commercial products with a high level of mitragynine (15.01 ± 0.20 mg/g). This work suggests that there are several *M. speciosa* chemotypes.

Keywords: kratom | *Mitragyna speciosa* | Rubiaceae | indole alkaloid | oxindole alkaloid | chemotype | UPLC-HRMS

Article:

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Kratom (*Mitragyna speciosa*) Validation: Quantitative Analysis of Indole and Oxindole Alkaloids Reveals Chemotypes of Plants and Products[#]

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ABSTRACT

Many consumers are turning to kratom (*Mitragyna speciosa*) to self-manage pain and opioid addiction. In the United States, an array of capsules, powders, and loose-leaf kratom products are readily available. Additionally, several online sites supply live kratom plants. A prerequisite to establishing quality control and quality assurance standards for the kratom industry, or understanding how alkaloid levels effect clinical outcomes, is the identification and quantitation of major and minor alkaloid constituents within available products and preparations. To this end, an ultra-high performance liquid chromatography-high resolution mass spectrometry method was developed for the analysis of 8 indole alkaloids (7-hydroxymitragynine, ajmalicine, paynantheine, mitragynine, speciogynine, isopaynantheine, speciociliatine, and mitraciliatine) and 6 oxindole alkaloids (isomitraphylline, isospeciofoleine, speciofoline, corynoxine A, corynoxeine, and rhynchophylline) in US-grown kratom plants and commercial products. These commercial products shared a qualitatively similar alkaloid profile, with 12–13 detected alkaloids and high levels of the indole alkaloid mitragynine (13.9 ± 1.1–270 ± 24 mg/g). The levels of the other major alkaloids (paynantheine, speciociliatine, speciogynine, mitraciliatine, and isopaynantheine) and the minor alkaloids varied in concentration from product to product. The alkaloid profile of US-grown *M. speciosa* "Rifat" showed high levels of the indole alkaloid speciogynine (7.94 ± 0.83–11.55 ± 0.18 mg/g) and quantifiable levels of isomitraphylline (0.943 ± 0.033–1.47 ± 0.18 mg/g). Notably, the alkaloid profile of a US-grown *M. speciosa* seedling was comparable to the commercial products with a high level of mitragynine (15.01 ± 0.20 mg/g). This work suggests that there are several *M. speciosa* chemotypes.

ABBREVIATIONS

|	
BEH	ethylene bridged hybrid
BLQ	below the lower limit of quantification
CH_3CN	acetonitrile
$CHCl_3$	chloroform
CYP	cytochrome P450
ECD	electronic circular dichroism spectroscopy
HESI	heated electrospray ionization
IT	injection time
LLOD	lower limit of detection
LLOQ	lower limit of quantitation
PDA	photodiode array detector
PVDF	polyvinylidene fluoride
RE	relative error
RSD	relative standard deviation
SE	standard error
SERS	surface-enhanced Raman spectroscopy
SFC	supercritical fluid chromatography
ULOQ	upper limit of quantitation

Introduction

Chronic pain affects an estimated 50 million adults in the United States (US) and is a prominent reason for seeking medical care [1]. The prevalence of pain and the highly addictive nature of opiates, the major therapeutics for chronic pain, have, in part, led to the current opioid crisis surging through the US [2]. An estimated 2 million Americans suffer from substance use disorders related to prescription opioids, and more than 130 die each day from opioid overdose [3]. In light of these concerns, an increasing number of US consumers are turning to plant-based medicines (i.e., botanical dietary supplements) as an alternative way to treat chronic pain [4]. Among the most popular of these is *Mitragyna speciosa* (Korth.) Havil. (Rubiaceae), commonly referred to as kratom [5], the use of which has risen dramatically in the US over the last decade [5, 6].

Kratom is an evergreen tree native to Southeast Asia [7, 8], where its medicinal use was interwoven into Thai and Malaysian cultures. Kratom exhibits a complex pharmacology illustrated by its traditional use both to thwart pain [7, 9] and ameliorate opioid addiction [7]. The earliest literature reports of kratom refer to its seemingly contradictory use by Malay and Thai people as a sedative opium substitute [10], an aid to opium-use cessation, and as a stimulant to combat fatigue and increase productivity [11]. These divergent applications could be explained by the mixture of structurally diverse alkaloids present in kratom leaf material, which exhibit differential binding affinities to neurochemical receptors and elicit concentration dependent effects [7, 12, 13]. While a body of literature largely attributes the effects of kratom to the major indole alkaloid constituent, mitragynine (**12**) (▶ **Fig. 1**), and the minor alkaloid, 7-hydroxymitragynine (**2**), the plant also produces at least fifty-eight other alkaloids with diverse structures [14].

Recent literature is beginning to evaluate the pharmacological importance of the other major and minor alkaloids present in *M. speciosa* [15–18]. Investigation of the opioid and adrenergic

binding affinities of five kratom alkaloids, including **2**, **12**, speciociliatine (**15**), corynantheidine, and 9-hydroxycorynantheidine, revealed that **15** exhibited stronger binding affinity to κ- and μ-opioid receptors than **12** [19]; interestingly, **12** and **15** only differ by their configuration at position C-3 [14]. In the same study, the indole alkaloids corynantheidine and 9-hydroxycorynantheidine showed measurable, albeit weaker, affinity to μ-opioid receptors than **12**. Mitraciliatine (**16**), a diastereomer of **12** with opposite configurations at the C-3 and C-20 positions, shows μ-opioid receptor partial agonism and κ-opioid receptor full agonism at both mouse and human receptors [18]. Our recent analysis of over 50 commercial kratom products identified two different chemotypes with either a high or low abundance of the oxindole alkaloid speciofoline (**6**) (▶ **Fig. 2**) [20]. While **6** does not exhibit measurable binding affinity at the μ-, δ-, or κ-opioid receptors, it does inhibit important cytochrome P450 enzymes involved in drug metabolism (i.e., CYP2C9, CYP2D6, CYP3A) [20]. Speciociliatine (**15**), a diastereomer of **12** also present in commercial kratom products, was shown recently in a preclinical pharmacokinetic study to exhibit a higher systemic exposure and lower clearance compared to **12** and corynantheidine [21].

