IMPROVED DETECTION OF KRATOM ALKALOIDS IN FORENSIC TOXICOLOGY

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A Dissertation

Presented to

The Faculty of the Department of Forensic Science

Sam Houston State University

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In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

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by

Stephanie Pauline Basiliere

December, 2019

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DEDICATION

"If you have faith as small as a mustard seed, nothing will be impossible for you."
-Matthew 17:20

To my Advisor, Dr. Sarah Kerrigan, I could not have done this without your faith, guidance, and support. Even when frustrations were high, we always pushed through and were able to complete this beautiful project. In addition, I wouldn't have experienced as much personal growth in terms of maturity and skill, had it not been for your influence. Thank you.

To my committee members, whom I've also had as professors during my undergraduate and graduate years, thank you for your time and guidance. Without you, I would not have gained the foundational knowledge necessary to complete this project. In addition, I also value the perspectives you bring to different subjects.

To my PhD cohorts, thank you for your support and encouragement. I especially want to thank Dr. Skillman, Polston (soon), and Winborn who have more than once uplifted me when I was at a low point in this journey.

To my parents, Elaine and Wayne Basiliere, thank you for your constant support and faith in me. We've come a long way from the days when I wanted to be a paleontologist at age 5. It's nice to see you helped keep my love for science alive. I hope I've made you proud.

To my family and friends, of whom there are too many to name, thank you for your support and all the laughs. Whether it's sending me memes or having a heart to heart conversation at 2 AM, you all have contributed to this journey and I couldn't have completed it without you. Although there are too many of you to name, I want to highlight Jackson, Hayden, Holly, Denise, Michael, Jordan, Erin, and Cathryn who have gone above and beyond in their support over the years.

ABSTRACT

Basiliere, Stephanie P., *Improved detection of kratom alkaloids in forensic toxicology*. Doctor of Philosophy (Forensic Science), December, 2019, Sam Houston State University, Huntsville, Texas.

Kratom is a botanical drug with psychoactive properties that produces both stimulant and opiate effects depending on the dosage. Its major psychoactive components are mitragynine and 7-hydroxymitragynine. The drug may not be included as part of routine toxicological screening and as such, its use may be underreported. This research seeks to improve the analysis of kratom alkaloids in toxicological specimens, and increase overall understanding related to their properties and drug metabolism.

Five *Mitragyna* alkaloids including speciociliatine (SC), mitragynine (MG), paynantheine (PY), speciogynine (SG), and 7-hydroxymitragynine (7-MG-OH) were analyzed in blood and urine using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q/TOF-MS). Each method was validated in accordance with published guidelines for forensic use. LC-Q/TOF-MS was used throughout the study due to its selectivity and sensitivity.

Recombinant human cytochrome P450 isoenzymes (rCYPs) were used to investigate the biotransformational pathways involved during drug metabolism. Phase I metabolism was attributed to four rCYPS (CYP3A4, CYP2D6, CYP2C19, and CYP2C18) producing a total of four metabolites (9-O-demethylmitragynine, 16-carboxymitragynine, 9-O-demethyl-16-carboxymitragynine, and 7-hydroxymitragynine). The pH and temperature dependent stability of MG, SC, SG, PY, and 7-MG-OH were also investigated. All five alkaloids were acid labile, with 7-MG-OH being the most unstable. However, SC,

SG, and PY were more stable than MG and 7-MG-OH. In addition, two degradation products of mitragynine were identified.

Chemical and enzymatic hydrolysis of conjugated metabolites of mitragynine were investigated using postmortem urine specimens. Acid and base hydrolysis, in addition to nine enzyme systems (β-glucuronidase: *Escherichia coli*, *Patella vulgata*, *Helix pomatia*; sulfatase: abalone entrails, *Aerobacter aerogenes*. *Patella vulgata, Helix pomatia*; recombinant systems; BGTurbo™, BGS™, ASPC™, IMCSzyme) were evaluated. Ultimately, hydrolysis did not improve the analysis of 9-O-demethylmitragynine and 7-hydroxymitragynine, but did improve the detection of 16-carboxymitragynine.

Postmortem blood (n=40), urine (n=16), and tissue (n=20) specimens from 20 fatalities were also evaluated. Speciociliatine and speciogynine were shown to be biomarkers of kratom use, with concentrations higher than mitragynine in the majority of specimens. In addition, the majority of blood and urine specimens contained several metabolites of mitragynine, with 9-O-demethylmitragynine and 7-hydroxymitragynine being the most prominent.

KEY WORDS: Kratom, LC-Q/TOF-MS, Mitragynine, 7-Hydroxymitragynine, Speciociliatine, Speciogynine, Paynantheine, High Resolution Mass Spectrometry

ACKNOWLEDGEMENTS

This project was supported by Award No. 2016-DN-BX-O006 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect those of the Department of Justice.

We gratefully acknowledge Dr. Ikhlas Khan and the National Center for Natural Products Research School of Pharmacy at The University of Mississippi for providing speciogynine.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER I

Introduction

In an effort to circumvent legislation, many drug users will seek drugs that can produce similar effects to illegal drugs, but are not yet regulated by law (1). There is also a preconceived notion that herbal or botanical drugs are a "safer" alternative to synthetic drugs, because they are naturally derived (2). One herbal drug that currently fits the criteria for a botanical "legal high" is kratom.

Kratom is traditionally used as an herbal medicine by the natives of Thailand and South East Asia due to its unique characteristic of being both a psychostimulant and a depressant, although the effects are dependent on dose (3). Kratom's dominant alkaloid is mitragynine, but it has many minor psychoactive alkaloids, most notably 7-hydroxymitragynine, which is a more potent analgesic than mitragynine (4). Recently, kratom usage has been increasing in the United States and Europe, as the plant is now widely available online and in retail outlets where it is being marketed as a safe, natural, and a legal alternative to opiates (2). The lack of legislation, wide availability, and the low cost of the drug when compared to other illicit substances has also increased the popularity of this drug.

History

Kratom, also known as Ketum or Baik-Baik, comes from the leaves of the Korth (*Mitragyna speciosa*) tree (5). The Korth tree is a large arboreal tree that can reach a height of 50 feet and a spread of 15 feet. The leaves of the tree are oval shaped and dark green in color. The veins of the leaves are either greenish-white or red, with red veins considered to be more potent (6). The tree is a member of the

Rubiaceae family which also includes the coffee plant (7). *M. speciosa* flourishes in hot, warm, and wet environments and grows in swamps, rubber plantations, rice fields, fruit gardens, ditches, and fishing ponds (8). The tree is indigenous to South East Asia, the Philippines, and Papua New Guinea, but is commonly seen in Thailand, Malaysia, and Myanmar (7). However, the plants can be cultivated in other regions of the world (8, 9).

Kratom usage was first recorded in the early twentieth century and it is a known herbal drug in Southeast Asia (10). Traditionally, kratom leaves have been chewed by the natives of Thailand to relieve pain and as an energy stimulant (10). It was also known to be used as an opium substitute (11). However, it has many other reported uses and is considered a cultural practice in Thailand and in some rural villages its use is even considered to be ritualistic (7). Since kratom is widely available for sale on the internet, marketed as a natural alternative herbal remedy for many conditions, and lacks strict legal restrictions, usage has increased dramatically in Western Europe and the United States (8). This use has further increased due to its opioid like properties, largely due to the growing opiate crisis in the United States (12). Kratom, and its major pharmacologically active component, mitragynine, have also been found in herbal blends sold in retail outlets (13). Although kratom usage has been reported for over 100 years, research on kratom is still ongoing and its chemistry and pharmacology are not well understood.

Scheduling

Kratom is illegal in Australia, Malaysia, Myanmar (Burma), and Thailand. It is also a regulated or a controlled substance in Finland, Denmark, Romania,

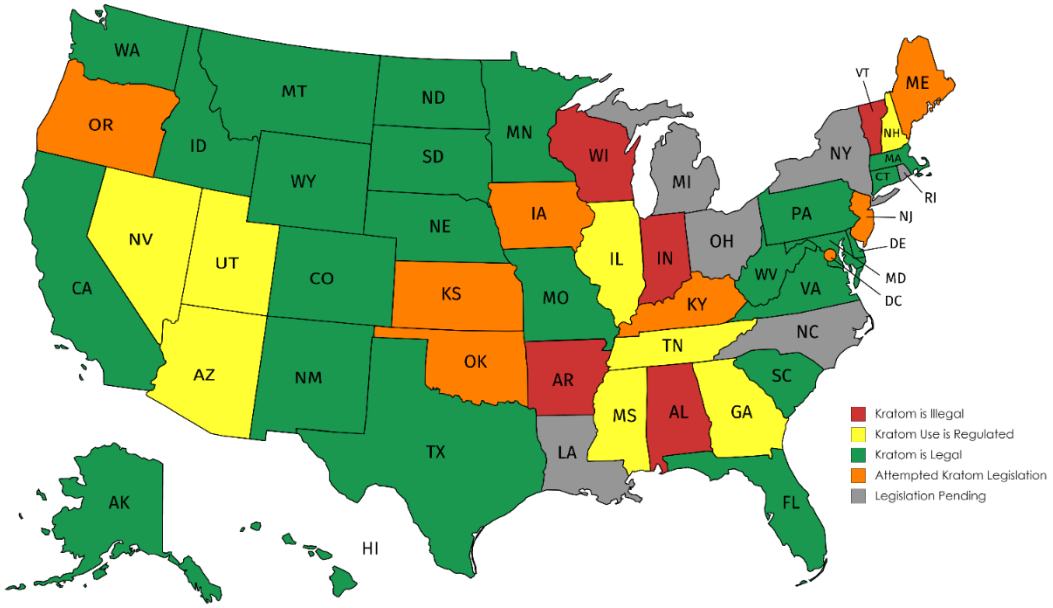
Germany, and New Zealand, but is still unregulated in most countries (6). Kratom was outlawed in Thailand by the Kratom Act in 1943, but it has not decreased usage (8). Kratom is one of the most widely abused illicit substances in Thailand and the use of the drug has been difficult to regulate due to the consumption of the drug being a culturally acceptable practice and because the plant grows naturally in the region with no human interference (14, 15). The plant is also easily cultivated which has led to it being grown in other regions of the world (9, 10). Although illegal in many countries in Southeast Asia, in Indonesia kratom is able to be grown legally and is shipped worldwide, making regulation even more difficult (8).

The legal status of kratom in the United States is somewhat controversial and has sparked much debate. At the federal level, the Drug Enforcement Administration (DEA) identified kratom as a "drug of concern" several years ago (16, 17). Following a recommendation from the US Department of Health and Human Services, in 2016 the DEA announced its intention to place kratom (and its primary psychoactive components, mitragynine and 7-hydroxymitragynine) into Schedule I of the Controlled Substances Act (17, 18). However this legislation was withdrawn in October 2016 due to intense public outcry from lobbyists, users, and medical professionals. Currently, the federal status of kratom regulation is uncertain pending additional research regarding its abuse potential, medical use, and overall health concerns (3). However, at the time of this study, the drug does not have any legitimate medical usage in the United States (16).

Regulation in the states is even more complicated with legislation varying by state (**Figure 1.1**). Kratom is illegal in Wisconsin, Indiana, Arkansas, Alabama,

and Vermont (19). There is currently pending legislation in Michigan, Louisiana, North Carolina, Ohio, New York, and Rhode Island. Kratom use is still legal in 39 states and Washington D.C., even though 8 of those states have attempted to pass (or had) legislation banning or regulating the drug. Interestingly, in 8 of the 39 states where kratom use is legal, the drug is regulated, usually by age. Three states (Arizona, Utah, and Georgia) have even passed legislation referred to as "The Kratom Consumer Protection Act" (20). This legislation is designed to protect consumer's access to the drug, but allow for the ban of adulterated products. It essentially requires vendors, creators, and sellers to properly label their products, prohibits the sale of certain products, creates specific penalties for violations of the act, mandates registration for kratom producers and creates standards for kratom production and sale. To summarize, kratom's legal status at both the state and federal level is still being defined and it is likely that it will be in a state of flux and uncertainty for many years to come.

Figure 1.1: Map of kratom legality in the United States.

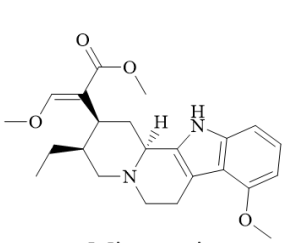


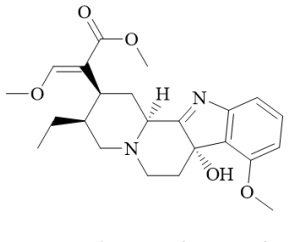
Chemistry

Forty-four compounds (**Figure 1.2**) have been isolated from the leaves of *M. speciosa* (3, 9). Although the alkaloid content varies due to geographical region and season, the total alkaloid content ranges from 0.5%-1.5% (8). The alkaloids in the leaves are a mixture of indole and oxindole alkaloids. Of these two types of alkaloids, the terpenoid indole alkaloid content remains the most stable in the leaves with little variation (7). The alkaloid content also varies with the age of the plant, with younger plants containing more of the mitragynine diastereoisomers, speciociliatine, and the paynantheine diastereomer, isopaynantheine than older leaves (8, 9). The alkaloid content also varies depending on several other factors including species, region of the plant, environmental factors and geographical location (9). Some specimens grown in the west have even reportedly contained alkaloids not seen in the specimens grown in Southeast Asia (21).

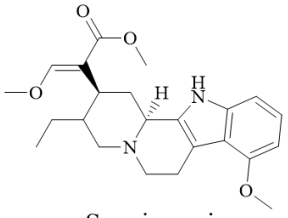
Figure 1.2: Indole alkaloids in *Mitragyna speciosa*.

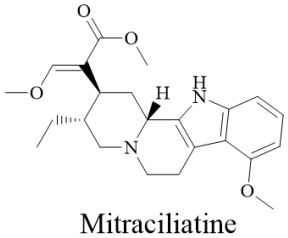
Major Psychoactive Alkaloids

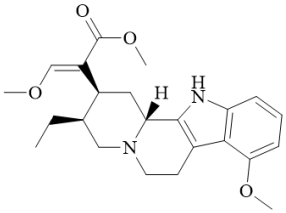

Mitragynine


7-Hydroxymitragynine

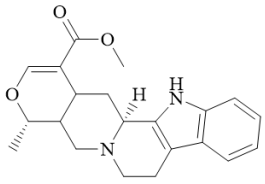
Diastereoisomers of Mitragynine

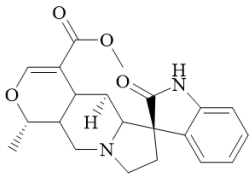

Speciogynine

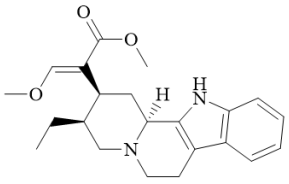

Mitraciliatine

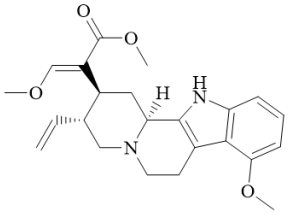

Speciociliatine

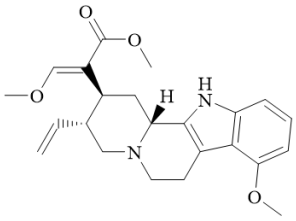
Indole Alkaloids in Kratom

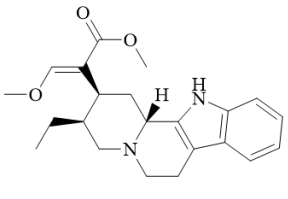

Ajmalicine


Mitraphylline


Corynantheidine


Paynanthenine


Isopaynantheine


3-Isocorynantheidiene

Mitragynine is the principal pharmacologically active compound in kratom, accounting for 60-66% of the total alkaloids present (5, 9, 22). The alkaloid content as described above, varies considerably, with one study by Takayama *et al*. reporting that speciogynine, speciociliatine, and paynanthenine account for 6.6%, 8.6%, and 0.8% of the total alkaloid content, respectively (5). Speciociliatine and speciogynine are both diastereoisomers of mitragynine while paynantheine is an alkaloid in kratom that is a dehydro analog of mitragynine. However, neither of these three compounds are thought to be significantly psychoactive (23). There is research that suggests that speciociliatine and speciogynine play a minor role in kratom's effects, but more research is needed to better understand these alkaloids. 7-Hydroxymitragynine is another prominent psychoactive compound present in kratom plant materials (2%) (5, 22). Remaining alkaloids such as mitraciliatine, mitraphylline, and isomitraphylline account for less than 1% collectively (8, 9). The primary target analyte for kratom in forensic toxicological analysis is mitragynine, although 7-hydroxymitragynine is sometimes targeted (**Table 1.1**). Other compounds in kratom such as, speciociliatine, speciogynine, and paynantheine are occasionally targeted, but published analytical methods for these compounds in biological specimens are uncommon and methods that target these compounds are usually limited to analysis of the plant materials (24-26).

Table 1.1: Overview of published analytical methods for *Mitragyna* alkaloids in biological matrices.

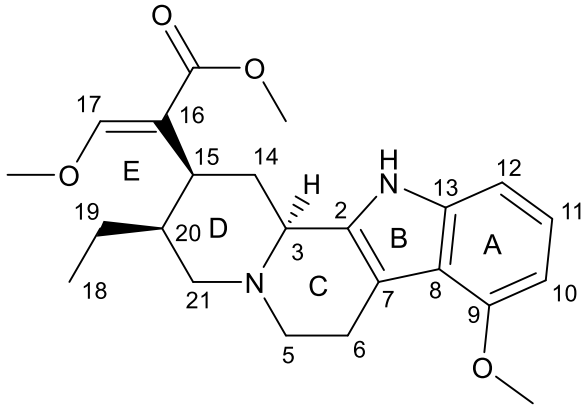
Matrix	Sample Volume	Extraction Method	LOD (ng/mL)	LOQ (ng/mL)	Linear Range (ng/mL)	Compounds Detected	Internal Standard(s)	Analytical Technique	Reference
Urine	0.2 mL	LLE	-	1	1-500	MG, 7-MG-OH	Mitraphylline	LC-MS/MS	(27)
Urine	3 mL	SPE	100	-	-	MG, PY, SC, SG	-	GC-MS	(28)
Urine	1 mL	BAμE	0.1	0.3	0.6-24	MG	-	LC-DAD	(29)
Blood, Tissue, Urine, Bile, Vitreous humor	1 mL or 1 g	LLE	0.25	1	1-10	MG	Proadifen	LC-MS/MS	(30)
Urine	2 mL	LLE	0.02	0.1	0.01-5	MG	Ajmalicine	LC-MS/MS	(31)

Matrix	Sample Volume	Extraction Method	LOD (ng/mL)	LOQ (ng/mL)	Linear Range (ng/mL)	Compounds Detected	Internal Standard(s)	Analytical Technique	Reference
Plasma	0.1 mL	LLE	0.2	1	1-5000	MG	Amitriptyline	LC-MS	(32)
Blood, Tissue, Urine	1 mL	SPE	30	50	50-1000	MG	MG-D_3	GC-MS	(33)
Plasma	0.1 mL	LLE	2	10	10-4000	7-MG-OH	Tryptoline	LC-MS	(34)
Blood	0.5 mL	LLE	0.16	-	5-500	MG	MG-D_3	LC-MS/MS	(35)
Plasma	0.5 mL	LLE	0.2	0.2	0.2-1000	MG	Amitriptyline	LC-MS/MS	(36)
Plasma	0.1 mL	SPE	25	50	50-10,000	MG	Mefloquine	LC-UV-DAD	(37)
Urine Blood Tissue Vitreous humor Bile	2 mL or 2g	LLE	0.25	1	1-100	MG	Proadifen	LC-MS/MS	(30)

BAμE, bar adsorptive microextraction; GC-MS, gas chromatography-mass spectrometry; LC-DAD, liquid chromatography-diode array detector; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LC-Q/TOF-MS, liquid chromatography-quadrupole time-of-flight-mass spectrometry; LC-DAD, liquid chromatography-UV-vis-diode array detector; LLE, liquid-liquid extraction; MG, mitragynine; MG-D_3, mitragynine-D_3: 7-MG-OH, 7-hydroxymitragynine; MG-OH-D_3, 7-hydroxymitragynine-D_3; PY, paynantheine; SC, speciociliatine; SG, speciogynine; SPE, solid phase extraction.

Mitragynine's structure was defined in 1963 and was later confirmed through crystallographic studies in 1965 (38, 39). It is a corynanthe-like alkaloid structurally similar to yohimbine (**Figure 1.3**) (40). Corynanthe-type alkaloids are known to be classified into four groups (normal, pseudo, allo, and epi-allo) based on chemical structure (9). Mitragynine belongs to the allo group, while its distereoisomers belong to the normal (speciogynine) and epi-allo (speciociliatine) groups. These differences in orientation on the chemical structure are thought play a major role in terms of its psychoactive effects (41). Mitragynine's chemical name is 9-methoxy-corynantheidine and its chemical structure is composed of an indole aromatic ring attached to two piperidine rings. Conformational studies suggest that the exocyclic groups have free rotation, but Rings A and B are thought to be planar due to being aromatic (42). Ring C is a piperidine derivative and the nitrogen is thought to be essential to mitragynine's ability to bind to opioid receptors (3). The methoxy-moiety at the positions C16 and the C9 have an open ring with substitution (42). Mitragynine has an empirical formula of $C_{23}H_{30}N_2O_4$ with a corresponding molecular weight of 398.50. Its pKa is 8.1 and the melting point is 104°C (7, 43).