The reports of variable biological effects of kratom alkaloids substantiate the need for further preclinical and clinical studies to determine the pharmacological and pharmacokinetic properties of consumer-utilized kratom products and the lesser studied kratom alkaloids contained therein. A prerequisite to such studies is the thorough identification and quantitation of the major and minor alkaloid constituents within commercially available products, traditional preparations, and those utilized for preclinical or clinical investigations. Analytical and bioanalytical methods over the last century have focused on the identification and quantification of **12** and **2** from different matrices (e.g., plant material, commercial products, blood, and urine) [22, 23] with varying chromatographic [e.g., HPLC, UHPLC, GC, and supercritical fluid chromatography (SFC)] and detection techniques [e.g., UV/DAD, MS, ELISA, and surface-enhanced Raman spectroscopy (SERS)] (see **Table 1S**, Supporting Information, for full comparison of methods and pertinent references). Recent efforts have pushed beyond the examination of **12** into the detection and quantification of several alkaloids present in *M. speciosa* and commercial kratom products. Kikura-Hanajiri et al. [24] applied LC-ESI-MS to simultaneously quantify five kratom alkaloids (**2**, paynantheine (**11**), **12**, speciogynine (**13**), and **15**). Wang et al. [25] compared three different chromatographic techniques (HPLC-DAD/MS, GC-MS, and SFC-DAD) to analyze eight kratom alkaloids (**2**, corynoxine A (**7**), **11**, **12**, **13**, isopaynantheine (**14**), **15**, and corynoxine B). Several methods arising from the Avery and McCurdy groups utilize a triple quadrupole mass spectrometer for UHPLC-MS/MS of kratom leaf extracts and commercial products. Initially, their method quantified simultaneously ten kratom alkaloids [**2**, mitraphylline (**4**), **7**, **11**, **12**, **13**, **15**, corynoxine B, corynantheidine, and isocorynantheidine] in leaf extracts and commercial products [26]. Later, Jeng-Yeou Chear et al. [17] quantified Malaysian *M. speciosa* samples using the method reported by Sharma et al. [26], including an additional four alkaloids [isospeciofoline, mitragynine oxindole B, speciociliatine-*N*(4)-oxide, and ajmalicine (**10**)] in the standard mixture. Recently, Kamble et al. used an optimized method
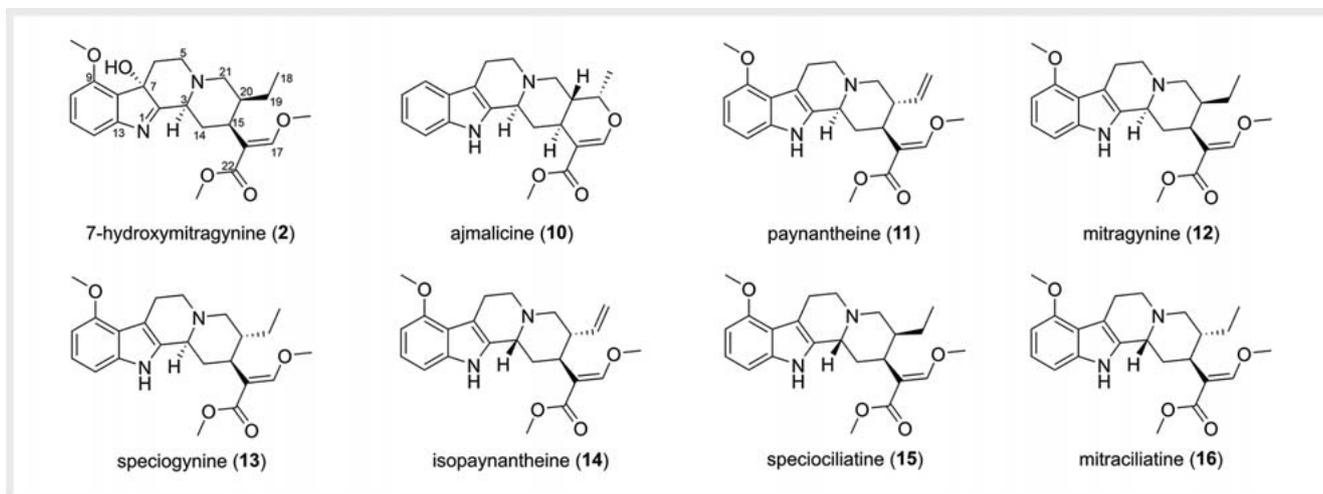
► **Fig. 1** Structures of the indole alkaloids 2, 10, and 11–16. The numerical order of the compounds corresponds to their elution order via ultra-high performance liquid chromatography in ► **Fig. 3**.
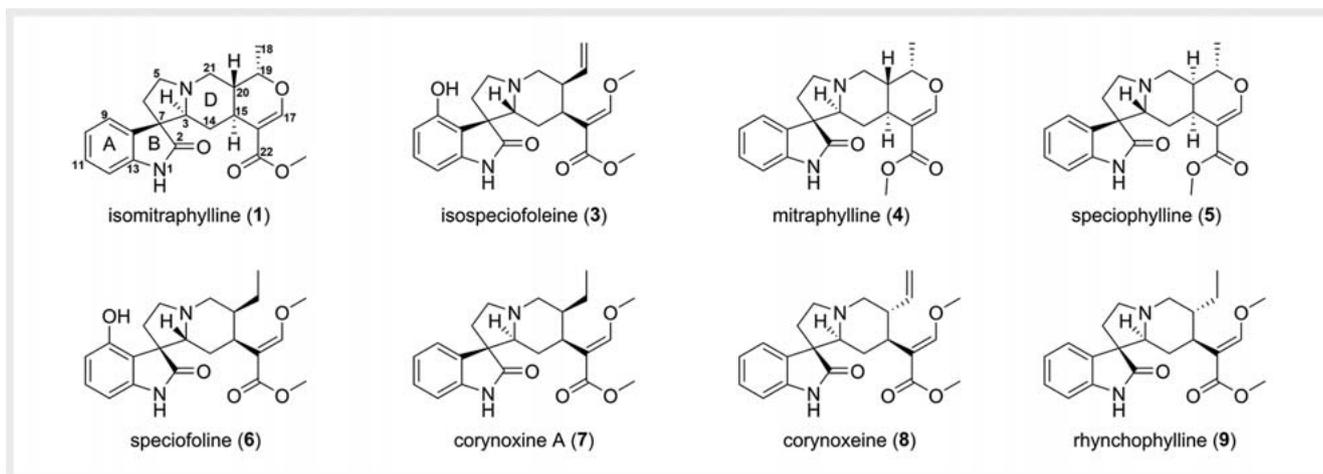
► **Fig. 2** Structures of the oxindole alkaloids 1 and 3–9. The numerical order of the compounds corresponds to their elution order via ultra-high performance liquid chromatography in ► **Fig. 3**.

with a shorter runtime to quantify 11 kratom alkaloids (2, 4, 7, 10, 11, 12, 13, 15, corynoxine B, corynantheidine, and isospeciofoline) in rat plasma [16].

As part of a project to study the potential for interactions between herbal medicines and drugs [27], our team recently generated a suite of kratom alkaloid reference standards [14]. The purpose was to incorporate these standards into a validated analytical method to quantify the indole and oxindole alkaloid constituents of commercially available kratom material. Compared to the published analytical methods for kratom alkaloid quantitation, this method was designed to leverage the superior resolving power of a hybrid quadruple-orbitrap mass spectrometer and a suite of both oxindole and indole alkaloid standards to generate a more comprehensive method for quantifying the major and minor alkaloids in kratom. Toward this goal, we developed a method that

identifies and quantifies 14 kratom alkaloids, including all four mitragynine diastereomers (12, 13, 15, 16) (► **Fig. 1**), and 5 oxindole alkaloids (1, 3, 6, 8, and 9) that have not previously been quantified in kratom (► **Fig. 2**). The method was then employed to describe the indole and oxindole alkaloid variability between commercial kratom products and living kratom plants, ultimately, to identify if US-grown kratom plants could exhibit a similar alkaloid profile to commercial kratom material purportedly originating from Southeast Asia.

Results and Discussion

Our initial aim was to develop a method capable of quantifying both indole and oxindole alkaloids simultaneously, including several compounds that are known to *M. speciosa* but never included
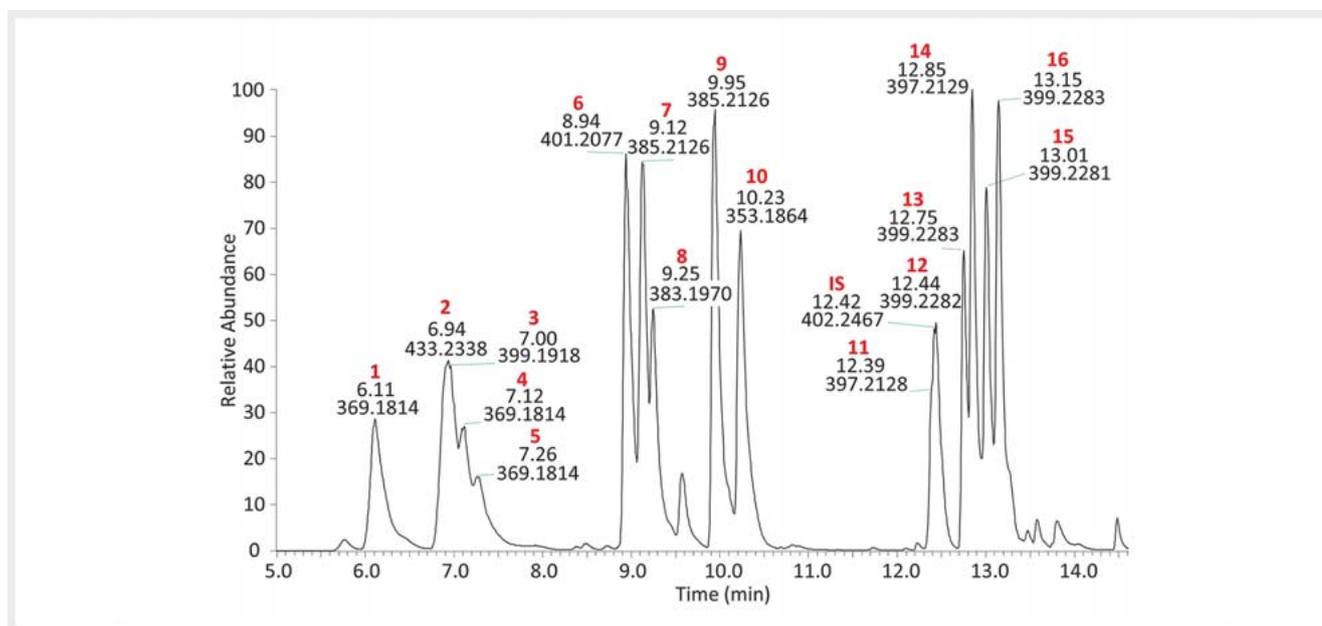
► **Fig. 3** Base peak MS chromatogram of an equimolar mixture of kratom alkaloids at 313 ng/mL. Structures corresponding to each peak (► **Figs. 1** and **2**) were assigned by matching retention time, fragmentation pattern, and accurate mass with authentic standards. Compounds are numbered in order of elution.

in prior quantification studies. Ultimately, a UPLC-HRMS method was developed for the quantitative analysis of 14 kratom alkaloids, including 8 indole alkaloids (i.e., **2, 10, 11, 12, 13, 14, 15,** and **16**) and 6 oxindole alkaloids [i.e., isomitraphylline (**1**), isospeciofoleine (**3**), **6, 7**, corynoxeine (**8**), and rhynchophylline (**9**)], in complex extracts.