Figure 1.3: Chemical structure of mitragynine showing ring positions and carbon numbering.



7-Hydroxymitragynine's structure (**Figure 1.2**) was identified relatively recently in 1993 (22). It is structurally similar to mitragynine with a hydroxyl group in the C7 position between rings 2 and 3. 7-Hydroxymitragynine's empirical formula is $C_{23}H_{30}N_2O_5$ with a corresponding molecular weight of 414.50, and a pKa of 12.20 (4). Speciociliatine and speciogynine share identical structures with mitragynine, differing only in spatial arrangement, and paynantheine has an unsaturated carbon at C18. Research on these three compounds is limited as much of the focus has been on 7-hydroxymitragynine and mitragynine. The structures of SC, SG and PY were confirmed between 1967 and 1975 (9, 44, 45). The molecular weights for these compounds are 396.5 (PY) and 398.5 (SC and SG) with corresponding molecular formulas of $C_{23}H_{28}N_2O_4$ (PY) and $C_{23}H_{30}N_2O_4$ (SC and SG).

Pharmacology

M. speciosa is a psychoactive plant, which means that it contains phytochemical compounds that affect the central nervous system and induce changes in behavior and/or mental activity (46). Kratom is not only known to be psychoactive, but it is also known to have several physiological effects (4, 5, 8, 9, 17, 40, 43, 47). Since kratom was recently introduced to western users, its pharmacological properties are not as well understood as many other illicit drugs. However, because of its increased popularity, comprehensive peer-reviewed studies on its unique properties are being further investigated.

Pharmacokinetics

Kratom is composed of more than 25 alkaloids, so the pharmacokinetics associated with the drug are complex. The drug is usually obtained as whole or crushed leaves, concentrated extracts, resin, or a loose or encapsulated powder that is typically consumed

orally (directly or prepared as a tea) (1, 8, 10). There are no studies that discus differences between doses of the more concentrated drug mediums such as the resin extract or the powder form. Also, although several internet forums do discuss what doses will give the desired effects, there are no published studies describing the relationship between dosage and the desired effect in humans (48, 49). Although there are many alkaloids in kratom, the primary alkaloid that has been the focus of the majority of pharmacokinetic studies is mitragynine. There have been several studies involving rats to determine its pharmacokinetic properties. After a 12 hour fasting period, 96 rats (8 rats per time interval) received a single oral dose of 20 mg/kg of mitragynine. Peak mitragynine concentrations of 424 ng/mL were reported at 1.3 hours in plasma (36). Another study involved 6 rats administered 1.5 mg/kg of mitragynine intravenously and 50 mg/kg orally. The mitragynine concentration in plasma peaked at 700 ng/mL at 4.5 hours following oral administration and 2,300 ng/mL at 1.2 hours following intravenous administration (37). Although pharmacokinetic studies involving humans are limited, Trakulsrichai *et al*. reported that mitragynine concentrations in ten male individuals peaked between 18.5-105 ng/mL with an average of 0.83 ± 0.35 hours (50).

The solubility of mitragynine has been reported to be 18.7 ± 0.4 μg/mL in buffer at pH 9, to 64.6 ± 1.2 μg/mL in water, 88.9 ± 1.6 μg/mL in buffer pH 7, and 3.5 ± 0.01 mg/mL in buffer at pH 4 (43). Since mitragynine is a weakly basic compound, it is not surprising that it is highly soluble at acidic pH since it is predominantly in its ionized form. In that same study, mitragynine was found to have an intermediate lipophilicity (logP value of 1.70). This intermediate lipophilicity indicates that mitragynine favors high blood-brain barrier penetration which could explain the psychotropic properties of the compound, but

there is currently no study that confirms this. In fact, since both mitragynine and 7-hydroxymitragynine are reported to be highly protein bound (>90%) they might actually have restricted passage across the blood-brain barrier (51). Information on postmortem redistribution (PMR) is limited at this time to two case reports. McIntyre *et al*. described PMR in a fatality caused by kratom (n=1). The central blood to peripheral blood (C/P) ratio was 0.83 and the liver to peripheral blood (L/P) ratio was 1.9 (33). Another study by Holler *et al*. described PMR from another fatality linked to kratom use and reported C/P and L/Ps of 0.39 and 0.12, respectively (30). These ratios indicate little to no propensity toward postmortem redistribution. However, due to the very limited sample size, inferences regarding PMR should not be made (52, 53).

The half-life following oral administration of mitragynine was reported to be between 3.9 and 6.6 hours in rats (36, 37). Half-lives in humans following oral administration of kratom tea was 23.24 ± 16.07 hours (50), highlighting important differences between species. The half-life following intravenous administration of mitragynine was reported to be 2.9 hours (37). That same study also found the oral bioavailability to be 3.03 ± 1.47%. The volume of distribution for mitragynine is widely variable in the literature and reported to be 38 L/kg (36) and 90 L/kg (54). However, the wide range might also be explained by the overestimation of mitragynine concentrations due to the lack of sensitivity of ultraviolet (UV) detection used by Janchachee *et al*. compared to the high sensitivity of liquid chromatography-tandem mass spectrometry (LC–MS/MS) used in the study by de Moraes *et al*. (36, 54). In another study by Trakusrichai *et al*. the estimated volume of distribution was 38.04 ± 16.07 L/kg. In that same study, the total clearance was 98.1 ± 51.34 L/h kg and the estimated half-life was 23

hours. Overall, only 0.14% of the drug was eliminated unchanged in urine (50). It is suspected from this study, along with the other pharmacokinetic studies, that mitragynine has a large volume of distribution and is mainly distributed outside of the circulation (36, 50, 54). The reported volumes of distribution in these three studies might suggest that the drug is a candidate for PMR, but limited reports to date have not confirmed this (30, 33).

With regard to the other *Mitragyna* alkaloids, the literature is much more limited in terms of their pharmacokinetic activity. 7-Hydroxymitragynine was reported to have a half-life of 23 mins and a much lower volume of distribution (1.5 L/kg) relative to mitragynine (34). Given the compounds increased polarity when compared to mitragynine, this is not surprising, but it is important to note that studies have only been performed using animal and in-vitro models.

Kratom is widely considered a "safe drug" by many users and one study suggests that it may be less toxic compared to similar drugs of abuse. Sabetghadam *et al*. found that using kratom extracts, the median effective dose (ED_{50}) was 22 mg/kg and the median lethal dose (LD_{50}) was 477 mg/kg in mice (55). The authors suggest that this high therapeutic index supports the contention that the drug is "safe" because it is unlikely to result in a fatal overdose from overconsumption of the drug alone (55). However, numerous fatalities have been reported (33, 56-58), albeit most frequently in combination with other drugs.

Pharmacodynamics

The pharmacodynamic behavior of kratom can be characterized by both physiological and psychological effects. Kratom has dual stimulating and sedative effects

that are dose-dependent (40). Low doses of kratom are reported to produce a stimulant effect similar to cocaine and high doses are reported to produce opiate-like effects (8). There are numerous studies detailing the positive and negative effects of kratom, including alertness, euphoria, and impaired memory (59). Acute usage may induce nausea and headaches and chronic usage has been shown to cause hepatic problems, hyperpigmentation, reduced mental prowess, and an induction of indolence that is usually associated with opiate use (47). Other side effects include seizures, coma, hypothyroidism, weight loss, pulmonary edema and congestion, increased blood pressure, increased heart rate, distended bladder, tremors, anxiety, irritability, aggression, constipation, itching, psychosis, weight gain, and insomnia (4, 8, 17, 30, 50, 55, 56, 60-64).

Kratom has various physiological effects depending on dosage. Muscle relaxation is a physiological effect often associated with opiate use, and is also widely reported in kratom users (40). A study by Watanabe *et al* indicates that mitragynine causes a decrease in muscle contractions by inhibiting the neurotransmitter release from the nerve endings by blocking the neuronal Ca^{2+} (65). This study indicates that mitragynine and other kratom alkaloids may act on the neuromuscular junction in a similar function to other opiates. However, the anecdotal and experimental evidence for muscle relaxation is contradicted by an earlier study, which states that mitragynine consumption may result in light tremors and tenseness in the hands and feet (64). This contradiction could be explained by the unique dose-dependent relationship that mitragyine has where studies have shown that low doses commonly cause stimulant like effects, such as tremors and muscle contractions (5, 27, 40). However, two case studies suggest that kratom usage may actually encourage seizures in some users which further supports the stimulant-like effects of mitragynine (61,

66). It should be noted that in the case studies, it is not known if a combination of drugs with kratom or the individual's health circumstances could have also helped instigated the seizure activity. However, seizures linked to kratom quadrupled in Thailand from 2005 to 2011 (67) further supporting the reports of kratom causing seizure activity.

Another physiological symptom commonly associated with kratom usage is constipation. In Thailand, natives often used kratom for gastrointestinal illness (40). A study by Chittrakarn *et al*. suggests that kratom has an inhibitory effect on the gastrointestinal tract (68). The study maintains that the effect is similar to a mu opioid receptor agonist (loperamide) used to treat diarrhea by decreasing potassium and sodium permeability giving more indication that kratom behaves like an opiate in certain doses (68). The study also showed that gastric acid secretion is inhibited by the same interactions with the opioid receptors in the gastrointestinal tract and leads to decreased motility (68). These interactions with the gastrointestinal tract may explain the anorexic effects users of kratom often experience (4, 8). However, there are conflicting reports on its anorexic effects, including a case study that suggests kratom may in fact cause weight gain (62). This suggests that the anorexic effect may depend on the individual and other mitigating factors and not solely the usage of kratom.

Kratom has been reported to cause a slight nystagmus and dilation of the blood vessels (64). A toxicity study by Harizal *et al*. indicated that high doses of over 1000 mg/kg in rats increased blood pressure and caused severe hepatotoxicity and nephrotoxicity (69). Another toxicity study reported that rats exposed to kratom at doses of 100 mg/kg, 200 mg/kg, and 500 mg/kg, showed a decreased body weight, toxicity in the kidneys, lungs, and liver, behavioral changes, and changes in blood chemistry. The authors noted that

adverse effects were more likely to be seen in the rats exposed to 200 mg/kg and 500 mg/kg (70). The hepatotoxicity seems to be supported by a case study where kratom usage caused intrahepatic cholestasis, although this effect may be dose dependent or dependent on the individual's health situation (71). However in an recent case study, cholestatic hepatitis was solely linked to kratom usage (72). However, Dorman *et al*. stated that hepatoxicity is only reported in western countries indicating that the hepatoxicity is potentially caused by contamination or as a result of an error in plant identification (72).

Kratom products have also been shown to be cytotoxic to both human neuronal and intestinal cells, as well as genotoxic in intestinal cells, but this is likely due to kratom products being mixed with other psychoactive substances (73). Glucose transport may also be affected by kratom usage. A study by Purintrapiban *et al*. indicated that kratom increases post-transcriptional expression of the glucose transporter 1 (GLUT1) gene, which increases glucose transport, suggesting that kratom has an anti-diabetic effect (74). Kratom's effect on glucose transport could explain why the plant is commonly used in traditional Thailand folk medicine for the treatment of diabetes (14).

In a case study involving a fatal overdose, an autopsy showed pulmonary congestion, edema, and a distended bladder thought to be caused by solely by kratom usage (56). Another study involving the multiple autopsies of deaths caused by kratom showed brain and lung edema and congestion in multiple organs (75). While these cases may suggest that kratom has a toxic effect on lung tissue, there is no existing toxicity study that confirms pulmonary tissue toxicity. Another study by Utar *et al*. showed that mitragynine decreases cyclooxygenase-2 (COX-2), which might explain the anecdotal descriptions of hot flashes, edema, and anti-inflammation effects of kratom (76).

Kratom also has several psychological effects that are unique to the mixture of compounds, primarily mitragynine, found in the plant. A study conducted by Boyer *et al*. reported that mitragynine inhibits radioligand binding at the central nervous system receptors including the adrenergic, dopamine, and opioid receptors (66). In that same study, mitragynine had an affinity for all opioid receptors, but had a high affinity μ-opioid receptor which could indicate why many users report its ability to reduce opiate withdrawal symptoms (66). In fact it has been reported that mitragynine has one fourth of the potency of morphine (5). 7-Hydroxymitragynine is reported to be 13-fold more potent than morphine and 46-fold more potent than mitragynine (3, 77). It has a high affinity for the mu opioid receptor, especially when kratom is consumed orally. 7-Hydroxymitragynine has reportedly been found to be more 46-fold more potent then morphine (77). In addition, a recent study by Kruegel *et al*. also reported that mitragynine and 7-hydroxymitragynine are partial agonists on the human opioid receptors, however other prominent alkaloids in kratom such as paynantheine, speciogynine, and speciociliatine, have no agonist activity and may in fact have partial antagonistic activity (78). Another study conducted by Matsumoto *et al*. showed that mitragynine at high doses inhibited the contraction of the vas deferens in mice, which indicates that mitragynine may be weakly agonistic towards the delta-opioid receptors (79). There is also evidence that crude extracts of kratom which contain large amounts of various alkaloids may both excite and inhibit the hippocampus through short term potentiation with a decrease of the intracellular calcium ion concentration (46).

It has frequently been reported that mitragynine has anxiolytic effects (80). A study conducted by Khor *et al*. indicated that mitragynine attenuated morphine withdrawal in

zebra fish by decreasing the production of G-protein coupled receptors that are associated with endocrine and behavioral responses to stress (81). The decrease in production led to a decrease in stress-induced behaviors (81). A similar study by Yusoff *et al*. showed that mitragynine's opiate like quality for reducing stress has a potential for addiction and abuse when given to mice in acute quantities (82). Mitragynine also has been shown to suppress the head twitch response in mice associated with the stimulation of serotonergic and adrenergic receptors, which adds more credibility to the idea that mitragynine also binds to these receptors (83). One study also indicates that mitragynine has an antidepressant effect in which the compound restores the monoamine neurotransmitter levels, specifically the serotonin, norepinephrine, and dopamine receptors (84). Studies in rats indicate that mitragynine may also cause neuropathological changes including local vacuolation, necrosis, an increase in lactate dehydrogenase, and the degeneration of neurons at high doses (63).

Both 7-hydroxymitragynine and mitragynine have been shown to have a similar response to morphine and cocaine when discriminative stimulus tests were performed on rats, further indicating the drugs stimulant and opiate like effects (85). Another study by Carpenter *et al*. showed behavior and biological activity in rats on par with oxycodone after mitragynine consumption (86). One study by Sufka *et al*. indicated that rats who were given mitragynine often showed place preference when consuming the drug (87). Another study involving human cancer cells and in vivo methods indicated that neuronal cells were highly sensitive to the cytotoxic effects of kratom (73). A study by Apryani *et al*. indicated that low doses of mitragynine may impair working memory (88) while another study indicated that it improved memory and learning behaviors (70). Finally, a study by Ismail *et al*.

disputed that claim by reporting that mitragynine can increase exploration activity, enhance punishment resistance, and impair learning behavior in mice exposed to it frequently (89).

Kratom has unique psychological effects when used with other drugs. A study by Fakurazi *et al*. showed that the combination of mitragynine and morphine increased the analgesic effects, but reduced the development of morphine tolerance (90). This indicates that the anecdotal reports on kratom being used for the treatment of opiate addiction may have some validity. Another study by Cheaha *et al.* reported that mitragynine extracts may also help with ethanol withdrawal symptom (91). Kratom also has been shown to be addictive and produces physiological and psychological withdrawal symptoms similar to opiate withdrawal (92). Withdrawal symptoms commonly reported are insomnia, anorexia, nausea, vomiting, muscle spasms, sweating, fever, diarrhea, hot flashes, watery eyes and nose, body aches, depression, restlessness, moodiness, coughing, fatigue, lack of motivation, anxiety, cravings, and sleepiness (60, 92, 93). At this time, one case study has been published describing a case of kratom addiction and withdrawal in the United States (94). While several surveys from Asia have reported addiction and withdrawal, this is the first case study of addiction and withdrawal from the United States. There have also been several case studies of neonatal abstinence syndrome on infants whose mothers consumed kratom frequently during pregnancy (95-98). These infants primarily displayed symptoms associated with opiate withdrawal. Although there are several reports of kratom being used to treat opiate addiction and discussions surrounding its potential for supervised medical use are ongoing, it is not an official treatment method in the United States for any medical condition.

Stability

At present, information regarding the stability of *Mitragyna* alkaloids is limited to only mitragynine and 7-hydroxymitragynine. Mitragynine has been reported to be stable in several processed samples and during freeze-thaw cycles (37, 50, 54, 99). Currently, only two studies have focused on the stability of mitragynine outside of method development and validation. A study by Manda *et al*. found that when incubated for two hours in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8), mitragynine degraded by 26% and 3.6% indicating that mitragynine is acid labile (51). Another study by Ramanathan *et al.* reported that the percentage of mitragynine recovered at 0.5 h, 6 h, and 24 h in multiple buffer systems were 100.6 ± 0.3%, 91.8 ± 1.0% and 88.8 ± 1.4% for pH 4 buffer; 99.2 ± 0.4%, 96.1 ± 1.9% and 101.9 ± 0.9% for pH 7 buffer; and 101.3 ± 2.6%, 97.8 ± 0.3% and 95.5 ± 2.5% for pH 9 buffer (43). In that same study, mitragynine was incubated in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8). Mitragynine was stable for 3 hours in the simulated intestinal fluid with less than 5% deviation from the original drug content. In contrast, mitragynine was highly unstable in the simulated gastric fluid, with over 20% deviation from the original drug content at 20 minutes after incubation (43).

In contrast to mitragynine, 7-hydroxymitragynine is known to be extremely unstable. Currently, methanolic 7-hydroxymitragynine certified accredited reference materials require special handling and must be stored in highly alkaline conditions at sub-freezing temperatures (-60 to -80°C) (100). Stability studies for 7-hydroxymitragynine are more limited than mitragynine, but Manda *et al*. determined that when incubated for two hours in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8), 7-

hydroxymitragynine degraded by 27% and 6%, respectively (51). However, 7-hydroxymitragynine has been reported to be stable in rat plasma for 12 hours at room temperature and for 30 days at -20°C (34), showing that 7-hydroxymitragynine may be stable in the short term under certain conditions.

Metabolism

The metabolism of the *Mitragyna* alkaloids is not fully understood. An early study by Zarembo *et al.* used *Helmin-thosporum sp.* to determine that oxidation and hydroxylation pathways are the primary mechanism for mitragynine metabolism (101). It is also thought that mitragynine is mainly metabolized by hepatic metabolism and that metabolism is linear on a two-compartment model (50). Although studies involving microbial transformations are useful, these studies are limited because they cannot fully mimic metabolism in humans in which case human and animal studies are important for identifying phase I and II metabolism. In phase I metabolism the primary mechanism is the hydrolysis of the methylester groups (C16) and the demethylation of the methoxy groups (C9 and C17) (102). The phase I metabolites of mitragynine found in studies involving human urine include 9-O-demthylmitragynine, 9-O-demethyl-17-carboxy-16, 17-dihydromitragynine, 9-O-demethyl-16-carboxymitragynine, 16-carboxymitragynine, 17-dihydromitragynine, 17-O-demethyl-16, 17-carboxy-16, 7-hydroxymitragynine, and 17-dihydromitragynine (102). While phase II metabolism has not been studied as thoroughly as phase I, one study by Philipp *et al.* has shown that mitragynine is heavily conjugated by both glucuronides and sulfates in human urine samples (102). The same study found that the phase II metabolites include 9-O-demethylmitragynine sulfate, 9-O-demthylmitragynine glucuronide, 9-O-demethyl-16-carboxymitragynine glucuronide, 9-

O-demethyl-16-carboxymitragynine sulfate, 9,17-O-demethyl-16,17-dihydromitragynine glucuronide, 16-carboxymitragynine glucuronide, 9,17-O-bisdemethyl-16,17-dihydromitragynine sulfate, and 17-O-bisdemethyl-16,17-dihydromitragynine glucuronide (102). During the course of this study, 7-hydroxymitragynine was reported to be a metabolite of mitragynine (103, 104). This finding is highly significant due to the increased potency of 7-hydroxymitragynine.

There are currently no studies that identifying the phase I metabolites of 7-hydroxymitragynine. Manda *et al.* reported that 7-hydroxymitragynine converted to mitragynine in the presence of human live microsomes (HLM) and in alkaline intestinal fluid (pH 6.8) (51). Although this is the first report of the metabolic conversion of 7-hydroxymitragynine into mitragynine, future studies are needed to evaluate this conclusion. In addition, a study by Kruegel *et al.* reported that 7-hydroxymitragynine may not be present in all plant samples but seems to be found more in dry leaf materials that have been exposed to air and sun for long periods of time (78). In conclusion, while 7-hydroxymitragynine is present in some plant samples and is psychoactive, very little is known about the compound. The phase I and II metabolites of speciogynine, speciociliatine and paynantheine have been identified (105-107) and they metabolize in a manner analogous to mitragynine, with many metabolites sharing identical structures.