The methodology benefits from the comprehensive nature of examining a suite of both indole and oxindole alkaloids, including all four mitragynine diastereomers (**12, 13, 15, 16**), and 5 oxindole alkaloids (**1, 3, 6, 8,** and **9**) which have not previously been quantified in kratom materials.

Method development began with the analysis of kratom alkaloids in both positive and negative electron ionization modes and UPLC separation using various chromatographic conditions. Positive mode ionization was suited for this analysis, but the chromatography proved to be a unique challenge. Kratom extracts were initially screened using a BEH C18 column and a binary mobile phase consisting of 0.1% formic acid in H_2O and CH_3CN (**Fig. 1S,** Supporting Information); however, the resolution between peaks was inadequate. Optimal chromatographic resolution was achieved by evaluating several chromatographic conditions, including alternative mobile phases [e.g., aqueous phase (A): water with added formic acid or ammonium formate; organic phase (B): MeOH or CH_3CN with added formic acid or ammonium formate] and alternative stationary phases (e.g., C18, amide, biphenyl, and pentafluorophenyl). The combination that showed the best chromatographic separation was the Kinetex F5 column (Phenomenex) with a binary mobile phase consisting of 0.1% formic acid in H_2O and CH_3CN at a flow rate of 0.6 mL/min. The alkaloids eluted sequentially over the course of 20.0 minutes with three primary groupings, specifically: compounds **1, 2, 3, 4,** and **5** (6.11,

6.94, 7.00, 7.12, and 7.26 min, respectively) in the first group; compounds **6, 7, 8, 9,** and **10** (8.94, 9.12, 9.25, 9.95, and 10.23 min, respectively) in the second group; followed by the internal standard (12.41 min) and compounds **11, 12, 13, 14, 15,** and **16** (12.39, 12.44, 12.75, 12.85, 13.01, 13.15 min, respectively) forming the last group (► **Figs. 3** and **4**).

The analytical method was then validated for linearity, precision, accuracy, repeatability, and sensitivity. A nine- (alkaloids **2, 3, 7, 9, 11, 13, 14, 15** and **16**) or ten-point (alkaloids **1, 6, 8, 10** and **12**) calibration curve was plotted using a weighted ($1/x^2$) least squares regression model. The curves were linear across a concentration range of 9.77–2500 ng/mL and 9.77–5000 ng/mL, respectively, with a coefficient of determination greater than 0.992 (► **Table 1**). The repeatability (intraday) and intermediate precision (interday) of the method was confirmed by evaluating the precision (RSD) at either nine or ten different concentrations for each alkaloid (**Table 2S,** Supporting Information). Intraday and interday accuracy was evaluated by calculating the relative error (RE), which is the percent difference between the measured concentration and the nominal concentration of each standard. Both interday and intraday RE and RSD were all below the acceptable cut-off value of 20% (**Table 2S,** Supporting Information) [28]. To evaluate potential matrix effects, an internal standard of heavy labeled **12** (mitragynine-d_3) was added to each of the samples at a final concentration of 125 μg/mL. The recovery of mitragynine-d_3 was determined using the standard curve of **12** (**Table 3S,** Supporting Information). Recoveries ranged from 80–96%, suggesting minimal matrix interference. Analysis of the standard mixture (9.77 ng/mL) at the start of each run verified a resolution greater than 2 for all isomeric compounds included in the quantitation, except isomers **15** and **16** with an adequate resolution of greater
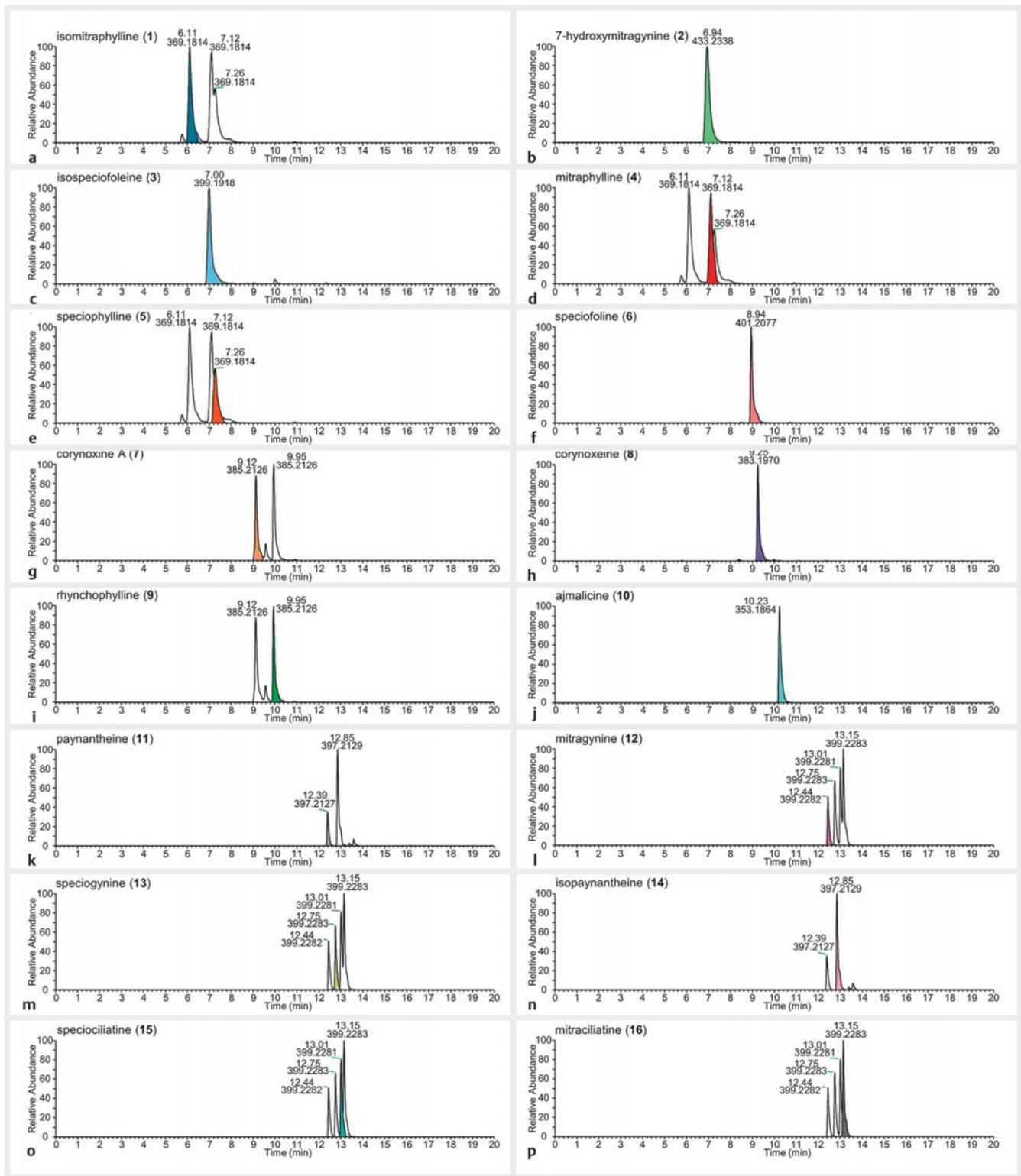
► Fig. 4 Extracted ion chromatograms of the protonated molecular ions m/z 369.1809 (panels **a**, **d**, and **e**), 433.2333 (panel **b**), 399.1915 (panel **c**), 401.2071 (panel **f**), 385.2122 (panels **g** and **i**), 383.1965 (panel **h**), 353.1860 (panel **j**), 397.2122 (panels **k** and **n**), and 399.2278 (panels **l**, **m**, **o**, and **p**) at 313 ng/mL, the central concentration of the analyzed calibration solutions. The diastereomers in the standard mixture share the same m/z value, thus several panels (**a**, **d**, **e**, **g**, **i**, **k–p**) have more than one peak present. The colors distinguish the alkaloid (**1–16**) being referenced by the panel name (e.g., isomitraphylline (**1**) is denoted by the color blue in panel **a**).

▶ **Table 1** Parameters of calibration curves for each alkaloid.

Analyte	Slope (± SE[a]) × 10^3	Intercept (± SE) × 10^5	r^2	LLOD[b] (ng/mL)	Linear Range of Quantitation (ng/mL)
isomitraphylline (**1**)	729.9 (9.0)	− 11.2 (2.4)	0.996	1.1	9.77–5000
7-hydroxymitragynine (**2**)	647 (11)	− 9.9 (2.8)	0.993	0.67	9.77–2500
isospeciofoleine (**3**)	307.1 (5.0)	− 4.9 (1.3)	0.993	0.79	9.77–2500
speciofoline (**6**)	1274 (15)	− 24.5 (4.1)	0.996	0.66	9.77–5000
corynoxine A (**7**)	1261 (16)	− 26.9 (4.1)	0.996	0.69	9.77–2500
corynoxeine (**8**)	817 (11)	− 20.7 (2.8)	0.995	0.77	9.77–5000
rhynchophylline (**9**)	1915 (27)	− 33.6 (6.8)	0.995	1.0	9.77–2500
ajmalicine (**10**)	1024 (17)	− 12.0 (4.6)	0.992	1.0	9.77–5000
paynantheine (**11**)	658.0 (7.3)	− 2.4 (1.8)	0.997	1.5	9.77–2500
mitragynine (**12**)	917 (11)	− 1.3 (2.8)	0.996	1.4	9.77–5000
speciogynine (**13**)	1009 (16)	− 23.1 (4.1)	0.994	1.3	9.77–2500
isopaynantheine (**14**)	668 (12)	− 5.7 (2.9)	0.993	0.94	9.77–2500
speciociliatine (**15**)	1110 (16)	− 21.1 (4.1)	0.995	0.59	9.77–2500
mitraciliatine (**16**)	1514 (23)	− 28.2 (5.8)	0.994	0.70	9.77–2500

[a] Standard error; [b] Lower limit of detection

than 1. While compounds **4** and **5** were omitted from quantitative validation due to poor chromatographic resolution and indistinguishable fragmentation spectra, this is the first method wherein both **4** and **5** are included and are identifiable. Our method surpasses previous methods by differentiating the isomers **1**, **4**, and **5**, and quantifying isomer **1**.

The landscape of commercially available kratom products in the US has grown significantly in the past decade. Currently, a vast array of powders, capsules, extracts, and loose-leaf kratom products are readily available to consumers via online sites and local retailers. The utility of the validated method was demonstrated by choosing a small sampling of products of different formulations and quantifying the indole and oxindole alkaloids contained therein (▶ **Table 2**). While powders representative of several different chemotypes (e.g., high/low speciofoline) were included in the group, this sampling does not epitomize the vast landscape of kratom materials and yet unknown chemotypes. Alkaloid content was measured by preparing methanolic extracts of two powdered plant products (K51 and K52), a loose-leaf product (K49), a liquid product (K76), and an encapsulated powder (K77) (▶ **Tables 2** and **3**). Alkaloid quantities revealed that **12** was the major alkaloidal constituent (0.53 ± 0.12–270 ± 24 mg/g of powdered material) (▶ **Table 2** and **Fig. 2S**, Supporting Information), which is consistent with the literature [17, 24, 26, 29]. The order of the next most abundant alkaloids varied among the samples with the general trend being **11**, **15**, **13**, **16**, **14**, **7**, and **2** with 5.79 ± 0.91–70.4 ± 5.2, 3.68 ± 0.32–41.7 ± 3.2, 3.18 ± 0.13–33.4 ± 2.7, 0.647 ± 0.035–4.75 ± 0.47, 0.512 ± 0.010–3.80 ± 0.26, 0.2322 ± 0.0044–11.40 ± 0.84, and 0.1240 ± 0.0014–1.10 ± 0.17 mg/g of dry material, respectively. Comparable to our pre-

vious analysis, K52 had a higher concentration of **6** (2.51 ± 0.19 mg/g of material) than the other two leaf products (K49 and K51), where the concentration of **6** was below the lower limit of quantitation (K49) and 0.1222 ± 0.0020 mg/g of material (K51), respectively [14, 20].

The encapsulated powder (K77) had the highest concentration of alkaloids among the commercial products (▶ **Table 2** and **3**; **Fig. 2S** and **3S**, Supporting Information). The ten-fold higher concentration of alkaloids in K77 suggests that the capsules were prepared from the alkaloidal fraction of kratom. Indeed, the alkaloid concentrations in K77 were comparable to those measured in an alkaloidal fraction prepared from product K51 (K51-2). This observation was supported by the prominent yellow color of the K77 powder, which closely resembled the color of the K51 alkaloidal fraction (K51-2). The alkaloid quantities of the liquid product (K76) were comparable to the methanolic extracts of K49, K51, and K52, suggesting the liquid product was an alcoholic extract of kratom material (▶ **Table 3** and **Fig. 3S**, Supporting Information).

Product K51 is being used in a Phase 1 clinical trial to evaluate the pharmacokinetics of kratom alkaloids (Clinical Trials.gov, NCT04392011, 2020). As part of the trial, participants consume a slurry of tea composed of 2 g of kratom dry leaf powder in 240 mL of hot water. This method of use mimics the typical consumption of kratom tea by US consumers. Considering this, a hot water extract of K51 was prepared (K51-3) for quantifying the alkaloidal constituents. Using this validated method, 13 kratom alkaloids were detected in the hot water infusion. The compounds **12**, **11**, **15**, **13**, **16**, **14**, **7**, **6**, **8**, **2**, and **3** were quantified at 15.3 ± 1.4, 4.3 ± 0.39, 3.20 ± 0.34, 2.18 ± 0.16, 0.377 ± 0.049, 0.301 ± 0.032, 0.275 ±

Table 2 Alkaloid content in commercial products and dried leaves (24 h maceration in MeOH), mg of compound/g of powder[a] (± SD[b]).

Sample	isomitraphylline (1)	7-hydroxymitragynine (2)	isospeciofoleine (3)	speciofoline (6)	corynoxine A (7)	corynoxeine (8)	rhynchophylline (9)	ajmalicine (10)	paynantheine (11)	mitragynine (12)	speciogynine (13)	isopaynantheine (14)	speciociliatine (15)	mitraciliatine (16)
K49		0.1590 (0.0077)	–	–	–	–		–	5.79 (0.91)	26.5 (4.0)	3.42 (0.52)	0.85 (0.13)	9.4 (1.7)	1.174 (0.079)
K51		0.1240 (0.0014)	–	0.1222 (0.0020)	0.2322 (0.0044)	–	–	–	5.86 (0.26)	19.48 (0.81)	3.18 (0.13)	0.512 (0.010)	5.12 (0.26)	0.647 (0.035)
K52		0.1383 (0.0053)	0.382 (0.034)	2.51 (0.19)	1.73 (0.13)	0.290 (0.017)	–	–	9.94 (0.88)	13.9 (1.1)	3.84 (0.32)	1.20 (0.11)	3.68 (0.32)	1.08 (0.12)
K55	1.47 (0.18)		–		0.451 (0.077)	–	0.239 (0.034)	0.192 (0.015)	1.46 (0.19)	0.53 (0.12)	7.94 (0.83)	–	0.185 (0.029)	0.872 (0.073)
K59	0.943 (0.033)		–		0.205 (0.045)	–	–	0.648 (0.053)	2.418 (0.020)	2.076 (0.068)	11.55 (0.18)	0.269 (0.018)	0.862 (0.027)	1.93 (0.13)
K77		1.10 (0.17)	1.675 (0.051)	5.90 (0.48)	11.40 (0.84)	1.217 (0.043)	–	–	70.4 (5.2)	270 (24)	33.4 (2.7)	3.80 (0.26)	41.7 (3.2)	4.75 (0.47)

Samples K49, K51, K52, and K77 are commercial kratom products that were purchased for this study. Sample K55 and K59 are cultivated kratom plants. Quantities were calculated by preparing methanolic extracts (24 h maceration) in triplicate and analyzing by LC-MS. [a] Quantity is denoted as mg of compound per g of dried kratom leaf or product material (± SD) and reflects the average of triplicate extractions; [b] Standard deviation of triplicate extractions, each analyzed separately. *Italicized* = BLQ, below the linear range and above the calculated lower limit of quantitation (LLOQ). – = below calculated LLOQ; No value = not detected or below lower limit of detection (LLOD)

▶ **Table 3** Alkaloid content in commercial products (24 h maceration in MeOH or H₂O), a liquid product, and an alkaloidal partition, mg of compound/g of dried extract[a] (± SD[b]).