Although several metabolites of mitragynine have been identified, there is little information regarding the cytochrome P450 isoforms (CYPs) responsible for metabolism. This is concerning, because understanding the isoforms involved is important from the standpoint of drug-drug interactions and potential adverse reactions due to genetic polymorphisms. A recent study identified CYP3A4 as the primary contributor to

metabolism with minor contributions from CYP2D6, CYP2C9, and CYP2C19 (104). Mitragynine and 7-hydroxymitragynine were also shown to inhibit P-glycoprotein. This indicates the possibility of a drug interaction if mitragynine and 7-hydroxymitragynine are coadministered with drugs that are P-glycoprotein substrates (51). Mitragynine has also been shown to have toxicokinetic interactions with permethrin by inhibiting hydrolysis, increasing the risk of developing neurotoxicity (108). The compound has also been found to inhibit hERG (human ether-à-go-go-related gene) and GIRK (G protein-coupled inwardly-rectifying) potassium channels which can cause a risk of cardiotoxicity (109). In addition, 7-hydroxymitragynine has also been reported to negatively interact with drugs that are metabolized by uridine 5'-diphospho-glucuronosyltransferase (UGTs) 2B7 and 1A1 (77).

Administration and Use

Kratom has been traditionally used by chewing on the leaves and brewing into a tea (10). In modern times, the leaves are also smoked, swallowed in a "gum" like substance, or taken by capsule (3). A typical user in Thailand will often consume ten leaves per day (110). Western users typically obtain the drug through vendors online or in retail outlets in herbal blends (40). However, several of these products are known to contain not only kratom, but several other psychoactive drugs (2, 75, 111).

Users claim that kratom has a variety of therapeutic uses. In Thailand, kratom was used by natives who performed manual labor because of its stimulant properties and its ability to treat chronic pain (3). It is also important to note that kratom usage in Thailand does not interfere with the social functioning of kratom users in Thailand and its usage is not seen as a negative (112). The only exception is the negative social stigma towards

female addicts and users which might explain why more male users are self-reported (112). Kratom usage in the United States and Western Europe is more recent and is often recreational because of its euphoric effects (3). However, a growing number of drug users are beginning to use kratom as an opioid alternative for the treatment of chronic pain and to treat opioid addiction (11). Other uses include treating intestinal infections, as an antidepressant, to reduce coughing, as an antidiarrheal, treatment for alcohol withdrawal, insomnia, diabetes, and to improve sexual performance (113). It also has been reportedly used as an analgesic, antidiabetic, antipyretic, to treat hypertension, and as an anxiolytic (8, 92, 93). Because of its recent introduction to Western society, there are few comprehensive studies on its potential medical uses and as of this time there are no official medical uses in the United States (16, 114).

Analytical Methods

There are few analytical methods for the detection of kratom in toxicological samples (**Table 1.1**). Most of the published methods for multiple *Mitragyna* alkaloids describe the analysis of the compounds directly from plant materials. In contrast, many of the methods for analysis of biological specimens only target mitragynine, and occasionally, 7-hydroxymitragynine. Current analysis methods are mostly centered on gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), but several other identification methods like thin layer chromatography (TLC) and ultraviolet-visible spectrometry (UV-VIS) have been reported.

As the drug is relatively new to the Western world, very few methods have been developed using toxicological specimens. Based upon the limited number of published case reports **(Table 1.2)** analytical methods must have a wide range of detection for

concentrations of these alkaloids in biological specimens. In a study involving urine from fifty recreational kratom users, mitragynine concentrations ranged from 1 to 50,000 ng/mL (27). Concentrations of mitragynine following overdose or fatalities have been reported in the range 10-3,470 ng/mL (**Table 1.2**). In another study, mitragynine concentrations in femoral blood (n=8) were 20-180 ng/mL (75). Another case detailing a fatality from kratom use reported mitragynine concentrations at 0.23 mg/L in peripheral blood, 0.19 mg/L in central blood, 0.43 mg/kg in liver, <0.05 mg/L in vitreous fluid, and 0.37 mg/L in urine (33). It is important to note that these studies (and most published analytical methods) focused only on the detection of mitragynine. It is also possible that kratom cases may be underreported due to the sole focus on mitragynine (115).

Table 1.2: Concentrations of Mitragynine in blood and urine specimens.

Investigation	Matrix	Internal Standard	Concentration (ng/mL)	Reference
Antemortem samples (n=50)	Urine	Mitraphylline	1.2 - >50,000	(27)
Intoxication (n=1)	Urine	Not described	167	(61)
Fatality (n=1)	Femoral Blood	Not described	600	(56)
Fatality (n=9)	Femoral Blood	LSD-D_3	20-180 (µg/g)	(75)
Fatality (n=1)	Heart Blood	Proadifen	390	(30)
Fatality (n=1)	Femoral Blood Heart Blood	Mitragynine-D_3	230 (Femoral) 190 (Heart)	(33)
Fatality (n=1)	Femoral Blood Urine	Amphetamine-D_3	1,060 ng/mL (Femoral) 3,470 ng/mL (Urine)	(116)
Fatality (n=2)	Femoral Blood Urine	MPPH	10-780 (Femoral) 10 - >400 (Urine)	(57)
Fatality (n=31)	Femoral Blood Iliac Blood Subclavian Blood	Mitragynine-D_3	11-3,300 (Femoral) 42-2,900 (Urine) 43 (Subclavian)	(35)

MPPH, 5-(p-methylphenyl)-5-phenylhydantoin.

Most of the published methods for the analysis of *Mitragyna* alkaloids have utilized LC-MS, while only three methods used GC-MS. Although GC-MS is the most commonly used analytical instrument in forensic laboratories, several issues with these alkaloids have made GC-MS methods undesirable. Phillip *et al.* developed a GC-MS method for toxicological samples using conventional electron ionization and full scan acquisition (28). Although the method was able to detect mitragynine, speciociliatine, paynantheine, and speciogynine, it lacked overall sensitivity (limit of detection; 100 ng/mL), was unable to quantify the alkaloids, and required derivatization due to the polarity of mitragynine (28).

Analytical methods that utilize LC-MS do not require derivatization for *Mitragyna* alkaloids and is also a more appropriate technique for the analysis of polar analytes. A study by de Moraes *et al.* used LC-MS/MS to detect mitragynine in rat plasma (36). This method used a mobile phase gradient of a 70:30 (v/v) solution of ammonium acetate and acetonitrile containing 0.5% formic acid coupled to a MS operating in positive electrospray ionization mode (36). This method was shown to be precise, accurate, and sensitive with a reported limit of quantitation (LOQ) of 0.2 ng/mL although the limit of detection (LOD) was not reported (36). Another study by Lu *et al.* developed a quantitative method for the analysis of mitragynine in urine using LC-MS/MS (31). This method used a mobile phase gradient of ammonium acetate with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) and methanol coupled to a MS operating in positive electrospray ionization mode and was able to detect and quantitate mitragynine with a limit of detection at 0.02 ng/mL and a limit of quantitation at 0.1 ng/mL. Several metabolism studies on multiple *Mitragyna* alkaloids have also used liquid chromatography-linear ion trap-mass spectrometry (LIT/MS/MS) (102, 105-107).

Some analytical methods have focused on detecting and quantifying 7-hydroxymitragynine is biological matrices. Vuppala *et al.* described an LC-MS/MS method for the detection and quantitation of 7-hydroxymitragynine in rat plasma with a LOQ of 10 ng/mL and a concentration range of 10-4,000 ng/mL (34). Le *et al.* also described an analytical method for 7-hydroxymitragynine in urine using LC-MS/MS. Although only a qualitative study, they reported that all specimens with mitragynine also contained 7-hydroxymitragynine (27). Studies involving the analysis of paynantheine, speciogynine, and speciociliatine in biological specimens are limited, but qualitative assays utilizing LIT/MS/MS were used to structurally identify potential metabolites of speciociliatine, paynantheine and speciogynine (105-107). Philipp *et al.* also described a quantitative GC-MS method that was able to detect speciociliatine, speciogynine, and paynantheine in human urine specimens (28). In addition, a study by Arndt *et al.* was able to qualitatively identify speciocilatine, speciogynine, and paynantheine using an LC-MS/MS method (111).

Recently a notable study for *Mitragyna* alkaloids by Avula examined kratom leaf extracts using liquid chromatography quadruple time-of-flight mass spectrometry (LC-Q/TOF-MS) and was able to successfully predict the characteristic fragmentation patterns of mitragynine and 7-hydroxymitragynine (25). While the method was effective, sensitive, selective, rapid, and allowed the identification of the indole group and other functional groups in kratom, this method was developed using plant extracts and has not been used in the identification of kratom's alkaloids in biological matrices.

Mitragyna Alkaloids in Forensic Toxicology

Kratom is new to the western world and is still legal in most of the United States. However, increased recreational use, interest in therapeutic use and the growing number of case reports involving fatal overdose and other serious side effects (58, 117) are a cause for concern. Between 2009-2013, over 14 exposures of kratom requiring hospitalization were reported in Texas (117). In fact, according to NFLIS (National Forensic Laboratory Information System), cases involving mitragynine were not reported until 2010. By December 2011, only 42 cases had been reported to NFLIS for that year and in 2013, 173 cases were reported. In total, over 745 cases were reported to NFLIS by June 2018 (118-120). In a recent report from Anwar *et al.* the number of poison control center calls related to kratom use in the US increased ten-fold from 2010 to 2015 (121). A more recent report by the Centers for Disease Control and Prevention (CDC), identified 152 kratom positive deaths in 27 states from July 2016-December 2017 (115). Of these fatalities, 91 of these deaths involved kratom, but it was the only drug present in just 7 cases. The majority of decedents (80%) had a history of substance abuse and the most frequently co-administered drugs were fentanyl (or its analogs), heroin or benzodiazepines. Overall, the majority of literature concedes that kratom use is growing in the United States.

As it relates to forensic analysis of kratom samples, there are many challenges. Most methods for kratom only focus on mitragynine, while literature suggests that other alkaloids might also be present (28, 111). The analysis itself is not trivial due to the need to separate structural isomers. Information on phase II metabolites and deconjugation is also limited. Lee *et al.* reported that sulfated conjugates of some of the metabolites were resistant to hydrolysis (122). The potential for metabolites and other *Mitragyn*a alkaloids

to be biomarkers of kratom use in order to improve analytical methods has not been fully investigated and with kratom use increasing, the ability to better detect the use of this drug is needed.

Statement of Problem

Kratom is a drug that is not readily detected using common toxicological screening, and as a consequence its use may be underreported in forensic laboratories (115). Previously published methods in biological fluids may not separate the structural isomers and other alkaloids present in the plant. In addition, some analytical methods lack the requisite sensitivity to identify the drug.

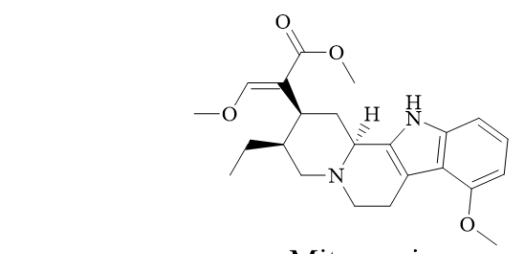
Another challenge involves knowledge gaps regarding metabolism and stability. As many kratom users are known to be poly drug users, this is a concern for both medical and forensic toxicology. When it comes to phase II metabolites, the efficiency of methods to deconjugate metabolites are relatively unknown and the benefits have not been explored. The limited information on stability is also an issue because this is critical in terms of sample preservation, storage, disposition and ultimately, interpretation.

To improve the detection of *Mitragyna* alkaloids in forensic toxicology, a comprehensive study of five prominent alkaloids (**Figure 1.4**) (speciociliatine, speciogynine, paynantheine, 7-hydroxymitragynine, and mitragynine) was undertaken. Analytical methods were developed, optimized, and validated in accordance with published guidelines (123). Simultaneous identification of the five alkaloids in urine and blood was achieved using LC-Q/TOF-MS. This is a highly sensitive technique that shows promise for these challenging analytes. Recombinant CYPs were also used to investigate the phase I metabolism of mitragynine and thoroughly investigate its biotransformation

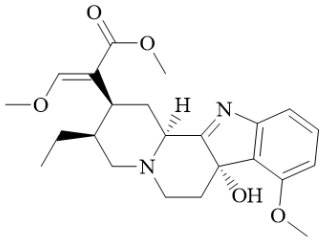
pathways. The stability of the target compounds under variable conditions of temperature and pH was also investigated in order improve sample preservation, sample collection, and toxicological interpretation. Finally, postmortem case samples were evaluated in order to identify potential biomarkers for kratom use, determine the efficiency and necessity for deconjugation, and provide insight regarding the distribution of the drug.

Figure 1.4: Targeted *Mitragyna* alkaloids and their deuterated analogs.

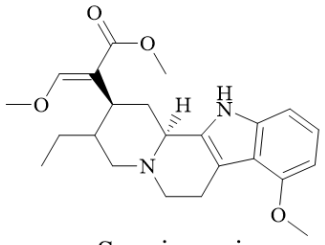
Targeted Analytes



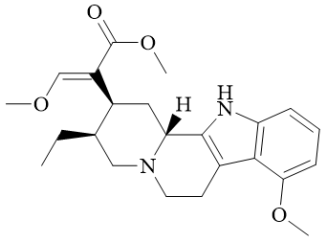
Mitragynine



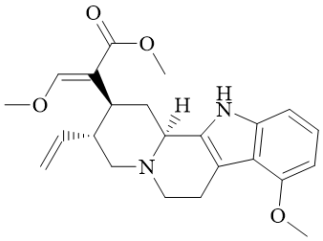
7-Hydroxymitragynine



Speciogynine

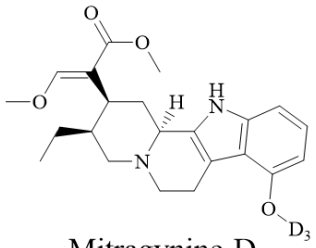


Speciociliatine

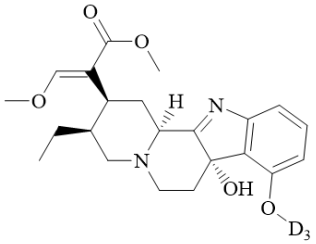


Paynanthenine

Deuterated Internal Standards



Mitragynine-D_3



7-Hydroxymitragynine-D_3

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CHAPTER II

Identification of Five *Mitragyna* Alkaloids in Urine using Liquid Chromatography-Quadrupole/Time of Flight Mass Spectrometry[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1]Basiliere, S., Bryand, K., Kerrigan S. (2018). Journal of Chromatography B, 1080, 11-19.

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Abstract

Mitragyna speciosa (Kratom) is a psychoactive plant that has recently emerged as a recreational drug. *Mitragyna* alkaloids are not within the scope of traditional forensic toxicology screening methods, which may contribute to under-reporting. Solid phase extraction (SPE) and liquid chromatography-quadrupole/time of flight mass spectrometry (LC-Q/TOF-MS) were used to identify five alkaloids in urine. Target analytes included the two known psychoactive compounds, mitragynine and 7-hydroxymitragynine, in addition to speciociliatine, speciogynine, and paynantheine. Two deuterated internal standards (mitragynine-D_3 and 7-hydroxymitragynine-D_3) were employed. Using traditional reversed phase chromatography all compounds and isomers were separated in 10 mins. The procedure was validated in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation. Extraction efficiencies were 63-96% and limits of quantitation were 0.5–1 ng/mL. Precision, bias and matrix effect were all within acceptable thresholds, with the exception of 7-hydroxymitragynine, which is notably unstable and unsuitable for quantitative analysis. In this paper we present a simultaneous quantitative analytical method for mitragynine, speciociliatine, speciogynine and paynantheine, and a qualitative assay for 7-hydroxymitragynine in urine using high resolution mass spectrometry (HRMS).

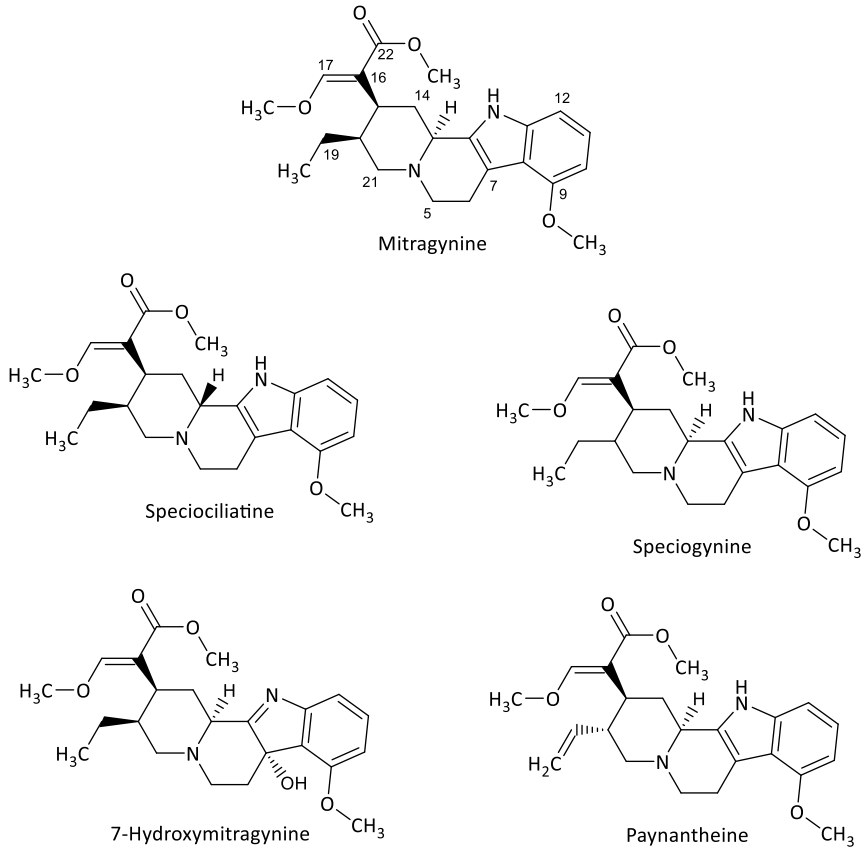
Keywords: Mitragynine, Kratom, Urine, LC-Q/TOF-MS, HRMS

Identification of Five *Mitragyna* Alkaloids in Urine using Liquid Chromatography-Quadrupole/Time of Flight Mass Spectrometry

Introduction

Kratom, also known as Ketum, Thang, Thom and Biak, comes from the leaves of *Mitragyna speciosa*, a tropical evergreen tree of the *Rubiaceae* family (1). The tree is indigenous to South East Asia, but is commonly seen in Thailand, Malaysia, and Myanmar (2). Forty-four compounds have been isolated from the leaves of *M. speciosa* (3), many of which include both indole and oxindole alkaloids. Mitragynine is the principal pharmacologically active compound in kratom, accounting for 66% of the total alkaloids present (4). Speciogynine, speciociliatine, and paynanthenine are estimated to account for 6.6%, 8.6%, and 0.8% of the total alkaloid content (1). Speciociliatine and speciogynine are both stereoisomers of mitragynine while paynantheine is a dehydro analog of mitragynine (**Figure 2.1**). Although these compounds are not believed to be pharmacologically active, they could serve as potential biomarkers of kratom use.

Figure 2.1: Target analytes included in this study.



Mitragynine is known to undergo hepatic metabolism (5). Hydrolysis of the methylester group (C16) and demethylation of the methoxy groups (C9 and 17) have been reported in humans (4). Hydroxylated and carboxylic acid metabolites are subsequently conjugated by both glucuronides and sulfates (4). Speciogynine, speciocilliatine, and paynantheine are reported to undergo similar biotransformations (6-8). The metabolic fate of 7-hydroxymitragynine has not been reported and may be complicated by the inherent instability of this compound. 7-Hydroxymitragynine is extremely labile, requiring storage at subzero temperatures under alkaline conditions (9). Metabolites of mitragynine are not yet commercially available, so the identification of kratom use in forensic toxicology specimens is currently limited to the parent drug and related alkaloids.

Kratom elicits both stimulating and sedative effects in a dose-dependent manner (10). Low doses of kratom are reported to produce a stimulant effect similar to cocaine, and high doses are reported to produce opiate-like effects (11). When used in a traditional setting as a mild stimulant, fresh or dried leaves of kratom are most commonly chewed or brewed into a tea. In the United States and Western Europe however, the drugs is used recreationally for its euphoric effects (3). However, a growing number of individuals also use kratom as an opioid alternative for the treatment of chronic pain, or to treat opioid addiction (12). Although mitragynine has an affinity for both mu and kappa opioid receptors, Boyer suggested that additional receptor affinities might augment its effectiveness, mitigating unpleasant effects associated with opioid withdrawal (13). More recent studies have indeed confirmed that *Mitragyna* alkaloids exhibit receptor binding activities distinct from classical opioids (14). Both mitragynine and 7-hydroxymitragynine act as partial agonists at mu opioid receptors and competitive antagonists at kappa and delta opioid receptors (15). Since both are G protein-based agonists at the mu receptor, their tendency to produce respiratory depression may be somewhat reduced. 7-Hydroxymitragynine is reported to be more potent than mitragynine, and has a high affinity for the mu opioid receptor, especially when kratom is consumed orally (3). Kratom is also reported to have adrenergic, serotonergic and dopaminergic effects, which may explain the relatively complex pharmacological profile of plant-based extracts (15). Given the current epidemic of opioid abuse in the United States, non-medical use of kratom is of growing concern (16). Although the Drug Enforcement Administration has identified kratom as a drug of concern, it is not currently scheduled at the Federal level (17).

The dose-dependent effects of kratom have been well documented throughout the literature (5, 11, 18-25). These range from increased energy and alertness, sociability and heightened libido at low dose, to drowsiness, confusion, seizures, loss of consciousness, coma and death at high doses. Regular kratom use is associated with drug dependency and withdrawal (26). In a series of 293 habitual kratom users estimated to use 276 mg/day, physical withdrawal effects included difficulty sleeping, muscle spasms, fever, watery eyes or nose and diarrhea (27). Psychological symptoms associated with withdrawal include restlessness, tension, anger, sadness and nervousness.

Since kratom is widely available and lacks strict control, usage has increased dramatically in Western Europe and the United States (11). The online marketplace has fueled the recreational use of novel or emerging psychoactive drugs. Using business-to-business and business-to-consumer marketing, the Internet and dark web make new psychoactive substances readily accessible (28). According to the National Forensic Laboratory Information System (NFLIS), mitragynine seizures in the U.S. have been on the rise since 2010 (29, 30). Kratom is illegal in Australia, Malaysia, Myanmar (Burma), and Thailand. It is also a regulated or a controlled substance in Finland, Denmark, Romania, Germany, and New Zealand, but is still unregulated in most countries (31). In August 2016, the DEA announced its intention to add kratom to Schedule I of the Federal Controlled Substances Act (32). However, the DEA promptly withdrew the request following significant public outcry by users, commercial vendors and researchers, citing the need for the therapeutic effects of the drug to be more thoroughly explored prior to any scheduling action.

Animal studies show that peak plasma concentrations following oral dosing (20 and 50 mg/kg) were reached at 1.3 (424 ng/mL) and 4.5 hours (700 ng/mL) (33, 34). In case studies following overdose or fatalities, concentrations of mitragynine in blood have been reported in the range 20-600 ng/mL (18, 19, 23). The distribution of mitragynine following a fatality involving kratom indicated concentrations of 0.23 mg/L in peripheral blood, 0.19 mg/L in central blood, <0.05 mg/L in vitreous fluid, and 0.37 mg/L in urine (35).

The vast majority of published reports describe the identification of mitragynine from plant material rather than biological fluids using either gas chromatographic (GC) or liquid chromatographic (LC) based techniques (36). Phillip was the first to describe the identification of kratom and its metabolites in urine using gas chromatography-mass spectrometry (GC-MS). However, due to the polar nature of the drug and its metabolites, derivatization was required and the limit of detection of the method was relatively high (50 ng/mL) (37). Lu was the first to describe the use of LC tandem mass spectrometry (LC-MS-MS) for the identification of mitragynine in urine, with enhanced sensitivity (38). Although LC-MS-MS procedures have been the most widely reported to date, none of the LC-based methods have utilized isotopically labelled internal standards or validated methods for forensic use (39).

In a study involving urine from fifty recreational kratom users, mitragynine concentrations ranged from 1 to 50,000 ng/mL (40). A subsequent controlled dosing study in humans indicated that elimination of unchanged drug in urine was relatively limited, accounting for only 0.14% of the total dose (5). For this reason, analytical methods capable of detecting low ng/mL concentrations are desirable in forensic investigations, because

specimen collection is sometimes delayed. Additionally, the identification of other *Mitragyna* alkaloids in a biological sample could potentially identify kratom usage.

In this report, we describe the simultaneous identification of mitragynine (MG), 7-hydroxymitraginine (7-MG-OH), speciogynine (SG), speciocilliatine (SC), and paynantheine (PY) in urine using liquid chromatography-quadrupole/time of flight mass spectrometry (LC-Q/TOF MS). Following careful optimization to eliminate problematic adduct ions and separate regioisomers, the method was validated in accordance with generally accepted guidelines in forensic toxicology (39).

Experimental

Chemicals and reagents

Reference standards for mitragynine, 7-hydroxymitragynine, mitragynine-D_3, and 7-hydroxymitragynine-D_3 were purchased from Cerilliant (Round Rock, TX USA). Reference standards for paynantheine and speciociliatine were purchased from Chromadex (Irvine, CA, USA) and speciogynine was provided by the National Center for Natural Products Research (NCNPR) at the University of Mississippi, (University, MS, USA). Reference standards were purchased as methanolic standards with the exception of paynantheine, speciociliatine and speciogynine (solids). 7-Hydroxymitragynine, which is known to be unstable, was purchased in ammoniated methanol (1% concentrated ammonium hydroxide in methanol, v/v) and stored at -80^{o}C. All working standards or mixtures containing 7-MG-OH (or its deuterated analog) were also prepared and stored accordingly, prior to use. Pooled drug-free urine was preserved with 1% preserved with sodium fluoride (w/v). Unless otherwise stated, solvents and inorganic reagents were LC or ACS grade, respectively. Concentrated ammonium hydroxide, ethyl acetate, hexane,

concentrated hydrochloric acid, and methanol (LCMS grade) were obtained from J.T. Baker (Center Valley, MA, USA). Acetonitrile (LCMS grade) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Glacial acetic acid was obtained from Mallinckrodt Chemicals (St. Louis, MO, USA) and ammonium acetate (LCMS grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA Deionized water was purified using a Millipore Direct-Q®UV Water Purification system (Billerica, MA, USA). PolyChrom ClinII 3 cc (35 mg) solid phase extraction (SPE) columns were obtained from SPEware (Baldwin Park, CA, USA).

Instrumentation

Nitrogen was generated using a Genius 3040 nitrogen generator (Peak Scientific, Billerica, MA, USA). SPE was performed using a J. T. Baker vacuum manifold and extracts were evaporated to dryness under nitrogen using a TurboVap LV®concentration workstation (Caliper Life Sciences, Hopkinton, MA, USA). After the samples were reconstituted, they were centrifuged using an Allegra™ X-22 Centrifuge (Beckman Coulter, Indianapolis, IN, USA). An Agilent Technologies 6530 LC-Q/TOF-MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 1290 Infinity autosampler was used to analyze samples. Separation was achieved using an Agilent Technologies Series 1200 LC system equipped with an Agilent Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 µm) and an Agilent Poroshell 120 EC-C18 guard column (2.1 × 5 mm, 2.7 µm) in a thermostatically controlled column compartment (35^{o}C).

The optimum mobile phase consisted of 5 mM ammonium acetate solution (A) and acetonitrile (B). A flow rate of 0.4 mL/min was maintained using the gradient elution profile as follows: 47% B (0–0.5 min), to 90% B (0.5–10 min), followed by re-

equilibration. The total acquisition time was 12 min and the target compounds eluted between 1.9 mins and 6 mins. The LC-Q/TOF-MS was equipped with an ESI source (positive mode) with Jet Stream technology under the following conditions: drying gas (N_2), 13 L/min; drying gas temperature, 350^{o}C; nebulizer, 45 psi; sheath gas temperature, 400^{o}C; nitrogen sheath gas flow, 12 L/min; capillary voltage, 4000 V; nozzle voltage, 0 V; fragmentor, 150 V; skimmer, 65 V. Agilent MassHunter software was used for acquisition, qualitative and quantitative analysis. Following optimization of collision induced dissociation (CID) voltages, a minimum of two transition ions were selected using targeted MS/MS acquisition. Precursor and product ions, collision energies, retention times and the internal standard for each drug are summarized in **Table 2.1**. Precursor ions were selected in the quadrupole using a 1 Da window. Data was acquired using a mass range of 100–1000 Da, with a MS scan rate of 8 spectra/s and a MS/MS Scan rate of 3 spectra/s. During the selection of product ions, fragments were structurally identified and significant emphasis was placed on specificity, rather than sensitivity (abundance).

Table 2.1: Transition ions, collision energies (CE), retention time (RT), and internal standard (IS) selection. Quantitation ions are underlined and ion ratios (relative to the quantitation ion) are shown in parentheses.

Alkaloid	Precursor Ion (*m/z*)	Product Ions (*m/z*)	CE (V)	RT (min)	IS
MG	399.2278	<u>174.0911</u> 226.1433 (36%) 238.1431 (33%)	30	4.61	MG-D_3
7-MG-OH	415.2227	<u>190.0862</u> 238.1434 (21%) 226.1434 (24%)	27	2.54	7-MG-OH-D_3
SC	399.2278	<u>174.0912</u> 226.1434 (28%) 238.1432 (28%)	30	2.00	MG-D_3
SG	399.2278	<u>174.0909</u> 226.1431 (23%) 238.1430 (30%)	30	3.32	MG-D_3
PY	397.2122	<u>174.0912</u> 236.1275 (25%) 224.1277 (33%)	26	3.79	MG-D_3
MG-D_3	402.2467	<u>177.1102</u> 226.1436 (39%) 238.1438 (36%)	30	4.54	-
7-MG-OH-D_3	418.2416	<u>193.1051</u> 226.1436 (24%) 238.1438 (22%)	27	2.49	-

Preparation of standards and reagents

Working standards containing all five target compounds were prepared in ammoniated methanol at 5, 0.5, and 0.05 µg/mL for the fortification of urine. The combined internal standard solution consisted of MG-D_3 and 7-MG-OH-D_3 in ammoniated methanol at 2 µg/mL. Ammoniated methanol was routinely prepared from concentrated ammonium hydroxide in methanol (1%, v/v). The elution solvent which was prepared daily, consisted of concentrated ammonium hydroxide in ethyl acetate (2%, v/v).

Urine extraction

Internal standard solution (50 µL) was added to 1.0 mL urine to achieve a final concentration of 100 ng/mL. Urine was acidified with 2 mL of 1 M hydrochloric acid and

briefly vortexed. Samples were transferred to SPE columns and allowed to flow through under gravity or sufficient vacuum to maintain constant flow (approximately 1 mL/min). Columns were rinsed with 1 mL deionized water followed by 1 mL of 1 M acetic acid. After drying columns for five minutes at full vacuum, samples were washed using 1 mL aliquots of hexane, ethyl acetate and methanol. MG, 7-MG-OH, PY, SC, and SG were eluted using 1 mL of elution solvent. Samples were then evaporated to dryness under nitrogen at 50^{o}C. Extracts were reconstituted in 25 µL of a 50:50 mixture of Mobile Phase A/B, briefly vortexed, and then centrifuged at 2500 rpm for 20 mins. After centrifugation, 1 µL was injected onto the LC-Q/TOF-MS for analysis.

Assay validation

Assay performance was evaluated in terms of extraction efficiency, calibration model, precision, bias, limit of detection (LOD), limit of quantification (LOQ), matrix effects, interference, ion suppression, and carryover in accordance with published recommendations (39). The extraction efficiency in urine was determined at 250 ng/mL by direct comparison of extracted and non-extracted samples. Samples containing internal standard were extracted in the presence and absence of the target compounds. Samples extracted without target compounds were fortified with equivalent drug post-extraction (prior to evaporation and reconstitution). Analytical recovery was calculated by comparing the relative peak area (drug/IS) for extracted samples (n = 6) with the mean relative peak area for the non-extracted samples (n = 6).

Limits of detection and quantitation were established using drug-free urine fortified with target analytes. Three sources of drug-free matrix were analyzed in duplicate over three independent runs. The LOD was the lowest concentration of drug that produced a

reportable result (signal to noise (S/N) ratio of 3:1 or more; retention time ± 2% of the standard; ion ratios ± 20%). The LOQ was determined contemporaneously and was defined as the lowest concentration of drug to produce a quantitative value within 20% of the expected value, a S/N ratio of 10:1 or more, retention time ± 2 % of the standard, ion ratios within 20%, and acceptable precision and bias. Precision and bias was evaluated at 5, 200 and 400 ng/mL using pooled fortified matrix (in triplicate), at three concentrations (low, medium,high), over five runs. Within-run precision was calculated for each concentration (n = 3) over each of the five assays. Between-run precision was calculated for each concentration over all five days (n = 15). Bias was evaluated contemporaneously with precision using the same concentrations over five days. Tolerance for bias and precision was ± 20%.

The calibration model was established using nine non-zero calibrators (2, 5, 10, 25, 100, 200, 300, 400, and 500 ng/mL) over five independent runs. Calibration models were evaluated using the coefficient of determination (R^2), standardized residual plots and the F-test to determine the significance of the quadratic term ($\alpha = 0.05$). Interferences associated with the biological matrix, isotopically labeled internal standards, common drugs and structurally related compounds were systematically evaluated. Matrix interferences were evaluated using ten drug-free urine samples from independent sources in the absence of internal standard. Ion contributions arising from the use of stable isotope internal standards were evaluated by fortifying drug-free urine with internal standard (100 ng/mL) and monitoring the signal of the target analytes. In a similar fashion, ion contributions associated with highest calibrator (500 ng/mL) were evaluated in the absence of internal standard.

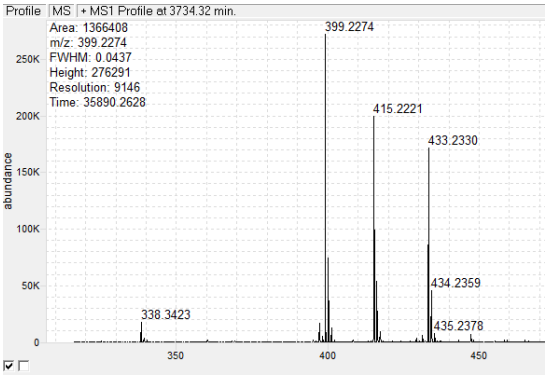
Drug interferences were evaluated using thirty common drugs and other therapeutic drugs of significance. A 10 to 100-fold excess of interferent (relative to the target drug) was routinely employed for interference testing. The negative control consisted of drug-free urine fortified with 1,000 ng/mL of interferent. Positive controls contained target analytes and interferents at 10 ng/mL and 1,000 ng/mL respectively (100X), and 50 ng/mL and 500 ng/mL (10X). Matrix effects were quantitatively assessed using post-extraction addition at two concentrations (5 ng/mL and 400 ng/mL). Ten drug-free matrices from independent sources (n = 2) were extracted in the absence of drug and fortified with drug post extraction. Ion suppression or enhancement was calculated by comparing the mean peak areas of drug in matrix with the drug in mobile phase (no matrix). Carryover was assessed by analyzing a negative control immediately following the injection of a high calibrator (500 ng/mL). Carryover was present if the analyte met the detectable reporting criteria (signal to noise ratio of 3:1 or more, retention time ± 2% and ion ratios within 20% of expected).

Results and discussion

During method development, significant emphasis was placed on the chromatographic separation of structural isomers (MG, SC and SG) and the elimination of adducts. To this end, several mobile phase systems were evaluated. 7-Hydroxymitragynine (m/z 415) has a tendency to form a very prominent water adduct (m/z 433), particularly when formic acid (0.1%) is used as a mobile phase additive (**Figure 2.2**). Adducts are relatively common in LC-MS analysis, but are known to reduce both sensitivity and reproducibility. Adduct formation was previously reported for *Mitragyna* alkaloids by Kikura-Hanajiri, who integrated the adduct into their method, rather than removing it (41).

In our study, traditional means to eliminate the adduct by modifying source conditions were unsuccessful. Mobile phase additives (formic acid and ammonium formate) at different concentrations and in combination were also evaluated without success. In the fully optimized method described here, it was possible to completely eliminate the water adduct described in other methods using 5mM ammonium acetate.

Figure 2.2: Prominence of water adduct (m/z 433) for 7-hydroxymitragynine (m/z 415) in the presence of mobile phase containing formic acid (0.1%). Mitragynine (m/z 399) is also shown.



The chromatographic separation of structural isomers (mitragynine, speciociliatine and speciogynine) also presents a challenge. These compounds fragment in an identical fashion, producing identical MS-MS spectra (**Figure 2.3**). The corynantheidine-type compounds undergo characteristic cleavage of the quinolizine ring to yield stable indole (m/z 174 and 190) and piperidine derivative fragments (m/z 238, 236, 226, 224). The 238 and 236 ions result from cleavage at C5, while 226 and 224 are formed via neutral loss of the methoxy indole group between C5 and nitrogen. HRMS allowed product ions to be structurally identified with mass accuracies within 0.5 ppm (**Table 2.2**). Using the optimized procedure, complete chromatographic resolution of the five *Mitragyna* alkaloids was achieved (**Figure 2.4**).

Figure 2.3: MS-MS spectra of *Mitragyna* alkaloids.

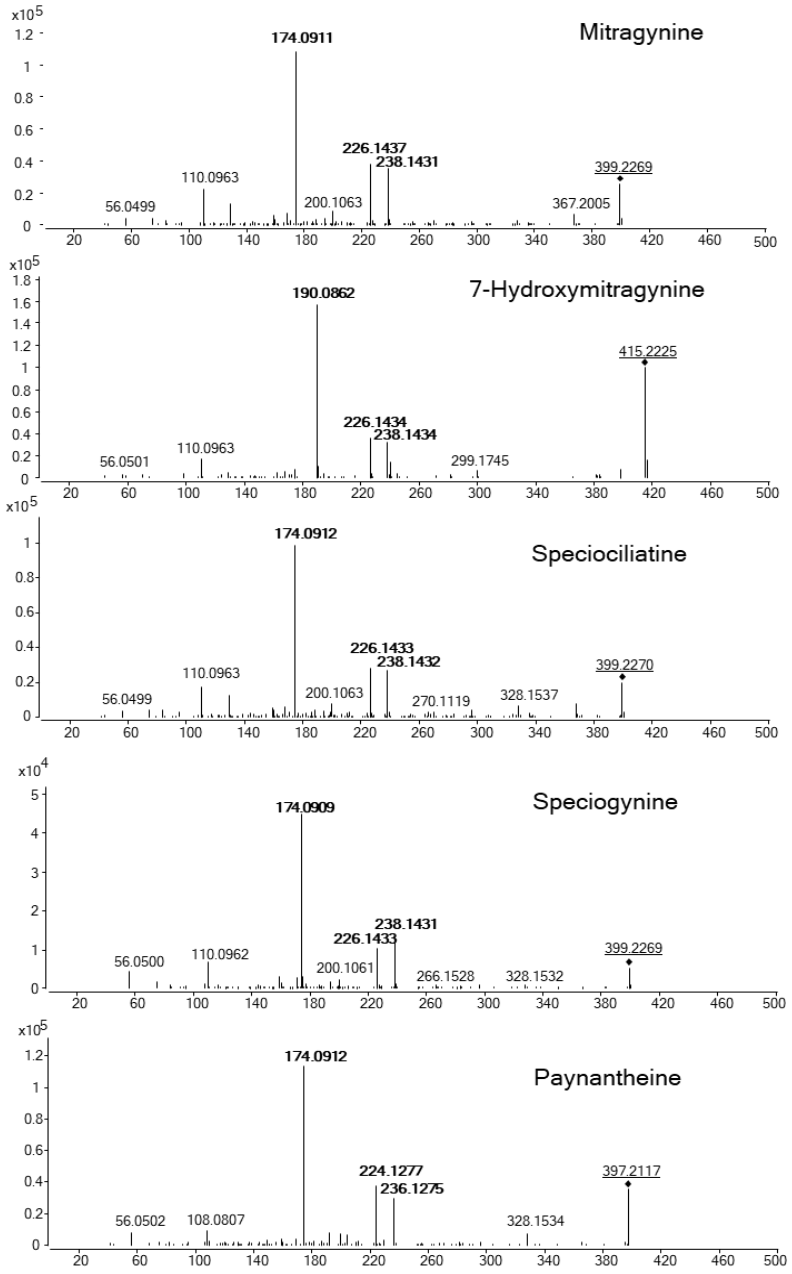
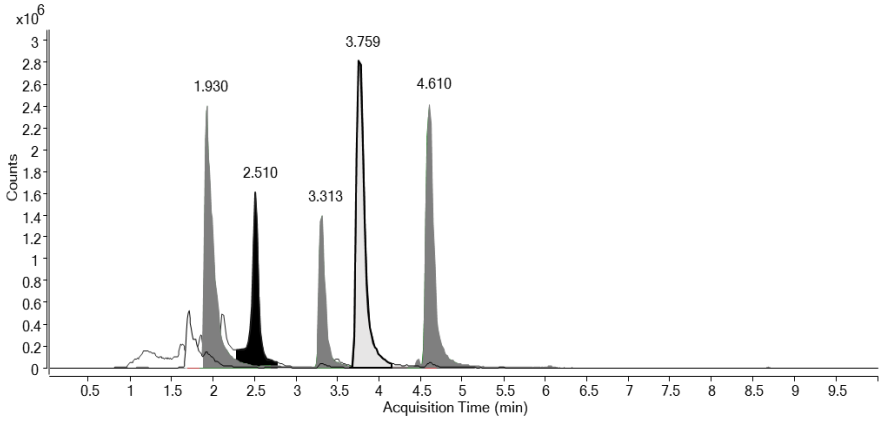


Table 2.2: Proposed fragmentation and mass accuracy of Mitragyna alkaloids using positive electrospray ionization.