Sample	isomitra-phylline (1)	7-hydroxy-mitragy-nine (2)	isospecio-foleine (3)	specio-foline (6)	coryno-xine A (7)	coryno-xeine (8)	rhyncho-phylline (9)	ajmalicine (10)	paynan-theine (11)	mitragy-nine (12)	specio-gynine (13)	isopay-nantheine (14)	specio-ciliatine (15)	mitra-ciliatine (16)
K49		0.449 (0.025)	–	–	–	–		–	16.3 (2.7)	75 (12)	9.7 (1.6)	2.41 (0.39)	26.4 (5.0)	3.31 (0.25)
K51		0.4585 (0.0069)	–	0.4516 (0.0030)	0.859 (0.014)	–	–	–	21.65 (0.80)	72.0 (2.7)	11.76 (0.41)	1.893 (0.052)	18.93 (0.76)	2.39 (0.11)
K51-2		10.3 (1.6)	–	3.05 (0.18)	8.81 (0.62)	2.686 (0.015)	–	–	102 (12)	387 (48)	57.7 (7.1)	10.9 (1.3)	106 (12)	13.1 (1.7)
K51-3		0.0939 (0.0028)	0.0528 (0.0021)	0.1114 (0.0076)	0.275 (0.036)	0.0949 (0.0051)	–	–	4.3 (0.39)	15.3 (1.4)	2.18 (0.16)	0.301 (0.032)	3.20 (0.34)	0.377 (0.049)
K52		0.420 (0.014)	1.160 (0.097)	7.62 (0.53)	5.27 (0.37)	0.881 (0.047)	–	–	30.2 (2.5)	42.2 (3.2)	11.68 (0.92)	3.65 (0.30)	11.17 (0.91)	3.28 (0.33)
K76		–	0.694 (0.013)	1.776 (0.041)	2.711 (0.016)	0.6096 (0.0054)	–	–	14.43 (0.11)	54.89 (0.37)	7.08 (0.13)	1.013 (0.034)	8.87 (0.17)	1.232 (0.022)
K77		2.88 (0.38)	4.40 (0.22)	15.5 (1.5)	30.0 (2.7)	3.20 (0.17)	–	–	185 (17)	709 (76)	87.8 (8.5)	10.00 (0.86)	110 (10)	12.45 (0.98)

Samples K49, K51, K52, K76, and K77 are commercial kratom products that were purchased for this study. K51-2 is the alkaloidal partition from a CHCl₃–MeOH extract of K51. K51-3 is a hot water (tea) extract of K51. Quantities were calculated by preparing extracts in triplicate and analyzing by LC-MS. [a]Quantity is denoted as mg of compound per g of dried extract (± SD) and reflects the average of triplicate extractions; [b]Standard deviation of triplicate extractions, each analyzed separately; *Italicized* = BLQ, below the linear range and above the calculated lower limit of quantitation (LLOQ); – = below calculated LLOQ. No value = not detected or below lower limit of detection (LLOD)

Table 4 Alkaloid content in commercial products and dried leaves (30 min sonication in MeOH), mg of compound/g of powder[a] (± SD[b]).

| Sample	isomitra-phylline (1)	7-hydroxy-mitragy-nine (2)	isospecio-foleine (3)	specio-foline (6)	coryno-xine A (7)	coryno-xeine (8)	rhyncho-phylline (9)	ajmalicine (10)	paynan-theine (11)	mitra-gynine (12)	specio-gynine (13)	isopay-nantheine (14)	specio-ciliatine (15)	mitra-ciliatine (16)
K51		–	–	0.1241 (0.0029)	0.1900 (0.0055)	0.1212 (0.0014)	–	–	4.97 (0.25)	16.56 (0.89)	2.86 (0.18)	0.387 (0.019)	4.32 (0.24)	0.611 (0.060)
K52		0.01288 (0.00034)	0.2271 (0.0050)	3.091 (0.043)	1.680 (0.042)	0.2500 (0.0029)	0.1561 (0.0026)	–	11.00 (0.23)	16.84 (0.14)	4.560 (0.010)	1.5074 (0.0050)	6.13 (0.23)	1.639 (0.057)
K59	1.230 (0.054)	0.01348 (0.00030)	–		0.113 (0.012)	–	0.0480 (0.0053)	0.883 (0.024)	3.034 (0.043)	2.782 (0.041)	12.69 (0.42)	0.251 (0.011)	1.536 (0.078)	2.522 (0.078)
K64	0.853 (0.032)	–	–		0.1556 (0.0054)	–	0.0525 (0.0022)	0.0665 (0.0015)	1.535 (0.049)	0.962 (0.010)	7.81 (0.33)	0.1052 (0.0079)	0.2181 (0.0069)	1.1042 (0.0081)
K68	0.476 (0.022)	0.01450 (0.00062)	0.01985 (0.00019)		0.1904 (0.0079)	0.4240 (0.0037)	0.1778 (0.0051)	0.0262 (0.0020)	3.53 (0.10)	15.01 (0.20)	4.52 (0.23)	0.0411 (0.0017)	0.4539 (0.0047)	0.400 (0.026)

Samples K51 and K52 are commercial kratom products that were purchased for this study. Sample K55, K59, K64, and K68 are cultivated kratom plants. Quantities were calculated by preparing methanolic extracts (30 min sonication) in triplicate and analyzing by LC-MS. [a] Quantity is denoted as mg of compound per g of dried kratom leaf or product material (± SD) and reflects the average of triplicate extractions; [b] Standard deviation of triplicate extractions, each analyzed separately; *Italicized* = BLQ, below the linear range and above the calculated lower limit of quantitation (LLOQ); – = not detected or below calculated LLOQ. No value = not detected or below lower limit of detection (LLOD)

0.036, 0.1114 ± 0.00076, 0.0949 ± 0.0051, 0.0939 ± 0.0028, and 0.0528 ± 0.0021 mg per g of dry extract, respectively. Compounds **9** and **10** were detected in the tea but were found below the LLOQ. One caveat to comparing the alkaloid levels from this specific water extraction to the tea preparation consumed in the referenced clinical trial is that the clinical trial participants consume both the liquid tea and the residual dregs (powder). The alkaloid levels reported here refer specifically to the water extractable amounts and may, therefore, be lower than the quantities ingested in the clinical study.

Quantitative analysis of leaves from a US kratom grower (K59) and a young kratom plant obtained from the same supplier and grown in Greensboro, NC (K55) yielded alkaloid profiles vastly different from the commercial products (▶ **Table 2** and **Fig. 2S**, Supporting Information). Compound **13** was the most abundant alkaloid in both samples (K55 7.94 and K59 11.55 mg/g powdered leaf material), with **12** content greater in K59 (2.076 ± 0.068 mg/g powdered leaf material) than K55 (0.53 mg/g powdered leaf material). Notably, both samples contained the oxindole alkaloid **1**, which was not detected in the commercial products.

The low levels of **12** observed in K55 and K59 were similar to previous analyses of US-grown *M. speciosa*. Specifically, a young (< 5 years old) *M. speciosa* plant grown in the gardens of the University of Mississippi yielded the oxindole alkaloid **4** as the major constituent [30], Florida grown *M. speciosa* cuttings yielded low levels of **12** with **4** and **13** as the major constituents [31], and a young *M. speciosa* plant grown in New York had the least amount of **12** per g of dried plant material compared to commercial kratom products [32]. The identity of the young kratom plants were not specified in the referenced articles, but it can be speculated that they are clones arising from either the "Rifat", "Bumblebee", or "Malay" plants available from several online suppliers of live kratom plants. Lesiak and Musah 2016 reported using direct analysis in real time-high resolution mass spectrometry (DART-HRMS) to analyze *M. speciosa* leaf samples [33]. The analysis compared a young "Rifat" plant to commercial kratom leaf and powder, wherein the relative intensity of the protonated mitragynine ion ($[M + H]^+$ = 399.2284) was 100% in the commercial samples, and 64.1% in the live "Rifat" sample. The protonated mitraphylline ion ($[M + H]^+$ = 369.1814) was the most abundant ion in the live "Rifat" sample (100% relative intensity) but was undetected in the commercial samples. The DART-HRMS method does not distinguish between diastereomers, thus the m/z of 369.1814 may represent any one of the stereoisomers of mitraphylline (i.e., speciophylline or isomitraphylline).