Compound	Molecular Formula	Accurate Mass	Exact Mass	PPM Shift
Mitragynine	$C_{23}H_{30}N_2O_4$	399.2269	399.2278	-0.2
	$C_{11}H_{12}NO$	174.0911	174.0913	-0.1
	$C_{12}H_{20}NO_3$	226.1433	226.1436	-0.1
	$C_{13}H_{20}NO_3$	238.1431	238.1438	-0.3
7-Hydroxymitragynine	$C_{23}H_{31}N_2O_5$	415.2225	415.2227	0.0
	$C_{11}H_{12}NO_2$	190.0862	190.0863	-0.1
	$C_{12}H_{20}NO_3$	238.1434	238.1438	-0.2
	$C_{13}H_{20}NO_3$	226.1434	226.1436	-0.1
Speciociliatine	$C_{23}H_{30}N_2O_4$	399.2270	399.2278	-0.2
	$C_{11}H_{12}NO$	174.0912	174.0913	-0.1
	$C_{12}H_{20}NO_3$	226.1434	226.1436	-0.1
	$C_{13}H_{20}NO_3$	238.1432	238.1438	-0.3
Speciogynine	$C_{23}H_{30}N_2O_4$	399.2269	399.2278	-0.2
	$C_{11}H_{12}NO$	174.0909	174.0913	-0.2
	$C_{12}H_{20}NO_3$	226.1431	226.1436	-0.2
	$C_{13}H_{20}NO_3$	238.1430	238.1438	-0.3
Paynantheine	$C_{23}H_{28}N_2O_4$	397.2117	397.2122	-0.1
	$C_{11}H_{12}NO$	174.0912	174.0913	-0.1
	$C_{12}H_{18}NO_3$	236.1275	236.1281	-0.3
	$C_{13}H_{18}NO_3$	224.1277	224.1281	-0.2

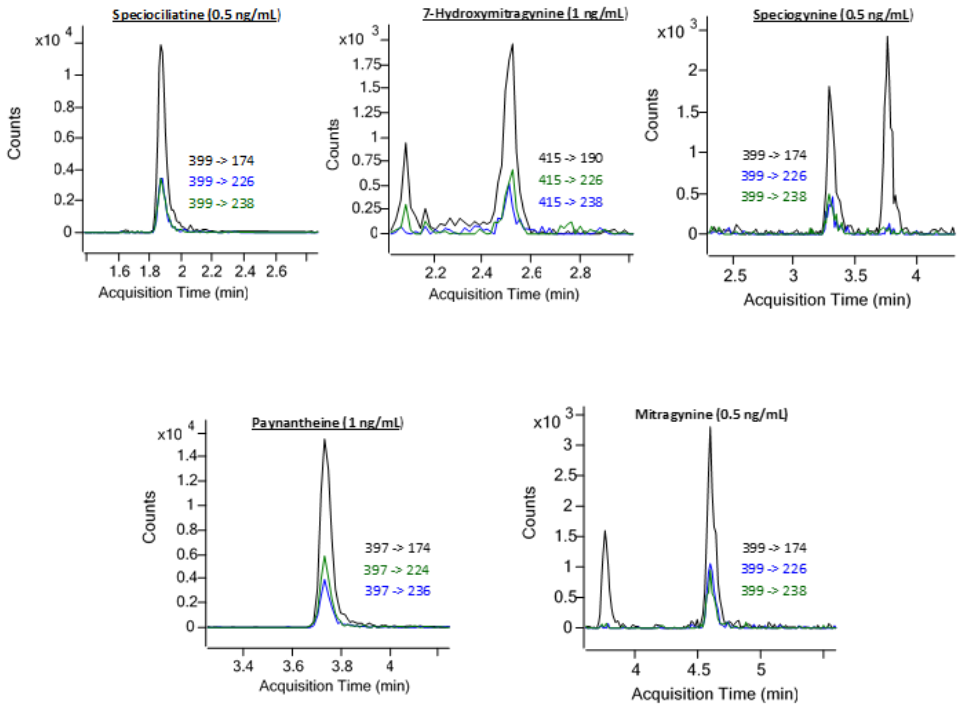
The extraction efficiency of mitragynine from urine was 96%, and 63–96% for remaining alkaloids (**Table 2.3**). It is perhaps not surprising that the compound with the lowest extraction efficiency was 7-hydroxymitragynine, which is known to be highly unstable. Following visual, analytical, and statistical evaluation of calibration models, a weighted (1/x) quadratic model was selected for all analytes. The coefficients of determination were above 0.99 or for all models. Upon visual assessment using residual plots, the data appeared to be randomly dispersed when a non-linear (quadratic) model was used. Statistical evaluation further indicated a weighted (1/x) curve was optimal.

Figure 2.4: Chromatographic separation of kratom alkaloids in a representative extract (200 ng/mL). Speciociliatine, 1.93 min; 7-hydroxymitragynine, 2.51 min; speciogynine, 3.31; paynantheine, 3.76 min; mitragynine, 4.61 min.



Limits of detection and quantitation were all within the range of forensic interest. The LOD and LOQ for mitragynine was 0.25 and 0.5 ng/mL, respectively. Limits of detection and quantitation for remaining alkaloids were 0.5–1 ng/mL (n = 18). Bias, precision and signal to noise ratios at the limits of detection and quantitation are summarized in **Table 2.4** and extracted ion chromatograms (EICs) for all compounds at the limit of quantitation in urine are shown in **Figure 2.5**. At the limit of quantitation, bias ranged from -1.0 to 5.0 and precision ranged from 10-16%.

Figure 2.5: Extracted ion chromatograms (EICs) in urine at the limit of quantitation.



Precision and bias were also evaluated at low, medium, and high concentrations in triplicate over five days. Intra-assay CVs were 0.5-15.1% (5 ng/mL); 0.3-7.5% (200 ng/mL); 0.2–6.0% (400 ng/mL) (n = 3) and inter-assay CVs over the same concentration ranges were 5.5-8.4%, 2.4-5.3% and 1.0-4.5% (n = 15). Bias and precision at all concentrations tested were within acceptable ranges (±20%) and are summarized in **Table 2.5**. Interferences from matrix, isotopically labeled internal standards, and other drugs were systematically evaluated. No interferences were present form either matrix or isotopically labelled internal standards. Although current validation standards in forensic toxicology only require potential interferences to be evaluated qualitatively (39), the potential for quantitative interference was also assessed.

Table 2.3: Extraction efficiencies from urine (250 ng/mL).

Analyte	Mean Extraction Efficiency (%) (n=6)
MG	96 ± 1
7-MG-OH	63 ± 13
SG	79 ± 16
SC	96 ± 9
PY	87 ± 12

No qualitative interferences were present for any of the target compounds. Even in the presence of 100-fold higher concentrations of interferents, all compounds met reporting criteria. However, a quantitative interference was observed for speciociliatine, whereby the concentration was not within 20% of the expected value. This interference was attributed to dextromethorphan, which coeluted with SC. Although the two drugs do not share identical transitions, the excess of dextromethorphan (relative to SC), decreases the ionization efficiency of the compound. Although this could be mitigated by the use of an isotopically labelled internal standard, none are commercially available for speciociliatine at this time.

Table 2.4: Limits of detection and assay performance at the limit of quantitation in urine. Signal to noise ratio is presented as the mean value for the quantitation ion.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Mean ± SD (ng/mL) (n=18)	Mean S/N Ratio	Bias (%) (n=18)	CV (%) (n=18)
MG	0.25	0.5	0.52 ± 0.09	276:1	4.0%	16
7-MG-OH	1	1	0.99 ± 0.11	108:1	-1.0%	11
SG	0.5	0.5	0.50 ± 0.05	128:1	0.0%	10
SC	0.5	0.5	0.53 ± 0.07	1750:1	6.0%	14
PY	0.5	1	1.05 ± 0.13	5735:1	5.0%	12

Table 2.5: Precision and bias (n=15) in urine at low (5 ng/mL), medium (200 ng/mL), and high (400 ng/mL) concentrations.

Alkaloid	Intra-Assay Precision (n=3, %CV)			Inter-Assay Precision (n=15, %CV)			Bias (n=15, %)		
	5	200	400	5	200	400	5	200	400
MG	0.5-10.9	0.3-2.8	0.2-1.1	5.5%	2.5%	1.0%	0.9	1.4	1.0
7-MG-OH	0.6-7.0	0.5-3.4	0.5-1.6	4.4%	2.4%	1.4%	2.2	1.5	0.1
SG	1.8-6.0	2.6-5.3	0.5-6.0	7.6%	5.2%	4.5%	-3.2	6.4	-1.7
SC	1.4-12.8	1.1-7.5	0.4-5.3	8.4%	5.3%	3.2%	-1	1.9	-0.4
PY	1.6-15.1	0.8-3.6	0.7-1.6	7.3%	2.8%	1.8%	1.2	-0.7	-0.2

The potential for ion suppression or enhancement was evaluated using ten independently sourced urine samples. Matrix effects were evaluated quantitatively using the post-extraction addition technique for all five analytes and two internal standards. Matrix effects for MG, MG-D_3, SC, SG, and PY were -4-31% at 10 ng/mL, and -8-30% at 400 ng/mL. Ion suppression was present for both 7-MG-OH and MH-OH-D_3 (-60 to -15% at 10 ng/mL and -53 to -25% in 400 ng/mL). However, given the instability of 7-hydroxymitragynine, quantitative reporting is not recommended. Corresponding CVs for SC, SG, PY, MG, and MG-D_3 were 5-7% and 5-7%, respectively. However, the corresponding CVs for 7-MG-OH and 7-MG-OH-D_3 were 30-27% and 23-25% (**Table 2.6**). Matrix effects and associated CVs for mitragynine, speciociliatine, paynantheine and speciogynine were well-within tolerable limits (39) . No carryover was present at 500 ng/mL for MG, 7-MG-OH, SC, and SG. Carryover of PY was present at 500 ng/mL, although given the relative abundance of this alkaloid in kratom, concentrations in this range may be unlikely.

Table 2.6: Matrix effect (%) and associated CVs in urine (10 and 400 ng/mL).

Alkaloid	CV (%) n=10		Matrix Effect (%)	
	10 ng/mL	400 ng/mL	10 ng/mL	400 ng/mL
MG	6%	8%	16%	15%
7-MG-OH	27%	23%	-45%	-39%
SG	7%	8%	19%	11%
SC	7%	5%	8%	1%
PY	6%	8%	7%	15%
MG-D_3	5%	7%	18%	19%
MG-D_3	20%	25%	-41%	-38%

Conclusion

Kratom is a novel psychoactive substance of natural origin that is becoming increasingly popular as a drug of abuse. In light of the current epidemic of opioid use in the United States and the tendency of users to substitute kratom for other mu receptor agonists, kratom is of growing concern. Increased vigilance among the clinical and forensic community in needed. The availability of analytical methods capable of detecting analytes at sufficiently low concentration will advance our understanding of both prevalence and trends associated with its use. The methods presented here offer distinct advantages over previously published work. Simultaneous identification of all five alkaloids, including isomers of mitragynine was achieved using solid phase extraction and LC-Q/TOF-MS

analysis at low to sub-ng/mL concentrations. Using deuterated internal standards, the method was fully validated for forensic use (39).

Acknowledgements

This project was supported by Award No. 2016-DN-BX-0006, awarded by the National Institute of Justice, Office of Justice Programs, and U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect those of the Department of Justice. We gratefully acknowledge Dr. Ikhlas Khan and the National Center for Natural Products Research School of Pharmacy at The University of Mississippi for providing speciogynine.

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CHAPTER III

CYP450-Mediated Metabolism of Mitragynine and Investigation of Unhydrolyzed Phase I Metabolites in Human Urine[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1]Basiliere, S., Kerrigan S. (2019). Journal of Analytical Toxicology, [In Press].

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Abstract

Mitragyna speciosa (Kratom) has emerged as a recreational drug and a substance of medicinal intrigue. Although the drug was initially used recreationally for its sedating and euphoric effects, more recently its use has been associated with the non-medically supervised treatment of opioid abstinence syndrome. Mitragynine is the principal pharmacologically active alkaloid in kratom. Although metabolites of mitragynine have been identified, the cytochrome P450 (CYP450) enzymes responsible for its biotransformation are still under investigation. The goal of this study was to contribute further knowledge regarding CYP450 activity as it relates to mitragynine. Recombinant cytochrome P450 enzymes (rCYPs) were used to investigate the isoforms involved in its metabolism. Biotransformational products were identified using liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS). Four rCYP enzymes (2C18, 2C19, 2D6 and 3A4) were found to contribute to the metabolism of mitragynine. 7-Hydroxymitragynine (which has an affinity for the mu opioid receptor >10-fold that of morphine) was produced exclusively by 3A4. 9-O-Demethylmitragynine, the most abundant metabolite *in vitro* (and the most prevalent metabolite in urine among kratom users) was produced by 2C19, 3A4 and 2D6. 16-Carboxymitragynine was produced by rCYPs 2D6, 2C19 and 2C18. 2C19 was solely responsible for the formation of 9-O-demethyl-16-carboxymitragynine. *In vitro* rCYP studies were compared with phase I metabolites in urine from cases involving mitragynine.

Keywords: Mitragynine, Metabolism, CYP450, Kratom, Urine

CYP450-Mediated Metabolism of Mitragynine and Investigation of Unhydrolyzed Phase I Metabolites in Human Urine

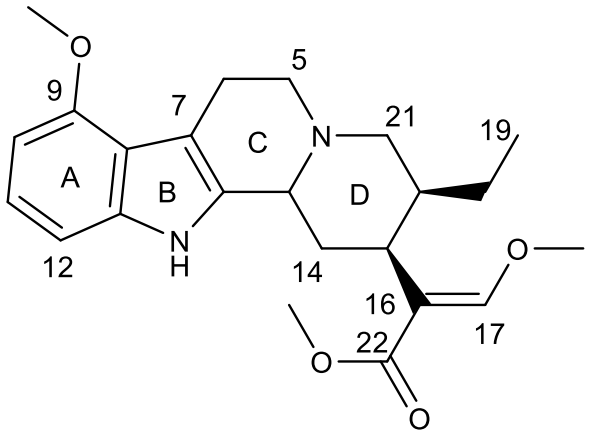
Introduction

Kratom is a psychoactive drug present in the leaves of the Korth tree (*Mitragyna speciosa*) (1). Traditionally, kratom leaves have been chewed by the natives of Thailand to relieve pain and as an energy stimulant (2). It has also been reported to have been used as an opium substitute (3). In the Western world kratom is predominately used as a recreational drug and more recently, for the non-medically supervised treatment of opioid abstinence syndrome (1, 3). In addition, since kratom is widely available for sale on the Internet and lacks regulatory control, usage has increased dramatically in Western Europe and the United States (4). According to the National Forensic Laboratory Information System (NFLIS), mitragynine seizures in the U.S. have been on the rise since 2010 (5-7). Kratom is a controlled substance in Australia, Malaysia, Myanmar (Burma), Thailand, Denmark, Romania, Sweden, Poland, Latvia, Lithuania, and New Zealand, but is still unregulated in most countries (8).

In August 2016, the Drug Enforcement Administration (DEA) announced its intention to add kratom to Schedule I of the Federal Controlled Substances Act (9). However, the DEA promptly withdrew the request following significant public outcry by users, commercial vendors and researchers, citing the need for the therapeutic effects of the drug to be more thoroughly explored prior to any scheduling action. In November of 2017, the Federal Drug Administration (FDA) issued a public health advisory related to mounting concerns regarding the risks associated with the use of kratom (10).

Forty-four compounds have been isolated from the leaves of *M. speciosa* (1). These include both indole and oxindole alkaloids. Mitragynine is the predominant pharmacologically active compound in kratom, and accounts for 66% of the total alkaloids present (11). Mitragynine's structure was defined in 1963 and was later confirmed through crystallographic studies in 1965 (12, 13). It is a corynanthe-like alkaloid, structurally similar to yohimbine. It is comprised of an indole aromatic ring (A and B) attached to two piperidine rings (C and D) (**Figure 3.1**). The piperidine moiety is believed to be central to its opioid activity (1) and the methoxy-moiety at the positions C16 and the C9 have an open ring with substitution (14).

Figure 3.1: Chemical structure of mitragynine.



Kratom elicits both stimulant and sedative effects in a dose-dependent manner. Low doses of kratom are reported to produce central nervous system stimulant effects similar to cocaine, and high doses are reported to produce opiate-like effects (4). As such, it is considered an "atypical opioid" due to its activity at both opioid and non-opioid (monoamine transporter) receptors. Mitragynine acts as partial agonist at mu opioid receptors and a competitive antagonist at both kappa and delta receptors (15). The dose-dependent effects of kratom have been well documented. Reported effects range from

increased energy and alertness, sociability and heightened libido at low doses, to drowsiness, confusion, seizures, loss of consciousness, coma and death at high doses (4, 16-24). Regular kratom use is associated with drug dependency and withdrawal (25, 26). A study by Elliot *et al*. reported that in a series of 293 habitual kratom users, physical withdrawal effects included difficulty sleeping, muscle spasms, fever, watery eyes or nose and diarrhea (27). Psychological symptoms associated with withdrawal include restlessness, tension, anger, sadness and nervousness.

There are relatively few studies detailing the metabolism of mitragynine, although recently there has been renewed interest in this area. An early study by Zarembo in 1974 used *Helmin-thosporum sp.* to determine that oxidation and hydroxylation were the primary metabolic routes (28). Mitragynine is known to undergo hepatic metabolism (16). Philipp *et al.* conducted the first comprehensive *in-vivo* study to date (11). Rats were administered a single 40 mg/kg dose of mitragynine by gastric intubation. Results were compared with human urine from known kratom users. They reported that phase I metabolism involved the hydrolysis of the methylester group at C16, demethylation of the methoxy groups at C9 and C17 positions, followed by oxidative and reductive transformations to produce carboxylic acid and alcohol derivatives. Given these transformative pathways, it is not surprising that mitragynine is reported to undergo extensive phase II metabolism to produce both glucuronide and sulfate conjugates. As many as seven phase I metabolites were identified in rat urine, and six in human urine following enzymatic hydrolysis (11). More recently a study by Lee *et al.* reported a quantitative liquid chromatography tandem mass spectrometry (LC-MS/MS) procedure for the identification of mitragynine, 16-carboxymitragynine, 9-O-demethylmitragynine and

glucuronides in human urine. They also reported that sulfate-conjugated metabolites were resistant towards enzyme hydrolysis (29).

Although some progress has been made identifying the metabolites of mitragynine in animal studies and in human specimens, insight regarding the specific enzymes responsible for its metabolism is just beginning to emerge. Most recently Kamble *et al.* used human liver microsomes (HLMs) and S9 fractions to identify metabolic pathways of mitragynine (30). They concluded that the major metabolites of mitragynine included four oxidative species (including 7-hydroxymitragynine) and one demethylated metabolite (9-O-demethylmitragynine). It was hypothesized that mono-oxygenation of the three remaining species occurred on the indolequinolizine moiety, although structural elucidation was not possible. Using rCYPs they concluded that CYP3A4 was the predominant cytochrome P450 isoform responsible for metabolism, with minor or negligible contributions from CYP 2D6, 2C9 and 2C19. Focusing exclusively on 7-hydroxymitragynine, Kruegel *et al.* showed that this metabolite was produced using both human and mouse liver microsomes (31). This is important given the increased pharmacological activity of 7-hydroxymitragnine, reported to have a ten-fold greater affinity for the mu opioid receptor compared to morphine (32). It has been suggested that first pass metabolism of mitragynine could explain why the drug has been shown to be paradoxically *more* potent as an analgesic when administered via oral and intraperitoneal routes, compared with subcutaneous injection (31). The heightened pharmacodynamic response of 7-hydroxmitragynine might explain why it was reported to have a high potential for abuse (33). Although 7-hydroxymitragynine is a minor alkaloid in *Mitragyna speciosa*, these findings suggest that routes of administration, or CYP isoform activities

that favor its formation, could have increased potential for addiction. Pharmacological activity has not been reported for other mitragynine metabolites has not been reported.

Understanding the specific isoforms involved can provide valuable information regarding the potential for adverse consequences due to genetic polymorphisms or drug-drug interactions. Published case reports suggest that multiple drug use is common among kratom users (**Table 3.1**). In addition, Anwar *et al*. reported that 35% of reports to poison centers involving kratom admitted to poly-drug use. Ethanol, botanical drugs, benzodiazepines, narcotics, and acetaminophen were the most commonly reported (46). In a recent report involving kratom from the Centers for Disease control (CDC), fentanyl and its analogs were the most commonly detected substances (65%) among a series of fatalities in twenty-seven states (47). Heroin was the second most frequently detected substance (33%), followed by benzodiazepines (22%), prescription opioids (20%) and cocaine (18%). Concomitant use of kratom with other opioids has been reported previously (1, 3). Given the extensive poly-drug use associated with kratom, additional studies regarding the CYP450-mediated metabolism of mitragynine are warranted.

Table 3.1: Poly-drug use among published kratom case reports.

Investigation	Matrix	Other Compounds Detected	Reference
Hospitalization (n=1)	Urine	Cannabinoids (not specified); Oxycodone; Tricyclic antidepressants (not specified)	(18)
Fatality (n=1)	Blood	7-Aminoclonazepam; Dextromethorphan; Diphenhydramine; Temazepam	(22)
Fatality (n=9)	Blood	Alimemazine; Alprazolam; Amphetamine; Buprenorphine; Citalopram; O-Demethyltramadol; Desmethylalimemazine; O-Desmethylvenlafaxine; Diazepam; Ethanol; Fluoxetine; Mirtazapine; Nordiazepam; Norfluoxetine; Olanzapine; Phenazon; Pregabalin; Tetrahydrocannabinol; Venlafaxine; Zopiclone	(34)
Fatality (n=1)	Blood; Tissues	Acetaminophen; Morphine; Promethazine; Propylhexedrine	(17)
Fatality (n=1)	Blood; Urine	O-Demethylvenlafaxine; Diphenhydramine; Ethanol; Mirtazapine; Venlafaxine	(35)
Antemortem Drug Screen (n=1)	Urine	O-Desmethyltramadol	(36)
Fatality (n=1)	Blood; Urine	Bupropion; Delorazepam; 3-Methoxyphencyclidine; Paroxetine	(37)
Fatality (n=1)	Blood; Urine	Citalopram; Lamotrigine; Zopiclone	(38)
Fatality (n=1)	Urine	Codeine	(39)
Hospitalization (n=1)	Urine	O-Demethyltramadol	(40)
Impaired Driver (n=1)	Blood	Amphetamine; Citalopram	(41)
Hospitalization (n=1)	Plant material	Modafinil	(42)

Investigation	Matrix	Other Compounds Detected	Reference
Fatality (n=1)	Blood; Urine	Acetaminophen; Amphetamine; Codeine; Etizolam; Fluoxetine; Gamma-Hydroxybutyric acid; Lorazepam; Methamphetamine; 3-Methylenedioxyamphetamine; 3,4-Methlenedioxymethylamphetamine; 2-Methylmethcathinone; 6-Monoacetylmorphine; Morphine; Olanzapine; Pipamperone; Pregabalin; Pseudoephedrine; Quetiapine; Triazolam	(43)
Fatality (n=1)	Blood; Tissues	Alprazolam, Etizolam; Fentanyl; Labetalol; Laudanosine; Lorazepam; Metoprolol; Mirtazapine; Morphine; Nicardipine; Norfentanyl; Normirtazapine; Norsertraline; Ofloxacine; Sertraline; U-47700	(44)
Fatality (n=1)	Blood	Quetiapine; Valproic Acid	(45)

A recent study by Kong *et al*. reported that methanolic extracts of kratom were potent inhibitors of 3A4 and 2D6 (48). Another study by Hanapi *et al*. reported that mitragynine inhibits 2C9, 2D6 and 3A4 (49). Additionally, a study by Lim *et al*. reported that mitragynine was a significant in-vitro 1A2 inducer, weak 3A4 inducer, and a weak 3A4 enzyme inhibitor (50). Studies have also shown that mitragynine and 7-hydroxymitragynine could have potential drug-drug interactions with drugs that are P-glycoprotein substrates and can activate the pregnane X receptor which could then lead to an increase in the activity of 3A4, 1A2, and P-glycoprotein (51, 52).

In this study, recombinant CYP450 isoenzymes were used to investigate the *in vitro* metabolic pathways of mitragynine. While this approach is useful, *in vitro* techniques do not fully emulate the complexity of metabolism in a living system. Nevertheless, given the increase in kratom use in the United States, enhanced knowledge regarding its biotransformation and the disposition of active metabolites can provide insight regarding the pharmacological and toxicological effects of this drug.

Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q/TOF-MS) is particularly useful for metabolite identification due to its mass accuracy and sensitivity (53). We previously reported a quantitative assay for mitragynine and related alkaloids using high resolution mass spectrometry (54). In this report, a modified assay was used to identify specific isoforms responsible for the metabolism of mitragynine. Several cytochrome P450 isoenzymes (2C8, 2C9, 2C19, 2D6, and 3A4) are known to be active in phase I metabolism for opioids (55-58) and other drugs. These and other recombinant CYPs (2C19, 3A4, 1A2, 2D6, and 2C9) were investigated as part of this study. Inhibition

studies were used to confirm CYP activity, and urine samples from sixteen fatalities were evaluated to identify metabolites of interest.

Methods

Chemicals and reagents

Reference standards for mitragynine, 7-hydroxymitragynine, mitragynine-D_3, ketoconazole, and fluvoxamine were purchased from Cerilliant in methanolic solutions (Round Rock, TX, USA). 7-Hydroxymitragynine, which is unstable, was purchased in ammoniated methanol (1% concentrated ammonium hydroxide in methanol, v/v) and stored at -80^{o}C. Unless otherwise stated, solvents and inorganic reagents were LC or ACS grade, respectively. Acetonitrile (LC-MS grade) was obtained from Fisher Scientific (Fair Lawn, NJ, USA) and ammonium acetate (LC-MS grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human cytochrome P450 (rCYP) isoenzymes expressed in *E. coli* (bactosomes) were obtained from Xenotech, LLC (Kansas City, KS, USA). Reduced nicotinamide adenosine di-phosphate (NADPH) regenerating system solution A (40 Ug/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate), solution B (26 mM NADP+, 66 mM glucose-6-phosphate and 66 mM magnesium chloride in aqueous solution) were obtained from Corning (Glendale, AZ, USA). All other chemicals and reagents (analytical grade) were obtained from VWR (Radnor, PA, USA). Deionized water was purified using a Millipore Direct-Q®UV Water Purification system (Billerica, MA, USA). Pooled drug-free urine used for the preparation of calibrators and controls, was preserved with 1% sodium fluoride (w/v) and PolyChrom ClinII 3 cc (35 mg) solid phase extraction columns were obtained from SPEware (Baldwin Park, CA, USA).

LC-Q/TOF-MS analysis

Nitrogen was generated using a Genius 3040 nitrogen generator (Peak Scientific, Billerica, MA, USA). An Agilent Technologies 6530 LC-Q/TOF-MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 1290 Infinity autosampler was used to analyze samples. Separation was achieved using an Agilent Technologies Series 1200 LC system equipped with an Agilent Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 µm) and an Agilent Poroshell 120 EC-C18 guard column (2.1 × 5 mm, 2.7 µm) in a thermostatically controlled column compartment (35^{o}C). The mobile phase consisted of 5 mM ammonium acetate solution in deionized water (A) and acetonitrile (B).

For the identification of metabolites, a flow rate of 0.4 mL/min was maintained using the gradient elution profile as follows: 10% B (0–0.5 min), 10-90% B (0.5–10 min), followed by re-equilibration. The total acquisition time was 12 min and the metabolites eluted between 1.3 min and 7.3 min. The LC-Q/TOF-MS was equipped with an ESI source (positive mode) with Jet Stream technology under the following conditions: drying gas (N_2), 13 L/min; drying gas temperature, 350^{o}C; nebulizer, 45 psi; sheath gas temperature, 400^{o}C; nitrogen sheath gas flow, 12 L/min; capillary voltage, 4000 V; nozzle voltage, 0 V; fragmentor, 150 V; skimmer, 65 V. Data was acquired in auto MS/MS (full scan) mode using a preferred list of suspected metabolites based upon previously published reports (11). Agilent MassHunter software was used for acquisition and qualitative analysis. Mass spectra were generated using collision induced dissociation (CID) energies of 25 and 28 eV. Data was acquired using a mass range of 100–1000 Da, with a MS scan rate of 8 spectra/s and a MS/MS Scan rate of 3 spectra/s. During the selection of potential metabolites, fragments were structurally identified, and mass accuracies were evaluated.

Mitragynine was quantitatively determined using a previously validated method (54). Briefly, the LC-Q/TOF-MS configuration described above was used with the following gradient elution profile: 47% B (0–0.5 min), 47-90% B (0.5–10 min), followed by re-equilibration. Source conditions, flow rate and scan rates were as described above. Targeted MS/MS acquisition was performed for mitragynine (*m/z* 399.2278 > **174.0913**, 226.1438, 238.1438) using mitragynine-D_3 as the internal standard. Quantitation ions are shown in bold and the collision energy was 30 eV.

Identification of CYP450 isoenzyme activity

Each rCYP isoenzyme (1A2, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6 and 3A4) was incubated separately to evaluate individual metabolic activity towards mitragynine. Fully optimized incubations were performed at 37°C in the presence of 50 µM mitragynine and 25 pmol/mL rCYP isoenzyme in accordance with manufacturer recommendations and previously published protocols (59). The incubation mixture also contained 100 mM potassium phosphate buffer (pH 7.4), 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM magnesium citrate and 0.4 U/mL glucose-6-phosphate dehydrogenase. Incubation mixtures were initially assayed at t = 0, 60, 120, 180 and 240 minutes. Each 25 µL aliquot was quenched using an equal volume of ice cold acetonitrile containing 5 µM internal standard (mitragynine-D_3). The solution was centrifuged at 10,000 g at 4°C for 3 minutes. The supernatant was mixed with an equal volume of a 50/50 mixture of mobile phase A/B and 1 µL was injected onto the LC-Q/TOF-MS. Control and blank samples were included in each assay. For the controls, rCYP isoenzymes were replaced with Xenotech control bactosomes, and for the blank (no drug) samples, mitragynine was replaced with phosphate buffer. Since multiple time stops were performed, a single aliquot of microsomal

incubation mixture was removed at 60, 120, 180 and 240 minutes. However, since 7-hydroxymitragynine was a novel metabolite at the time of this study, additional experiments to confirm the activity of CYP 3A4 were performed using six replicates.

Inhibition studies

For the inhibition study, active rCYPs (2C18, 2C19, 2D6, and 3A4) were incubated side by side, in the presence and absence of inhibitor (n=4). Conditions were identical to those described earlier, with the exception of the addition of the inhibitor. Ketoconazole (20 µM) was used for rCYPs 2C9, 2C18, 2C19 and 3A4 and fluvoxamine (20 µM) was used for rCYP 2D6. Inhibitor concentrations were selected based on Ki values and previously published work (59). For the inhibition studies, aliquots were removed at t = 0 and 120 minutes and samples were analyzed using LC-Q/TOF-MS. For 7-hydroxymitragynine, 6 replicates were utilized to remain consistent with the previous incubation study.

Metabolite identification

Potential metabolites were first identified by observing changes in each rCYP incubation mixtures over time. Using the optimized LC separation, the abundance of each potential metabolite was normalized to the internal standard (mitragynine-D_3). Measurement of the relative peak area (RPA) minimized random errors associated with volumetric steps, injection volume and ionization efficiency. The exact masses of predicted metabolites were monitored using a preferred list, and mass spectra of compounds that appeared to increase over time were further investigated over a range of CID voltages. Since many of these metabolites lack a certified reference standard, identification relied upon MS/MS spectra and mass accuracy.

Postmortem specimens

Mitragynine and potential metabolites were identified in 16 postmortem urine samples in accordance with an IRB-approved study (Sam Houston State University, IRB#: 2016-05-29903). Mitragynine was quantitatively determined using a previously validated method (54) and metabolites, including those identified in the *in vitro* study, were investigated qualitatively.

Mitragyna alkaloids were isolated from urine using solid phase extraction (SPE) as previously described (54). Briefly, internal standard solution was added to 1 mL urine to achieve a final concentration of 100 ng/mL. Following acidification with 2 mL of 0.1 M hydrochloric acid, samples were transferred to PolyChrom Clin II SPE cartridges. Following the addition of 1 mL deionized water and 1 mL of 1 M acetic acid, columns were dried for five minutes at full vacuum. Additional washes using 1 mL aliquots of hexane, ethyl acetate and methanol were performed prior to elution with 1 mL ethyl acetate/concentrated ammonium hydroxide (98:2). Extracts were evaporated to dryness under nitrogen at 50ºC and reconstituted in 25 µL of 1:1 Mobile Phase A/B prior to injection (1 µL) on the LC-Q/TOF-MS. The limits of detection and quantitation for mitragynine were 0.25 ng/mL and 0.5 ng/mL and the calibration range of the assay was 0.5 -500 ng/mL.

Due to the high concentration *Mitragyna* alkaloids in actual case samples, specimen dilution was necessary. Using the previously published procedure, dilution integrity for urine was evaluated using 2, 10 and 100-fold dilutions of matrix in 0.1 M HCl. The total volume of diluted urine was 1 mL and the SPE was performed as described earlier. The initial concentration of mitragynine prior to dilution was 400 ng/mL and quantitative

results within ±20% of the expected concentration were deemed acceptable for extracts analyzed in triplicate.

Mitragynine metabolites were also investigated in postmortem urine samples using the extraction protocol described above. However, in order to improve the detectability of the metabolites identified in the *in vitro* study, urine was not diluted prior to extraction. Metabolites were acquired using the auto MS/MS LC-Q/TOF-MS assay described for metabolite identification above. In the absence of commercially available metabolite reference standards, rCYPs (3A4, 2D6, 2C19, and 2C18) were used to prepare 9-O-demethylmitragynine, 16-carboxymitragynine, and 9-O-demethyl-16-carboxymitragynine *in-situ* for qualitative identification purposes. Retention times, MS/MS spectra and mass accuracy were used for identification purposes.

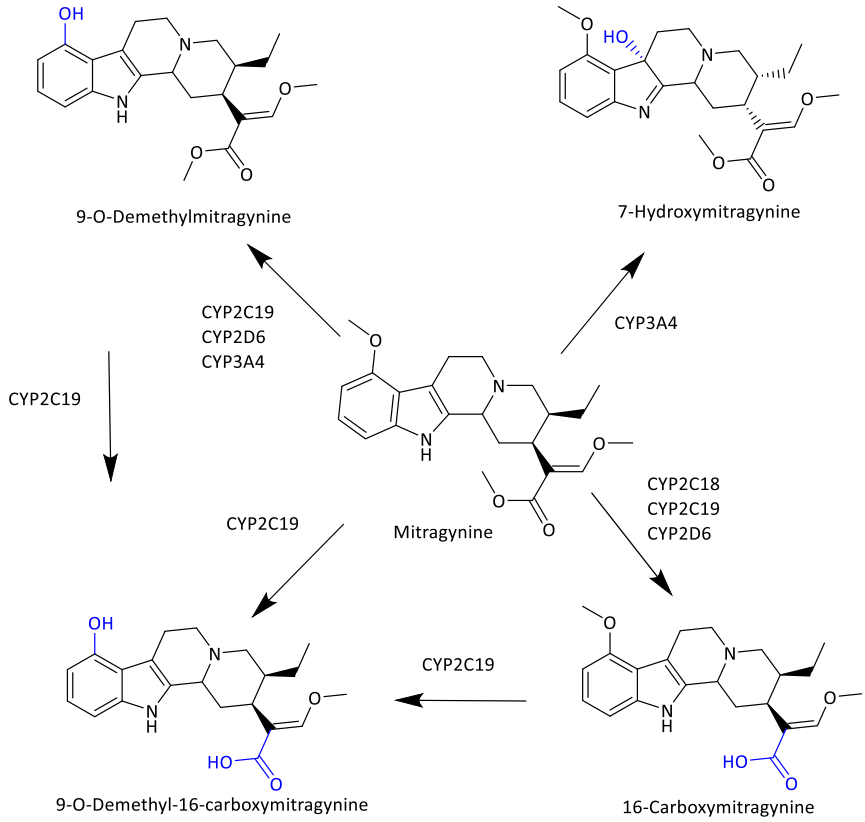
Results and discussion

In vitro metabolites

Of the eight rCYPs investigated, metabolic activity was observed for 2C18, 2C19, 2D6 and 3A4A. 9-O-Demethylmitragynine was produced by CYPs 2C19, 3A4 and 2D6 and 16-carboxymitragynine was produced by 2D6 and 2C19. 9-O-Demethyl-16-carboxymitragynine, which was identified in lower abundance, was attributed to 2C19 alone, and 7-hydroxymitragynine was only produced by rCYP 3A4. **Figure 3.2** summarizes the CYP450 activity observed for mitragynine. The extracted ion chromatograms for representative incubation mixes at 120 min are shown in **Figure 3.3** and the associated MS/MS spectra are depicted in **Figure 3.4**. Molecular formula, retention times, exact and accurate masses are summarized in **Table 3.2**. Fragmentation of the

metabolites and mass accuracies of diagnostic ions shown in bold are also summarized in **Table 3.2**.

Figure 3.2: Proposed biotransformational pathways of mitragynine.



The mass fragmentation of mitragynine has been described in the literature (54, 60). Loss of the piperidine moiety is responsible for the formation of *m/z* 174, which contains the relatively stable indole fragment, while the *m/z* 238 and 226 ions contain the nonaromatic portions of the molecule (**Figure 3.5**). The *m/z* 238 ion is formed from the cleavage at the C5 to form the dihydropyridine derivative with the loss of the methoxy indole moiety. The *m/z* 226 ion is formed by the neutral loss of the methoxy indole group between C5 and nitrogen in piperidine. Similar fragmentation patterns occur for the remaining metabolites to produce ions with *m/z* 174 and 160 corresponding to the loss of

the piperidine, and 238, 224, 226, and 212 resulting from the C-ring cleavage. Mass accuracies of the proposed metabolite fragments were within 5.4 ppm (**Table 3.2**). Although microsomal incubations were assayed at 60, 120, 180 and 240 minutes, no significant increases in metabolite were observed beyond 180 minutes and 7-hydroxymitragynine plateaued by 60 minutes (**Figure 3.6**). The abundance of each metabolite was measured relative to the internal standard in the incubation mixture. The rate of metabolite formation was not estimated because *in vitro* rates of reaction using rCYPs do not necessarily reflect *in vivo* kinetics due to biological and environmental factors (61).

Figure 3.3: Extracted ion chromatograms (120 min) of 9-O-demethyl-16-carboxymitragynine (*m/z* 371.1965; 1.3 min, rCYP 2C19), 16-carboxymitragynine (*m/z* 385.2122, 3.05 min, rCYP 2C19), 9-O-demethylmitragynine (*m/z* 385.2122, 4.6 min, rCYP 2C19), 7-hydroxymitragynine (*m/z* 415.2227, 5.3 min, rCYP 3A4) and mitragynine (*m/z* 399.2278, 7.25 min, rCYP 2C19).

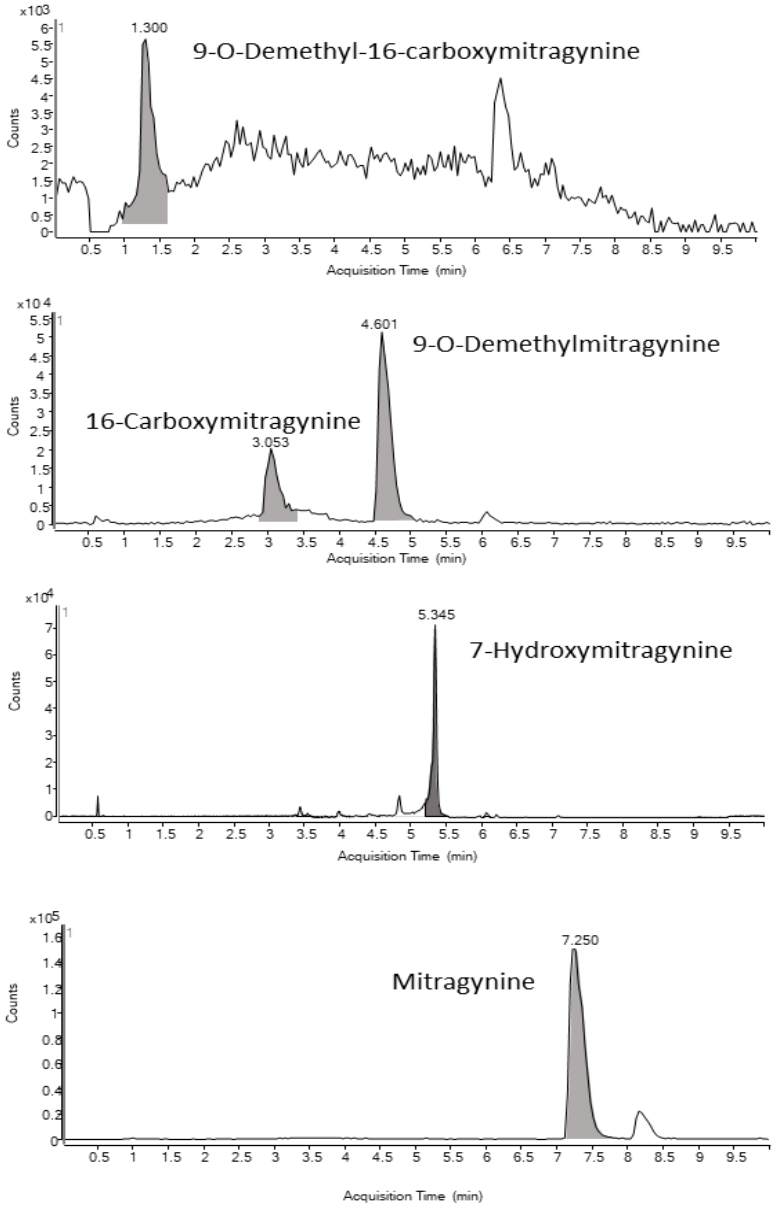
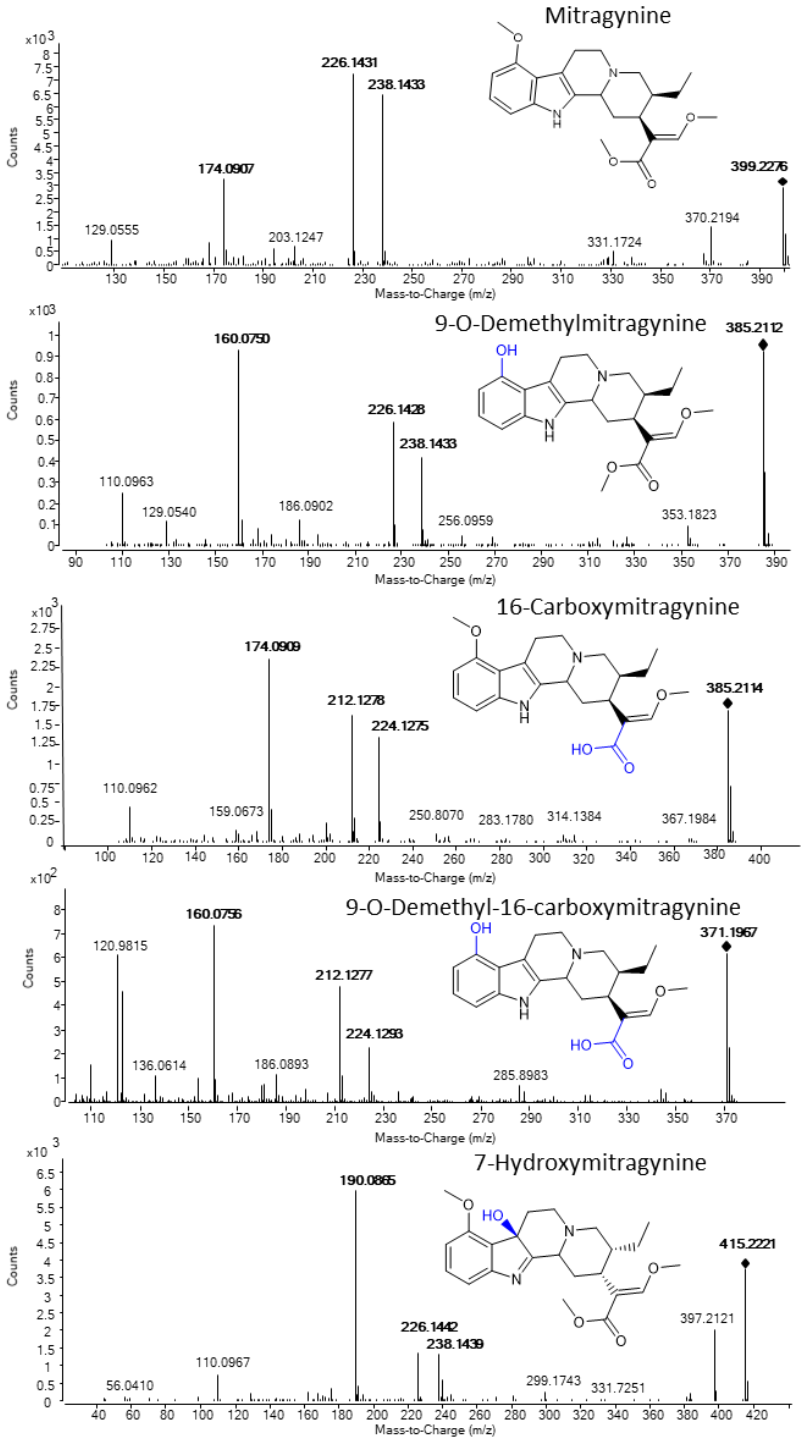