Previous studies hypothesized that compared to commercially obtained kratom samples, US-grown *M. speciosa* were a chemical variant (chemotype) [17, 32], a genetic variant (genotype) [31], or that a key environmental factor responsible for biosynthesis of **12** was not present in their study [31]. Assuming that previous work done on US-grown *M. speciosa* was conducted using rooted cuttings of a "Rifat" plant, we tested the hypothesis that the ratio of **12** to **13** in *M. speciosa* "Rifat" is due to underlying chemotype differences and not due to growing conditions. To test this hypothesis, we quantified the indole and oxindole alkaloid levels of a rooted cutting of *M. speciosa* "Rifat" (K64) and a *M. speciosa* seedling (purportedly from Mempawah, West Kalimantan, Indonesia)
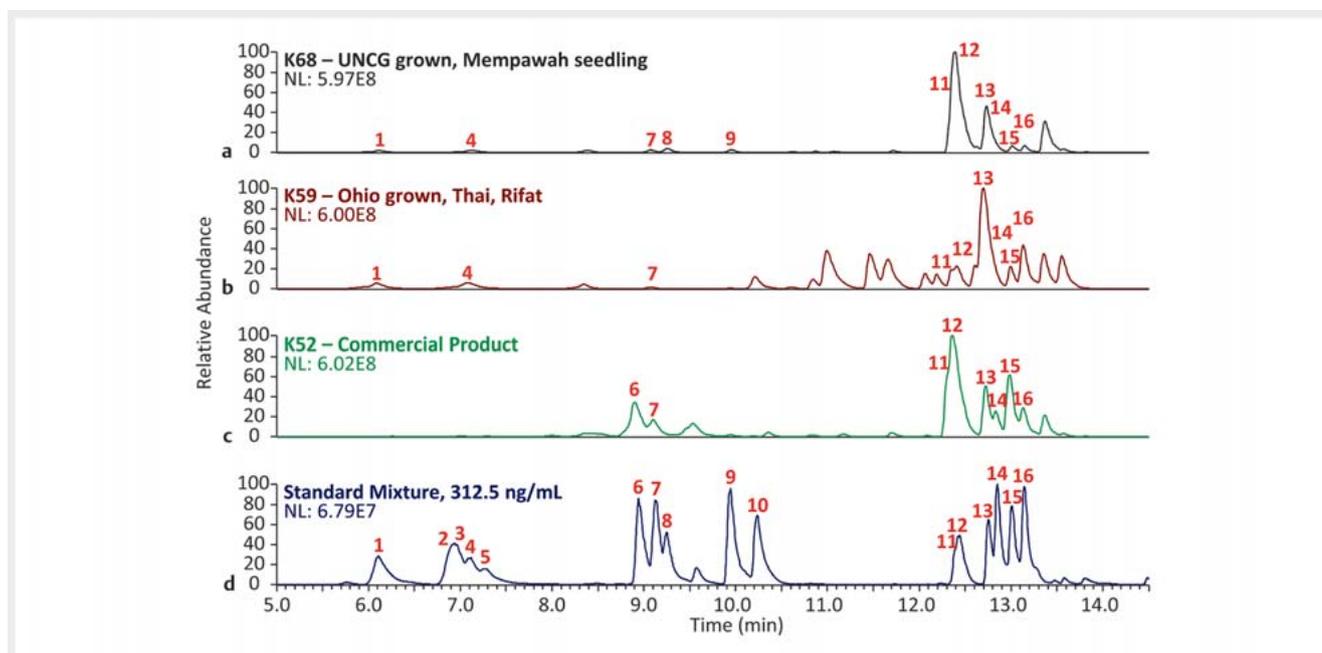
► **Fig. 5** Base peak MS chromatograms of, from top to bottom, (panel a) methanolic extracts of a UNCG grown kratom seedling (K68), (panel b) an Ohio grown Thai-Rifat cutting, (panel c) a commercial kratom powder (K52), and (panel d) an equimolar mixture of kratom alkaloids at 313 ng/mL. Structures corresponding to each peak (► **Figs. 1** and **2**) were assigned by matching retention time, fragmentation pattern, and accurate mass with authentic standards. Compounds are numbered in order of elution. NL is the normalization level, i.e., the base peak intensity.

(K68) that were cultivated under the same growing conditions in Greensboro, North Carolina, USA (► **Table 4**, ► **Fig. 5**, and **Fig. 4S**, Supporting Information). For this analysis we performed a rapid methanolic extraction utilizing 30 min of ultrasound sonication. Other literature has established sonication as a rapid and useful method to extract alkaloids from *M. speciosa* [24, 34]. This method allows a higher throughput of plant samples, which will prove useful in future analyses of many kratom plants and products. The **12/13** ratio for K64 was 1:8 (0.962 ± 0.010 : 7.81 ± 0.33 mg/g powdered leaf material), while that of K68 was 3:1 (15.01 ± 0.20 : 4.52 ± 0.23 mg/g powdered leaf material). The **12/13** ratio of K64 resembled that of K59 (1:5, 2.782 ± 0.041 : 12.69 ± 0.42 mg/g powdered leaf material), which was obtained from a different supplier of *M. speciosa* "Rifat" leaf material. Conversely, the **12/13** ratio of K68 was similar to the commercial products K51 and K52 (6:1, 16.60 ± 0.89 : 2.95 ± 0.17 mg/g powdered material and 4:1, 16.84 ± 0.14 : 4.560 ± 0.010 mg/g powdered material, respectively) (► **Table 4**). This is the first literature example of a US-grown *M. speciosa* plant exhibiting a high-mitragynine alkaloid profile equivalent to Southeast Asian kratom products. Moreover, these results substantiate the claim that the *M. speciosa* "Rifat" plants used in this study (and likely previous studies in the literature) are a different chemotype (i.e., chemical phenotype) than *M. speciosa* plants growing in Southeast Asia.

The scientific evidence supporting the existence of *M. speciosa* chemotypes is growing. In addition to literature reports of alkaloid levels varying considerably between geographical localities, such as Thailand [13, 35], Malaysia [13, 17, 35], Indonesia [35],

and the Philippines [36], a recent report has shown significant alkaloid variation from *M. speciosa* within the same Malaysian plantation [17]. Further research is needed to reveal the breadth of *M. speciosa* chemical diversity in wild and/or cultivated plants and the prevalence of different chemotypes in kratom products circulating in the worldwide market. Toward that goal, this quantitative method provides an effective tool, and may be used for future efforts in quality control, quality assurance, and standardization of kratom materials.

This method would also prove useful to the chemical analysis of kratom products prior to pharmacological and clinical studies. The literature on the pharmacology of kratom continues to expand with several studies evaluating the "major" constituents of kratom such as **12, 11, 13** and **15** [12, 19], studies focused on the metabolic products of kratom alkaloids such as **2** and mitragynine pseudoindoxyl [37, 38], and a few recent studies that have characterized the pharmacological contributions of low abundance alkaloids such as **4**, isorhynchophylline, **6, 7**, corynoxine B, **14, 16**, and corynantheidine [17–20, 39].

Pharmacological analyses were not incorporated into this study; however, it is worth noting that the powder and loose-leaf products K49, K51, and K52 are composed of alkaloid quantities slightly higher (due to alcoholic extraction), but comparable to traditional tea preparations (i.e., 37.8 mg of **12** per g of extract) [15, 40]. Thus, tea-based preparations of these products would likely mimic the pharmacological activity of teas consumed in Southeast Asia. Alternatively, the alkaloid quantities of the commercial capsules far exceed those used traditionally. Thus, these products may pose a heightened health risk as compared to kra-

tom tea, particularly given that adverse interactions can occur when kratom alkaloids are co-consumed with other pharmaceutical and illicit substances [41].

The commercial products evaluated herein, K51 and K52, have comparable levels of **12**; however, K52 has a greater concentration of the other alkaloids (e.g., **6**, **7**, and **11**). The oxindole alkaloid **7** is reported to have high binding affinity to *µ*-opioid receptors and exhibit antinociceptive activity equal to morphine [17, 18]. Alkaloids **6** and **11** have both been shown to moderately inhibit CYP2D6, while **6** also has inhibitory activity against CYP3A and CYP2C9, which may affect the pharmacokinetics and pharmacodynamics of the other alkaloids [20, 42]. Pharmacokinetic differences between **12** administered alone (as an HCl salt), as an organic extract, as a lyophilized kratom tea, or as a commercial liquid sample have been reported in the literature [16].

Chemical analysis of the living kratom samples K55, K59, K64, and K68 shows that there are, at least, two different chemotypes in kratom plants being cultivated within the US. The high-**12** producing variety (K68) exhibits a chemical profile resembling the commercial products obtained from Southeast Asia, thus the biological activity may also be similar. The high-**13** producing variety (K55, K59, and K4), with low levels of **12**, could be predicted to exhibit weaker analgesic effects, based on the lower binding affinity of **13** compared to **12** [12, 39]. However, a recent analysis by Buckhalter et al. demonstrated that "Rifat" kratom (purchased from the same supplier as K55 and K59) does exhibit antidepressant-like and analgesic effects [43]. This activity may be from the two major constituents, **13** and 3-isoajmalicine, or may be attributed to metabolism products, as has been shown with 7-hydroxyspeciogynine [44] and 9-*O*-desmethylspeciogynine (i.e., gambirine) [45]. Further experiments would be needed to verify any predictions of biological activity based on differences in alkaloid content.