Figure 3.4: MS/MS spectra of mitragynine, 9-O-demethylmitragynine, 16-carboxymitragynine, 9-O-demethyl-16-carboxymitragynine and 7-hydroxymitragynine from rCYP incubation mixes.



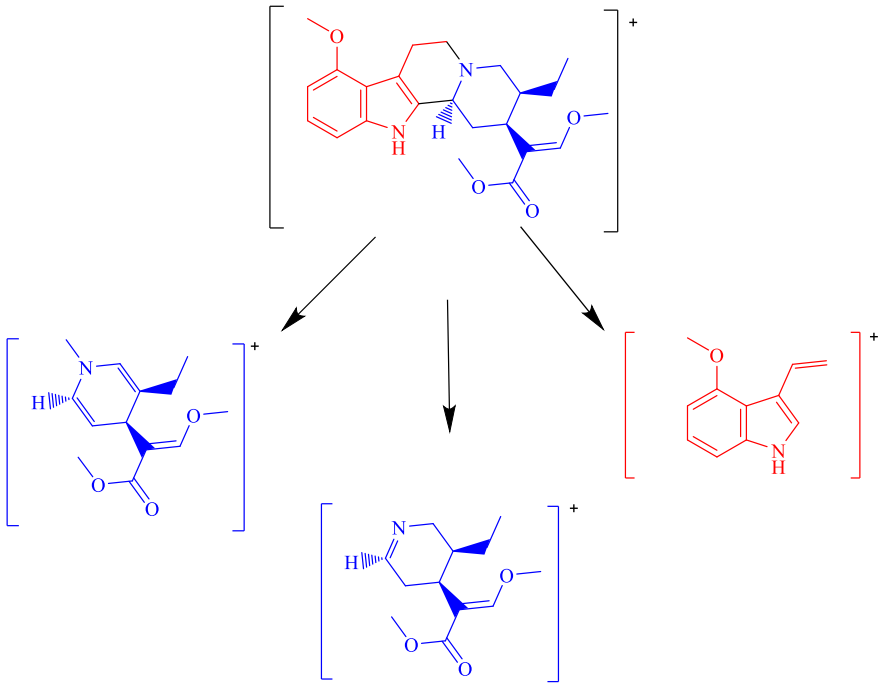
The relative contribution of each CYP towards the formation of each metabolite (determined by abundance, *i.e.* RPA) is shown in **Figure 3.7**. 9-O-Demethylmitragynine was the most abundant metabolite in the *in vitro* study and was produced by three isoenzymes (2C19, 3A4 and 2D6). This is in contrast to Kamble *et al.* who reported negligible activity for 2C19 and no activity for 2D6. This might be attributed to differences in reactivity between rCYP isoforms or experimental approaches (*i.e.* reduced incubation times). Nevertheless, it highlights the need for comparative studies and additional research. Not surprisingly, 9-O-demethyl-16-carboxymitragynine, which is formed in a step-wise fashion, was the least abundant and was produced by 2C19 alone. This metabolite was not reported by Kamble *et al.* Although the relative abundance observed using recombinant isoforms may not reflect those observed in humans, it does provide important insight regarding the overall role of the CYP450 enzymes. No metabolites were observed in any of the controls or blanks.

Table 3.2: Molecular formula, retention time, and mass accuracies for mitragynine (MG), 7-hydroxymitragynine (7-OH-MG), 9-O-demethylmitragynine (9-O-DM-MG), 16-carboxy mitragynine (16-COOH-MG) and 9-O-demethyl-16-carboxymitragynine (9-O-DM-16-COOH-MG). Precursor ions are shown in bold.

Compound	Retention Time (mins)	Molecular Formula [M+H$^+$]	Accurate Mass [M+H$^+$]	Exact Mass [M+H$^+$]	Mass Error (PPM)
MG	7.2	**$C_{23}H_{31}N_2O_4$**	**399.2276**	**399.2278**	**-0.5**
		$C_{11}H_{12}NO$	174.0907	174.0913	-3.4
		$C_{12}H_{20}NO_3$	226.1431	226.1438	-3.1
		$C_{13}H_{20}NO_3$	238.1433	238.1438	-2.1
9-O-DM-MG	4.6	**$C_{22}H_{29}N_2O_4$**	**385.2112**	**385.2122**	**-2.6**
		$C_{10}H_{10}NO$	160.0750	160.0757	-4.4
		$C_{12}H_{20}NO_3$	226.1428	226.1438	-4.4
		$C_{13}H_{20}NO_3$	238.1433	238.1438	-2.1
16-COOH-MG	3.0	**$C_{22}H_{29}N_2O_4$**	**385.2114**	**385.2122**	**-2.1**
		$C_{11}H_{12}NO$	174.0909	174.0913	-2.3
		$C_{11}H_{18}NO_3$	212.1278	212.1281	-1.4
		$C_{12}H_{18}NO_3$	224.1275	224.1281	-2.7
9-O-DM-16-COOH-MG	1.3	**$C_{21}H_{27}N_2O_4$**	**371.1967**	**371.1965**	**0.5**
		$C_{10}H_{10}NO$	160.0756	160.0757	-0.6
		$C_{11}H_{18}NO_3$	212.1277	212.1281	-1.9
		$C_{12}H_{18}NO_3$	224.1293	224.1281	5.4
7-OH-MG	5.3	**$C_{23}H_{31}N_2O_5$**	**415.2221**	**415.2227**	**-1.4**
		$C_{11}H_{12}NO_2$	190.0865	190.0863	1.1
		$C_{12}H_{20}NO_3$	226.1442	226.1438	1.8
		$C_{13}H_{20}NO_3$	238.1439	238.1438	0.4

Inhibition studies were performed to verify observed isoenzyme activity. The results of the inhibition study are shown in **Table 3.3**. Significant inhibition (>20%) was observed for 3A4, 2C19, 2C18, and 2D6 for all identified phase I metabolites. Inhibition rates of 63-100% were observed, confirming all metabolic pathways proposed in **Figure 3.2**. Although our results confirm the importance of CYP 3A4-mediated metabolism of mitragynine (30-31), they also identify the importance of other isoforms.

Figure 3.5: Fragmentation of mitragynine.



Although *in vitro* assays using recombinant enzymes can identify possible pathways, they do not always mimic *in vivo* biotransformations in humans. Despite the convenience of *in vitro* studies, further *in vivo* studies are needed. A total of 16 urine samples from confirmed kratom users were evaluated. No hydrolysis step was performed prior to analysis. Therefore, the species identified represents free (unconjugated) phase I metabolites, or unstable phase II metabolites that might be hydrolyzed during exposure to acidic or basic conditions during the extraction. The investigation of phase II metabolites and the efficiency of various enzymatic and chemical deconjugation was investigated, but is reported separately.

Figure 3.6: 7-Hydroxymitragynine production by rCYP 3A4 (n=6). Data represents the mean relative peak area (RPA) ± 1SD.

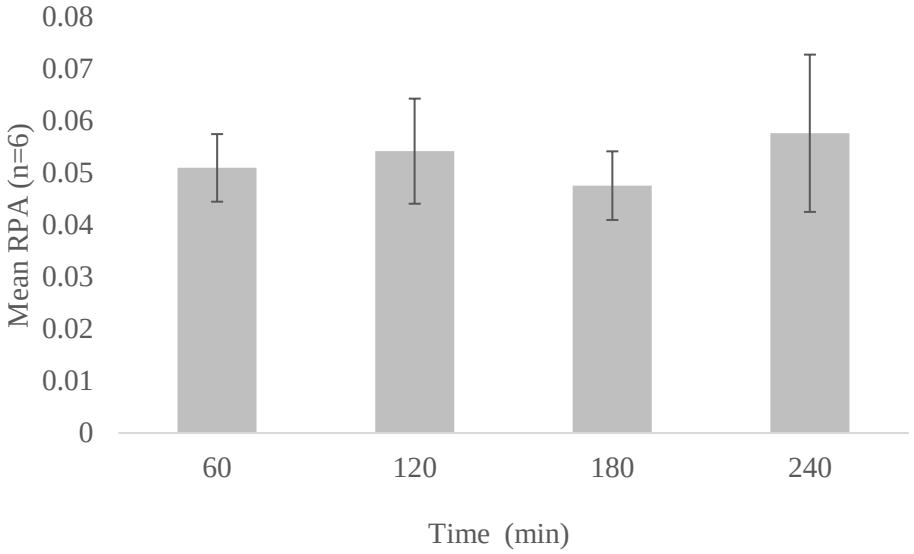
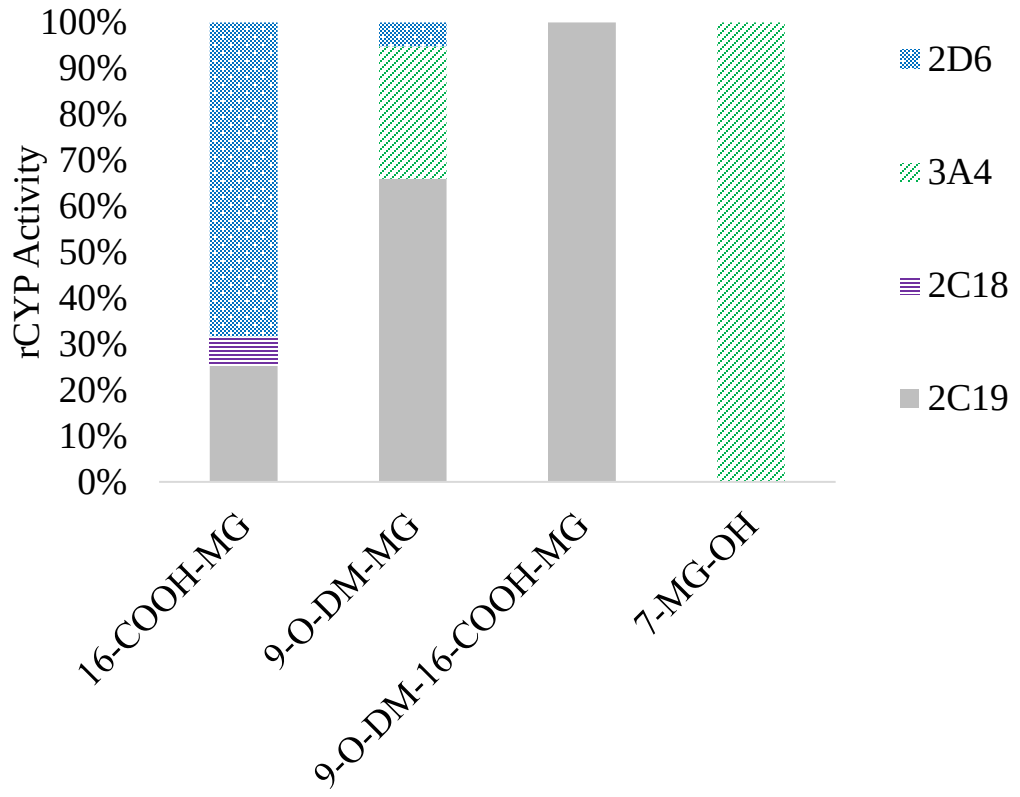


Figure 3.7: rCYP activity for 16-carboxymitragynine (16-COOH-MG), 9-O-demethylmitragynine (9-DM-MG), 9-O-demethyl-16-carboxymitragynine (9-O-DM-16-COOH-MG) and 7-hydroxymitragynine (7-OH-MG).



Human urine samples

Postmortem urine samples (n=16) were analyzed to determine the presence of mitragynine and metabolites. The concentration of mitragynine in each of the urine samples is shown in **Table 3.4**. Appropriate dilutions were performed where necessary prior to extraction. Evaluation of dilution integrity at 1:1, 1:10 and 1:100 produced results within 1-16% of the expected concentration and CVs of 1.5 – 2.9% (n=3). Drug concentrations in human urine ranged from 26 to 1,987 ng/mL. Interestingly, some specimens with elevated concentrations of parent drug had no detectable metabolite (e.g., case #1).

Although mitragynine was readily detected in all specimens, deconjugation of phase II metabolites might be necessary if metabolites are of interest.

Table 3.3: Inhibition of mitragynine metabolism using rCYPs at t=120 mins. Results reflect replicate measurements (n=4) for all metabolites, with the exception of 7-hydroxymitragynine (n=6).

rCYP	Metabolite	% Inhibition (Mean ± SD)
2C18	16-Carboxymitragynine	100 ± 0
2C19	16-Carboxymitragynine	77 ± 14
	9-O-Demethylmitragynine	78 ± 10
	9-O-Demethyl-16-carboxymitragynine	100 ± 0
2D6	16-Carboxymitragynine	63 ± 3
	9-O-Demethylmitragynine	100 ± 0
3A4	9-O-Demethylmitragynine	100 ± 0
	7-Hydroxymitragynine	93 ± 1

Metabolites were identified in 14 (88%) of the 16 postmortem urine samples tested (**Table 3.4**). The most prevalent metabolite present was 9-O-demethylmitragynine, which was present in 12 (75%) of the 16 case samples. 7-Hydroxymitragynine was the next most prevalent compound, present in 10 (63%) of the samples. However, since this alkaloid is also present in the plant material, its presence cannot be attributed solely to metabolism (62). 16-Carboxymitragynine was identified in only one sample (indicating that it may be a minor metabolite or highly conjugated) and 9-O-demethyl-16-carboxymitragynine was not detected in any of the urine samples.

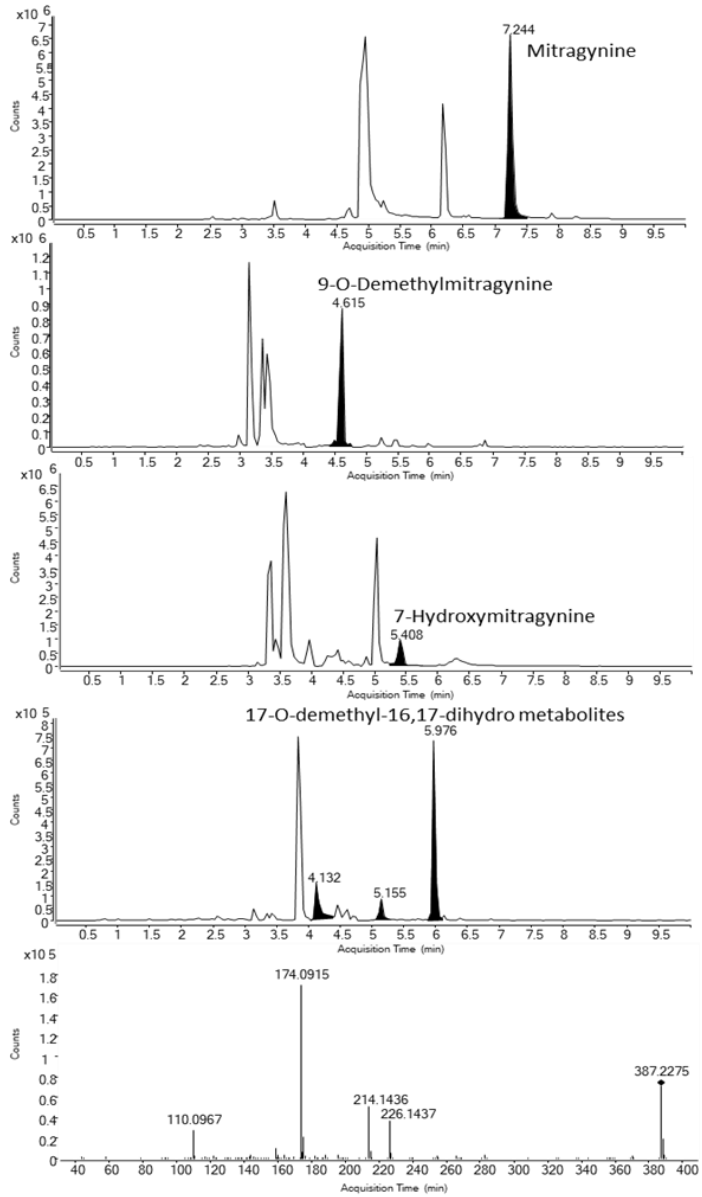
Table 3.4: Identification of mitragynine and other metabolites in human urine. Identification of 17-O-demethyl-16, 17-dihydro (17-O-DM-16,17-DH) metabolites was tentative.

Case Number	Mitragynine Concentration (ng/mL)	9-O-DM	7-OH-MG	16-COOH-MG	17-O-DM-16,17-dihydro Metabolite
1	1,987				
2	26				+[1]
3	461	+	+		
4	176	+	+		+[1]
5	1,271	+	+		+[1,3]
6	677	+			+[1,3]
7	642		+		
8	402	+	+		+[1,2,3]
9	540	+	+		+[1,2,3]
10	938	+			+[1,3]
11	258	+	+		+[1]
12	54	+		+	+[1,3]
13	254	+	+		+[1,3]
14	249	+	+		+[1,3]
15	980	+	+		+[1,3]
16	520				

[1] *m/z* 387, 4.1 min; [2] *m/z* 387, 5.1 min; [3] *m/z* 387, 5.9 min;

In the absence of reference materials, the presence of 9-O-demethylmitragynine, 16-carboxymitragynine and 9-O-demethylmitragynine was confirmed using rCYP-generated controls which were analyzed contemporaneously within the run. Extracted ion chromatograms for a representative case sample (#8) are shown in **Figure 3.8**.

Figure 3.8: Extracted ion chromatograms for mitragynine (7.2 min), 9-O-demethylmitragynine (4.6 min), 7-hydroxymitragynine (5.4 min) and 17-O-demethyl-16-17-dihydro metabolites (4.13, 5.15 and 5.97 min) from case #8. The tentatively identified mass spectrum of 17-O-demethyl-16-17-dihydro-mitragynine [M+H]+ *m/z* 387 is also shown.