Collectively, the results presented herein build on previous literature to show that kratom is a botanical supplement composed of a variable mixture of alkaloids. The precise composition and quantitative amounts of each alkaloid may determine the cumulative biological effects of the whole. Therefore, comparative pharmacological evaluation of disparate mixtures of kratom alkaloids in both *in vitro* and *in vivo* models is imperative to fully elucidate the pharmacological potential of products containing *M. speciosa*. Quantitative analysis using the method described herein could serve as a useful aspect of such biological studies.

Materials and Methods

Materials and chemicals

Six of the indole alkaloids (**11**, **12**, **13**, **14**, **15**, and **16**) and four of the oxindole alkaloids (**3**, **6**, **7** and **8**) were isolated from commercial kratom powders and characterized in detail using proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance spectroscopy (NMR), high-resolution electrospray ionization mass spectrometry (HRESIMS), and electronic circular dichroism spectroscopy (ECD) data, as described previously [14], and were all of a high purity (≥98%) as determined by ultra-high-performance liquid chromatography-ultraviolet spectroscopy (UHPLC-UV) analysis. Corynox-

ine B was not included as a reference standard due to instability, as noted previously [14].

The indole alkaloids [**2** (>98%, Clearsynth) and **10** (≥98%, Adipogen)] and the oxindole alkaloids [**1** (97.5%, BOC Sciences), speciophylline (**5**) (90.3%, Chromadex), **4** (91.6%, Chromadex), and **9** (>98%, Carbosynth)] were all of a high purity. The purity and identity of these standards were verified by ^{1}H NMR, ^{13}C NMR, HRESIMS, and ECD. Data obtained with these methods were consistent with literature values (**Table 4S**, Supporting Information).

Mitragynine-d_3 (purity ≥98%; internal standard) was purchased from Sigma-Aldrich. Optima LC-MS grade acetonitrile (CH_3CN), formic acid, methanol (MeOH), and water (H_2O) were purchased from Fisher Scientific. Chemical grade chloroform ($CHCl_3$) was purchased from Fisher Scientific.

Constituent levels were quantified in MeOH extracts obtained from five commercial kratom products, which were termed "Green Maeng Da", "Yellow Indonesian", "White Jongkong", Kratom Extract, and "*Mitragyna speciosa* Botanical Extract" by the suppliers, and were internally coded as K49, K51, K52, K76, and K77, respectively (**Table 5S**, Supporting Information). Product K49 was a dried and cut leaf material, K51 and K52 were powdered leaf material, K76 was a liquid extract, and K77 was a capsule containing powdered kratom extract. Three powdered, dry leaf samples (coded as K55, K59, and K68) from live *M. speciosa* plants were also included in the analysis. Powdered, dry leaf material (coded as K55) was obtained from a young (ca. 9-month-old), cultivated *M. speciosa* plant ("Rifat"), as previously described [20]. Fresh leaves obtained from a 7-year-old cultivated *M. speciosa* plant ("Rifat"), generously donated by the same supplier as the K55 plant, were lyophilized, powdered (high-speed grinder, Newtry, Amazon), and sifted (400 µm) to yield sample K59. A 2-month-old rooted cutting (coded as K64) from a 4-year-old *M. speciosa* tree ("Rifat"-Thai) and an approximately 8-month-old *M. speciosa* seedling (purportedly from Mempawah, West Kalimantan, Indonesia) (coded as K68) were purchased from a US-based online vendor (Texas Coast Botanicals LLC). Upon receipt, the plants were re-potted in a 3-gal container using Happy Frog potting soil (FoxFarm Soil & Fertilizer Co.) and exposed to 24 h full spectrum LED light (GHodec, Amazon) with relative humidity maintained at >90% for 4 weeks. The plants were then transferred to a grow tent (VIVOSUN) and grown with a 24 h photoperiod of indirect light from a full spectrum LED light source (BP-1000, Bloom Plus, Amazon). The temperature and humidity were maintained above 15 °C and 60%, respectively. At 6 months, 3 leaves were harvested, dried at 25 °C, powdered using a high-speed grinder (Newtry, Amazon) and sifted (400 µm). Samples K49, K51, K52, and K55 were previously identified as *M. speciosa* based on DNA barcoding and maximum likelihood phylogenetic analysis [14, 20]. A BLAST search in the NCBI GenBank database using the Internal Transcribed Spacer (ITS) region from K64 and K68 showed these samples had ≥99% sequence similarity with *M. speciosa* (**Fig. 5S** and **6S**, Supporting Information). Phylogenetic analysis using the ITS region also placed K64 and K68 in a strongly supported clade (≥78% PhyML bootstrap support) with *M. speciosa* sequences (**Fig. 7S**, Supporting Information). All living

specimens grown at UNCG were also identified morphologically by authors M. K. or P. K. M.

Preparation of calibration standards

A 1 mg/mL primary stock solution was prepared for each of the kratom alkaloids [**1**, **3**, **4**, **5**, **6**, **7**, **8**, rhynchopylline (**9**), **10**, **11**, **12**, **13**, **14**, **15**, and **16**] by dissolving an accurately weighed quantity of each standard in an appropriate volume of MeOH. Compound **2** was purchased and used as a 100 μg/mL solution in MeOH. A combined stock of the 16 kratom alkaloids was prepared with the concentration of each constituent at 20 μg/mL in MeOH. The combined stock was further diluted with MeOH and H_2O to form a combined stock of 10 μg/mL of each alkaloid in MeOH–H_2O (90:10, v/v). A mitragynine-d_3 internal standard was prepared at 0.25 μg/mL in MeOH–H_2O (90:10, v/v). A working stock was prepared by diluting the 10 μg/mL combined stock two-fold with the internal standard. This combined working stock (5000 ng/mL) was then serially diluted two-fold with a 1:1 (v/v) mixture of the internal standard and the dilution solution (MeOH–H_2O, 90:10, v/v) to produce calibration curve solutions containing 2500, 1250, 625, 313, 156, 78.1, 39.1, 19.5, and 9.77 ng/mL of each analyte and 125 ng/mL of mitragynine-d_3.

UPLC-HRMS quantitative analysis

Chromatographic analyses of the kratom alkaloids were conducted utilizing a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system (Waters) coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). This system was operated using Thermo Scientific Xcalibur software version 3.0 (Thermo Fisher Scientific). UPLC system consisted of the following modules: a sample manager, photodiode array detector (PDA), column manager, and binary solvent manager. Chromatographic separation was achieved using a Kinetex F5 column (Phenomenex, 100 mm × 2.1 mm, 1.7 μm) at a column temperature of 35 °C, and a binary mobile phase consisting of 0.1% formic acid (A) and 0.1% formic acid in CH_3CN (B). Samples were eluted from the column at a flow rate of 0.6 mL/min using the following gradient: 95% A and 5% B were held isocratically for 1.0 min, followed by a linear decrease in solvent A from 95% to 88% over 2.0 min, a slight decrease to 87% in 4.0 min, a decrease to 75% in 4.0 min, a linear decrease to 55% in 4.0 min, and a sharp decrease to 0% in 1.0 min. The gradient was held at 0% A and 100% B for 0.9 min followed by a sharp increase to the 95% A and 5% B starting conditions over 0.1 min. The column was re-equilibrated at the starting conditions for 3.0 min. Total analysis time per sample was 20.0 minutes (▶ **Fig. 3**). All samples and standards were analyzed in triplicate using 2 μL injections via a 10 μL sample loop. The strong needle wash was comprised of isopropanol; the weak needle wash consisted of H_2O–CH_3CN (90:10, v/v). Isopropanol was injected between each sample to minimize carryover.

The Q Exactive Plus was equipped with a heated electrospray ionization (HESI) source operated in positive ionization mode using the following parameters: spray voltage of 3.5 kV, heater temperature of 450 °C, capillary temperature of 275 °C, S-Lens RF level of 50, sheath gas, auxiliary gas, and spare gas of 55, 15, and 3 (arbitrary units), respectively. Nitrogen was used as the source gas and as the collision gas.

Detection of the alkaloids was achieved using one full scan event followed by up to five data-dependent scans, based on the top five most abundant ions found on the inclusion list (**Table 6S**, Supporting Information). The full scan event included a mass range from m/z 250 to 1200 with a resolving power of 35 000, an AGC target of 1.0×10^6, and a maximum injection time (IT) of 50 milliseconds. The data dependent acquisition occurred with a resolving power of 17 500, an AGC target of 1E5, a maximum IT of 50 milliseconds, an isolation window of 1.8 Da, a collision energy of 43, and an intensity threshold of 1.6×10^5.

The spectrometer was calibrated weekly using Thermo Scientific Pierce LTQ Velos ESI Positive Ion Calibration Solution (Thermo Fisher Scientific).

Data acquisition and quantitative analysis of the alkaloids were accomplished using Thermo Scientific Xcalibur software version 3.0 (Thermo Fisher Scientific). All calibration curves were generated in Xcalibur Quan Browser. The measured uncertainty for the quantified alkaloids was reported with two significant figures, and the mean was rounded to the same decimal as the uncertainty [46]. Retention times, accurate mass, and MS-MS fragmentation spectra were compared with those of the known standards to confirm identities of the alkaloids in the *M. speciosa* samples.

Method validation

The analytical method to quantify indole and oxindole alkaloids was validated using guidance from the Association of Official Analytical Collaboration (AOAC) International guidelines for single-laboratory validation of chemical methods for dietary supplements and botanicals for linearity, precision, accuracy, repeatability, and sensitivity [47, 48].

Extracted-ion chromatograms were plotted for each of the alkaloids using the calculated m/z with a mass tolerance of 5.0 ppm. Peak integration was performed using peak picking algorithms built into the Thermo Scientific Xcalibur software. Settings used for peak integration are included in **Table 7S** (Supporting Information). The peak area was then plotted against the standard concentration and a weighted ($1/x^2$) least-squares regression was performed to determine the linear portion of the calibration curve.

Precision and accuracy were determined by calculating the percent RSD and percent RE, respectively, for replicate injections [49, 50]. RSD is defined as the percent of the standard deviation divided by the mean of sample replicates, where the standard deviation is the square root of the sum of squared residuals divided by the degrees of freedom [47]. In this work, RE is defined as the percent difference between the average measured concentration of three replicate injections of each standard concentration, and the nominal concentration of that standard.

Repeatability was evaluated based on the RSD and RE for triplicate analysis in a single day (i.e., triplicate injections of the standard solution at set concentrations), while intermediate precision was determined based on the interday RSD and RE of standard solutions at set concentrations (freshly prepared each day of analysis). Thus, intraday RSD and RE (repeatability) was determined using triplicate injections of the same solution, while interday RSD and RE (intermediate precision) used triplicate injections from

each of the replicate solutions prepared and analyzed on the three separate days (total of 9 injections).

System suitability was verified at the start and end of each analysis by analyzing a reverse-phase HPLC Gradient System Diagnostics Mix (Sigma-Aldrich). System suitability was also addressed by analyzing the lowest concentration of the calibration curve solution of alkaloids (9.77 ng/mL) at the start of each analysis. The AOAC half-height equation was used to determine the resolution of isomers in the system suitability mixture [48]. Additionally, mitragynine-d_3 was included as an internal control in all standard concentrations and samples to monitor consistency in retention times, linearity of instrument response, and to evaluate potential matrix effects.

The lower limit of detection (LLOD) was defined as the estimated lowest concentration that would give a measurable response [51]. This value was calculated according to Equation 1, where $\bar{x}$ is the average measured response and s is the standard deviation of the measured response of three experimental replicates of the lowest measured concentration, 9.77 ng/mL. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were defined as the lowest and highest concentration where the given analyte could be measured with an intraday accuracy between 80–120%. The linear dynamic range for each alkaloid was defined as being in the range between (and including) the LLOQ and the ULOQ. The linearity of all standard curves was verified by each having an $R^2 > 0.990$. An extrapolated lower limit of quantitation was calculated as three times the LLOD. Analytes beneath the linear range and above the extrapolated lower limit of quantitation were quantified and denoted as below the lower limit of quantification (BLQ).

$$LLOD = 3.3 \times (((lowest\ measured\ concentration)/\bar{x}) \times s)$$

Preparation and analysis of commercial kratom products and *M. speciosa* leaf samples

Triplicate extractions of the cut leaf and powdered kratom materials K49, K51, K52, K55, and K77 were conducted by adding 50 mg of kratom material and 5 mL of MeOH to a 20 mL scintillation vial, consistent with previously published methods [24, 27]. The mixtures were shaken 24 h at 20 °C and 150 rpm, decanted, and dried under a stream of nitrogen. Triplicate extractions of the powdered kratom products and *M. speciosa* leaf samples K51, K52, K59, K64, and K68 were conducted with a similar solvent to powder ratio (1 : 10), subjected to 30 minutes of sonication (FS110 Ultrasonic Cleaner, Fisher Scientific), decanted, and dried under a stream of nitrogen. An aqueous preparation of K51 (coded K51-3) was included in our quantitative analysis by adding 5 mL of 90 °C pure H$_2$O (NANOpure, Barnstead) to 50 mg powdered material in a 20 mL scintillation vial, performed in triplicate. The mixture was shaken 24 h at 20 °C and 150 rpm, decanted, and dried under a stream of nitrogen. The liquid kratom product K76 was filtered using 0.22 µm PDVF syringe filters (Fisherbrand, Fisher Scientific) and dried under a stream of nitrogen. In addition to the methanolic and aqueous preparations of K51, an alkaloidal fraction was prepared using the method described by Flores-Bocanegra et al. [14]. Briefly, 10 g of powdered material was macerated with 20 mL of 10% aqueous potassium hydroxide and 180 mL of CHCl$_3$

and MeOH (1 : 1, v/v) for 24 h at room temperature. The mixture was filtered, and the solvent was evaporated under reduced pressure. The majority of the dried extract was reconstituted in a 400 mL mixture of 1 M HCl and hexanes (1 : 1, v/v), filtered through a cotton plug into a separatory funnel, and partitioned. The aqueous layer was separated and basified (pH 9) with dropwise addition of concentrated NH$_4$OH, and the alkaloids were extracted with CHCl$_3$ (200 mL). The organic phase was washed with neutral H$_2$O and dried under reduced pressure and a stream of nitrogen to yield the alkaloidal fraction (35.6 mg) (K51-2).

The dried methanolic extracts of the commercial products and *M. speciosa* leaf samples, K51 tea, and K51 alkaloidal fraction were reconstituted to 2 mg/mL in an appropriate volume of Optima LC-MS grade MeOH (Fisher Scientific), sonicated, and centrifuged. Samples K76, K77, and K51 alkaloidal fraction were further diluted to 20 µg/mL prior to sample preparation, due to their high concentration of alkaloids. All samples (50 µL) were diluted with 450 µL of Optima LC-MS grade MeOH and 500 µL of the internal standard solution and analyzed with freshly prepared calibration standards (i.e., the primary stock solutions used to make the calibration standards were prepared from dry material within 24 h of the analysis) using the validated UPLC-HRMS method.

Supporting Information

The following are available in the Supporting Information: List of publications covering the analytical analysis of kratom plants and products (**Table 1S**), LC-MS chromatograms comparing a generic screening method using a BEH C18 column with the developed method using a Kinetex F5 column (**Fig. 1S**), precision and accuracy of the method for kratom alkaloid quantification (**Table 2S**), concentration and percent recovery of internal standard (**Table 3S**), base peak MS chromatograms representing kratom materials quantified in ▶ **Tables 2**, **3** and **4** (**Fig. 2S, 3S,** and **4S**, respectively), literature references for purchased indole and oxindole alkaloid standards (**Table 4S**), the commercial kratom products and *M. speciosa* specimens analyzed in this study (**Table 5S**), graphical overview of BLAST results in NCBI GenBank database using the Internal Transcribed Spacer (ITS) region for K64 (**Fig. 5S**) and K68 (**Fig. 6S**), phylogram showing that K64 and K68 group with other *M. speciosa* (**Fig. 7S**), the precursor ion inclusion list used for MS^2 analysis (**Table 6S**), and the constrain peak width settings used for peak integration (**Table 7S**).

Contributors' Statement

Conception and design of the studies: P. K. Manwill, H. A. Raja, D. A. Todd, N. B. Cech, N. H. Oberlies, L. Flores-Bocanegra, M. Khin. Data collection: P. K. Manwill, M. Khin, L. Flores-Bocanegra, H. A. Raja, D. A. Todd. Analysis and data interpretations: P. K. Manwill, H. A. Raja, L. Flores-Bocanegra, D. A. Todd, N. B. Cech, M. Khin. Provision of key study materials: L. Flores-Bocanegra. Manuscript drafting: P. K. Manwill, H. A. Raja, D. A. Todd, N. B. Cech, N. H. Oberlies, M. Khin. Critical revisions of manuscript: P. K. Manwill, H. A. Raja, D. A. Todd, N. B. Cech, N. H. Oberlies.

Conflict of Interest

The authors declare that they have no conflict of interest.

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