Analysis of human urine from kratom users is beneficial due to the limitations of *in vitro* metabolism studies. Additional phase I metabolites that have been reported in literature (11) were tentatively identified in the postmortem urine samples. Notably, these

were not identified *in vitro* using the recombinant CYPs. At least one of three compounds with an *m/z* of 387 were identified in 12 of the 16 samples, each having MS/MS spectra consistent with 17-O-demethyl-16, 17-dihydromitragynine (11). Speciogynine and speciociliatine are diastereoisomers of mitragynine and form analogous 17-O-demethyl-16, 17-dihydro metabolites (63, 64). Due to their structural similarity, MS/MS spectra for all three compounds are indistinguishable and they can only be identified by retention time. Although it is highly likely that the intense peak at 5.9 mins is 17-O-demethyl-16,17-dihydromitragynine, it cannot be confirmed due to the absence of a reference standard. The mass spectrum is depicted in **Figure 3.8**. The remaining peaks are likely due to 17-O-demethyl-16,17-dihydrospeciogynine and 17-O-demethyl-16,17-dihydrospeciociliatine since these *Mitragyna* alkaloids are also present in the urine. Although 17-O-demethyl-16,17-dihydromitragynine was only tentatively identified, mass accuracies (<5 ppm) and fragment assignments in a representative human urine sample are shown in **Table 3.5**. Although 17-O-demethyl-16,17-dihydromitragynine has not been produced in any of the *in vitro* studies to date, it has been identified in human urine (11, 65). Philipp *et al.* attributed its formation to O-demethylation, carboxylation and subsequent reduction to the alcohol (11). This study also identified important differences in metabolism between rats and humans.

Table 3.5: Molecular formula, retention time, and mass accuracies for 9-O-demethylmitragynine (9-O-DM-MG), 7-hydroxymitragynine (7-OH-MG), 17-O-demethyl-16,17-dihydromitragynine (17-O-DM-DH-MG) and mitragynine (MG) in a representative case sample (#8).

Compound	Retention Time (min)	Molecular Formula [M+H]	Exact Mass [M+H]	Accurate Mass [M+H]	Mass Error (PPM)
9-O-DM-MG	4.6	$C_{22}H_{29}N_2O_4$	385.2122	385.2126	1.0
		$C_{10}H_{10}NO$	160.0757	160.0763	3.7
		$C_{12}H_{20}NO_3$	226.1438	226.1441	1.3
		$C_{13}H_{20}NO_3$	238.1438	238.1442	1.7
7-MG-OH	5.4	$C_{23}H_{31}N_2O_5$	415.2227	415.2244	4.1
		$C_{11}H_{12}NO$	190.0863	190.0871	4.2
		$C_{12}H_{20}NO_3$	226.1438	226.1444	2.7
		$C_{13}H_{20}NO_3$	238.1438	238.1445	2.9
17-O-DM-DH-MG	5.9	$C_{22}H_{31}N_2O_4$	387.2278	387.2275	-0.8
		$C_{11}H_{12}NO$	174.0913	174.0916	1.7
		$C_{11}H_{20}NO_3$	214.1438	214.1436	-0.9
		$C_{12}H_{20}NO_3$	226.1438	226.1437	-0.4
MG	7.2	$C_{23}H_{30}N_2O_4$	399.2278	399.2273	-1.3
		$C_{11}H_{12}NO$	174.0913	174.0919	3.4
		$C_{12}H_{20}NO_3$	226.1438	226.1442	1.8
		$C_{13}H_{20}NO_3$	238.1438	238.1441	1.3

A comprehensive understanding of the isoenzymes involved in mitragynine's metabolism is important in terms of the potential for toxicity due to genetic polymorphisms and drug-drug interactions. CYP450 2C19, 2D6 and 3A4 are known to metabolize many drugs including opiates, antidepressants, benzodiazepines and other central nervous system depressants (66). Existing case reports have established that poly-drug use is common among kratom users, notably with opioids. Despite the increase in popularity of kratom for recreational or self-medication purposes, the safety profile and potential for drug-drug interactions are still poorly understood (50).

Conclusion

CYP 3A4 mediated metabolism of mitragynine to 7-hydroxymitragynine was confirmed in this study. Although the compound is known to be unstable, biotransformation of mitragynine to 7-hydroxymitragynine is notable because its mu opioid receptor activity is reported to be more than ten-fold greater than morphine (32). Four CYP 450 isoenzymes were involved in the metabolism of mitragynine (2C19, 3A4, 2D6 and 2C18). 9-O-Demethylmitragynine was the most prominent phase I metabolite in both *in vitro* studies and in human urine samples. Although 9-O-demethyl and 7-hydroxy metabolites were readily identified in the majority of urine samples tested, the formation of the latter has important pharmacodynamic implications. Due to its increased potency, *in vivo* production could explain the reported increased analgesic potency of mitragynine when administered orally, rather than parenterally in animals. Differences in metabolism have been observed between species, and between *in vitro* based studies. Given increased kratom use, additional pharmacokinetic studies involving both mitragynine and 7-hydroxymitragynine are warranted.

Acknowledgements

This project was supported by Award No. 2016-DN-BX-0006, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect those of the Department of Justice. We gratefully acknowledge Dr. Ruth Winecker and Dr. Justin Brower of the North Carolina Office of the Medical Examiner (NCOME) for providing urine samples.

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CHAPTER IV

Temperature and pH-Dependent Stability of *Mitragyna* Alkaloids[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1]Basiliere, S., Kerrigan S. (2019). Journal of Analytical Toxicology, [In Press].

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Abstract

Mitragynine is the principal psychoactive alkaloid in kratom. The drug produces a variety of dose-dependent effects that appeal to recreational drug users and individuals seeking therapeutic benefits in the absence of medical supervision. In light of documented intoxications, hospitalizations and fatalities, mitragynine and other alkaloids from *Mitragyna speciosa* are of growing importance to the forensic toxicology community. However, the chemical stability of these compounds has not been thoroughly described. In this report, the stability of mitragynine (MG), 7-hydroxymitragynine (7-MG-OH), speciociliatine (SC), speciogynine (SG) and paynantheine (PY) are investigated. Short-term stability of the *Mitragyna* alkaloids was determined over a range of pH (2-10) and temperature (4-80°C) over 8 h. Liquid chromatography time-of-flight mass spectrometry (LC-Q/TOF-MS) was used to estimate half-lives and identify degradation products where possible. The stability of mitragynine and other alkaloids was highly dependent on pH and temperature. All of the *Mitragyna* alkaloids studied were acid labile. Under alkaline conditions mitragynine undergoes chemical hydrolysis of the methyl ester to produce 16-carboxymitragynine. 7-Hydroxymitragynine was the most unstable alkaloid studied, with significant drug loss at 8 hours experienced at temperatures of 40°C and above. No significant drug losses were observed for mitragynine in aqueous solution (pH 2-10) at 4, 20 or 40°C. Diastereoisomers of mitragynine (speciociliatine and speciogynine) demonstrated even greater stability. These findings are discussed within the context of the identification of *Mitragyna* alkaloids in toxicological specimens.

Keywords: Mitragynine, 7-Hydroxymitragynine, Speciociliatine, Speciogynine, Paynantheine, Stability

Temperature and pH-Dependent Stability of *Mitragyna* Alkaloids

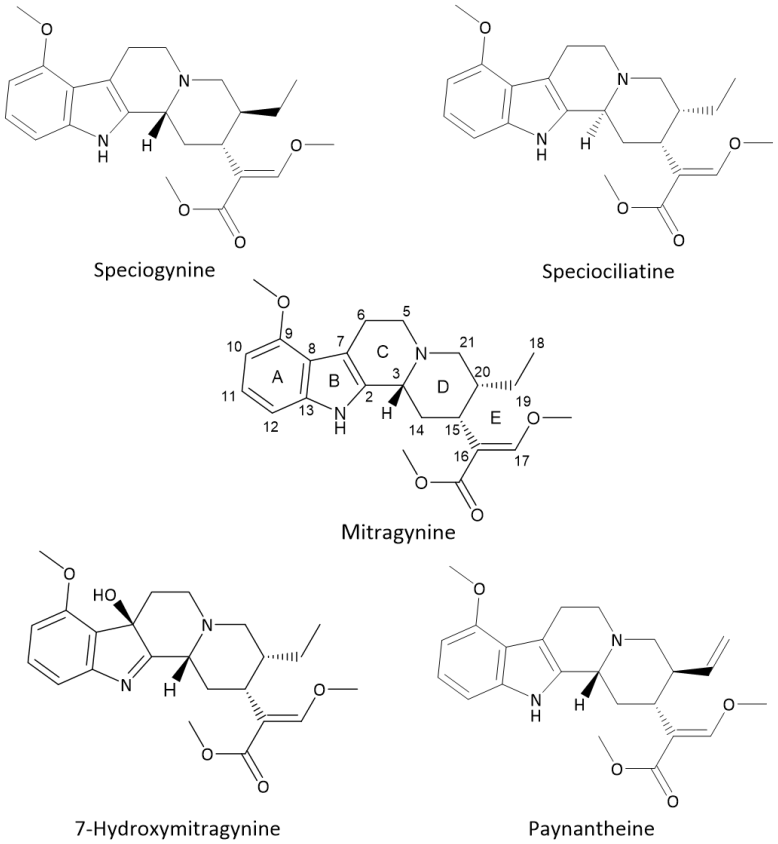
Introduction

Kratom is a drug that comes from the Korth tree (*Mitragyna speciosa*) (1). The drug is widely available on the Internet and in retail outlets in the form of a leaves, powder, capsules, tablets, resin or concentrated extracts (2). Kratom's physiological and psychological effects are known to be dose dependent, with low doses producing stimulant effects and high doses producing opiate like effects (3). The alkaloids of *M. speciosa* are atypical opioid receptor modulators with distinct pharmacological properties (4). The drug is reportedly used to treat chronic pain, intestinal infections, depression, insomnia, and even to improve sexual performance (3, 5-7). Kratom is also used for the non-medically supervised treatment of opioid abstinence syndrome (8, 9). Its rise in popularity has led to growing concern regarding its safety, unregulated use, and overall ease of access. In August 2016 the Drug Enforcement Administration published its intent to place kratom alkaloids mitragynine and 7-hydroxymitragynine into Schedule I of the federal Controlled Substances Act (10). However, due to successful lobbying from kratom users, researchers and the dietary supplement industry, the temporary placement was rescinded and kratom's legal status at the federal level is still uncertain. At the time of this report, it is illegal to possess or sell kratom in some states (Alabama, Arkansas, Indiana, Tennessee, Vermont, Wisconsin and the District of Columbia) and legislation is pending elsewhere.

Mitragyna speciosa is the most well described species of the *Mitragyna* genus (11). The leaves are reported to contain over forty compounds, including mitragynine, 7-hydroxymitragynine, speciociliatine, speciogynine and paynantheine. Chemical structures of these five alkaloids are shown in **Figure 4.1**. Only two alkaloids are believed to be

psychoactive: mitragynine and 7-hydroxymitragynine (2, 3). Mitragynine is the most abundant compound in kratom, comprising of about 66% of the total alkaloid content (1). 7-Hydroxymitragynine is less abundant and accounts for only 2%, but it is considerably more potent than mitragynine (12). 7-Hydroxymitragynine is reported to have a 13-fold greater mu opioid agonist effect compared to morphine (46-fold greater than mitragynine) (11). In addition to its mu opioid receptor activity, mitragynine is also reported to bind to the α-2 adrenergic receptor (α2R), dopamine (D_2) and serotonin receptors (5-HT_{2c} and 5-HT_7) (4). Although less studied, its affinity for these receptors could explain its unique pharmacological and dose-dependent effects. Several published case reports have described fatalities, overdoses, and hospitalizations following kratom use (13-27).

Figure 4.1: Chemical structures of the *Mitragyna* alkaloids.



Mitragynine is a basic drug (pKa 8.1) of moderate lipophilicity (log $P_{oct/water}$ 1.73) which is poorly soluble in water (28). Drug stability is an important consideration that influences many factors, including bioavailability and absorption, as well as analytical and pre-analytical factors, such as storage. Although biological specimens can be exposed to a wide variety of conditions before sample collection, proper handling, storage and transport of the specimen is critically important. Information regarding the stability of the *Mitragyna* alkaloids is relatively sparse. Manda reported the stability of mitragynine and 7-hydroxymitragynine in simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) (29). Both matrices were fortified with 5,000 ng/mL of MG and 7-MG-OH and for up to 2.5 hours at 37°C. For mitragynine, samples in SGF (pH 1.2) were reported to be unstable (>26% loss) in 2 hours. Conversely, samples in SIF (pH 6.8) were stable, with a <4% loss

at 2.5 hours. 7-Hydroxymitragynine showed similar results with the samples being unstable in SGF (27% loss in 2 hours), but stable in SIF (6% loss at 2.5 hours). Interestingly, they reported that the decrease in 7-hydroxymitragynine concentration was accompanied by an increase (23%) in mitragynine, possibly attributed to the presence of enzymes. Ramanathan reported similar results regarding the stability of mitragynine in simulated gastric and intestinal fluids (28). Ramanathan also investigated the stability of mitragynine at various pH. Although they reported that mitragynine was stable at pH 4, 7, and 9 for 24 hours, the temperature of the study was not specified. A study by Fu et al indicated that both mitragynine and 7-hydroxymitragynine were stable at refrigerated temperature for two weeks, however the pH of the specimen was not indicated (30). Although methods describing the analysis of mitragynine have addressed stability in processed samples and during freeze-thaw cycles (31-34), the chemical stability of the *Mitragyna* alkaloids are not well described. Published stability studies to date are summarized in **Table 4.1**.

Table 4.1: Published literature involving *Mitragyna* alkaloid stability. The matrix, pH, temperature and concentration (if stated) are reported.

<table>
<tr><th>Compound</th><th>Matrix</th><th>pH</th><th>Temperature (°C)</th><th>Concentration (ng/mL)</th><th>Findings</th><th>Reference</th></tr>
<tr><td>MG</td><td>Methanol</td><td>-</td><td>4</td><td>1,000</td><td>Stable for 1 month</td><td>(31)</td></tr>
<tr><td rowspan="4">MG</td><td rowspan="3">Human Plasma</td><td rowspan="3">7.4</td><td>F/T</td><td rowspan="4">400-4,000</td><td>Stable for 3 freeze/thaw cycles</td><td rowspan="4">(32)</td></tr>
<tr><td>RT</td><td>Stable for 6 hours</td></tr>
<tr><td>-20</td><td>Stable for 1 month</td></tr>
<tr><td>Processed Sample</td><td>-</td><td>-20</td><td>Stable for 48 hours</td></tr>
<tr><th>Compound</th><th>Matrix</th><th>pH</th><th>Temperature (°C)</th><th>Concentration (ng/mL)</th><th>Findings</th><th>Reference</th></tr>
</table>

MG	Methanol	-	4	5,000 and 50,000	Stable for 1 month	(33)
MG	Kratom Tea	-	4	104,200-191,700	Stable for 14 days	(34)
MG	Simulated gastric fluid	1.2	37	5,000	Unstable at 2 hours	(29)
	Simulated intestinal fluid	6.8			Stable for 2.5 hours	
MG	Simulated gastric fluid	1.2	37	20,000	Unstable at 1 hour	(28)
	Simulated intestinal fluid	6.8			Stable at 2.5 hours	
	Aqueous buffer	4, 7, 9	-		Stable for 24 hours	
MG	Urine	NR	4	1	Stable for 14 days	(30)
7-MG-OH	Simulated gastric fluid	1.2	37	5,000	Unstable at 2 hours	(29)
	Simulated intestinal fluid	6.8			Stable for 2.5 hours	
7-MG-OH	Rat Plasma	-	FT	20 - 3200	Stable for 3 cycles	(35)
			25		Stable for 12 hours	
			-20		Stable for 1 month	
	Processed Sample		25		Stable for 48 hours	
7-MG-OH	Urine	NR	4	1	Stable for 14 days	(30)

FT, Freeze/Thaw; MG, Mitragynine; 7-MG-OH, 7-Hydroxymitragynine; NR, Not reported; RT, Room Temperature.

Vuppala explored the stability of 7-hydroxymitragynine during analytical method development (35). They reported that the compound was stable at various concentrations in rat plasma (20, 600, 3200 ng/mL), including 12 hours at 25°C, 1 month at -20°C, three

freeze/thaw cycles, and processed samples stored for 48 hours at 25°C. In addition to those published studies, certified reference materials for 7-hydroxymitragynine state that the compound should be stored at -80°C in strongly alkaline media (0.1N ammonia in methanol) due to its unstable nature (36). Fundamental knowledge of pH-dependent stability can provide insight from the standpoint of pharmacokinetics, analysis and interpretation. Based upon previously published studies and anecdotal reports, it was hypothesized that mitragynine and its alkaloids degrade in a pH- and temperature dependent manner. The stability of the *Mitragyna* alkaloids was investigated under accelerated conditions, in an effort to better understand the potential for degradation of these species in toxicological specimens. Use of aqueous media lends itself to tighter pH control and improves the possibility of identifying degradation mechanisms and products than more complex biological matrices. The pH and temperature-dependent stability of five *Mitragyna* alkaloids were systematically evaluated in aqueous solutions. In this short-term stability study, half-lives for both species were determined over a wide range of pH (2 – 10) and temperature (4 - 80°C).

Methods

Chemicals and reagents

Reference standards for mitragynine and 7-hydroxymitragynine were purchased from Cerilliant (Round Rock, TX USA) in methanol and ammoniated methanol, respectively. Paynantheine and speciociliatine were purchased from Chromadex (Irvine, CA, USA) in solid form. Speciogynine was provided by the National Center for Natural Products Research (NCNPR) at the University of Mississippi, (University, MS, USA). Boric acid and acetic acid were purchased from Mallinckrodt Chemicals (St. Louis, MO,

USA). Concentrated hydrochloric acid and sodium borate were purchased from J.T. Baker (Center Valley, MA, USA). Acetonitrile (LCMS grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). LCMS grade formic acid, ammonium formate (10 M), and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was purified in-house using a Millipore Direct-Q® UV Water Purification system (Billerica, MA, USA). Acetate (pH 4), formate (pH 6) and borate (pH 8 and 10) buffers were prepared at 10 mM from solutions of the salt and its corresponding acid. The aqueous pH 2 solution consisted of 10 mM hydrochloric acid.

LC-Q/TOF-MS analysis

An Agilent Technologies 6530 LC-Q/TOF-MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 1290 Infinity autosampler was used for analysis. Separation was achieved using an Agilent Technologies Series 1200 LC system equipped with an Agilent Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 µm) and an Agilent Poroshell 120 EC-C18 guard column (2.1 × 5 mm, 2.7 µm) in a thermostatically controlled column compartment (35^{o}C). Nitrogen was generated using a Genius 3040 nitrogen generator (Peak Scientific, Billerica, MA, USA).

The LC-Q/TOF-MS conditions were based upon a previously validated method for MG, MH-OH, SC, SG and PY (37). The mobile phase consisted of 10 mM ammonium acetate solution (A) and acetonitrile (B). A flow rate of 0.4 mL/min was maintained using the following gradient elution profile: 10% B (0–0.5 min), 10-90% B (0.5–10 min), followed by a 2 minute re-equilibration. The total acquisition time was 10 min. The mobile phase gradient was modified from the previously validated method in order to assist with the identification of polar degradation products. The LC-Q/TOF-MS was equipped with an

ESI source (positive mode) with Jet Stream technology under the following conditions: nitrogen drying gas, 13 L/min; drying gas temperature, 350°C; nebulizer, 45 psi; sheath gas temperature, 400°C; nitrogen sheath gas flow, 12 L/min; capillary voltage, 4000 V; nozzle voltage, 0 V; fragmentor, 150 V; skimmer, 65 V. Data was acquired in full scan (auto) mode using a preferred list that contained precursor ions for all five alkaloids. Agilent MassHunter software was used for data analysis. MS/MS spectra were generated using collision induced dissociation (CID) energies of 10-60 eV. Data was acquired using a mass range of 100–1000 Da, with a MS scan rate of 5 spectra/s and a MS/MS scan rate of 2 spectra/s. Structural identification of potential degradation products was performed for mitragynine.

Sampling for Stability Study

Dilute acid (pH 2) or aqueous buffer (pH 4, 6, 8, 10) was fortified with MG, 7-MG-OH, SG, SC or PY to achieve a final concentration of 2000 ng/mL. Aqueous samples (1.5 mL) were sealed in tightly capped borosilicate glass tubes and protected from light. Solutions were maintained at 4°C, 20°C, 40°C, 60°C and 80°C. A total of twenty-five conditions were tested (five pHs at five temperatures) for all five *Mitragyna* alkaloids. Each compound was evaluated independently to reduce the potential for interferences caused by degradation products.

Short-term stability was evaluated over a period of eight hours. Immediately following fortification, aliquots (50 µL, n=2) of each sample were removed and analyzed to establish the abundance of each alkaloid at T_0 (0% loss). Sampling intervals following T_0 were 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hours. Although the autosampler was maintained at 4°C, T_0 samples were reinjected throughout the run to verify that no changes in abundance

took place while samples were awaiting analysis. During each sampling interval, aliquots (50 µL, n=2) were removed and diluted 1:1 with the associated buffer for samples at pH 2, 4, and 6. Due to degradation in the autosampler, samples at pH 8 and 10 were reconstituted 1:1 with 10 mM acetate buffer (pH 4) prior to injection. Samples were then transferred to the refrigerated autosampler and 1 µL was injected onto the LC-Q/TOF-MS. The analyte was considered unstable when the mean peak area of the precursor ion decreased by more than 20% (relative to T_0). Assuming first-order kinetics, rate constants (*k*) and half-lives ($T_{1/2}$) were estimated for alkaloids with significant decreases in abundance (>20%) over consecutive measurements.

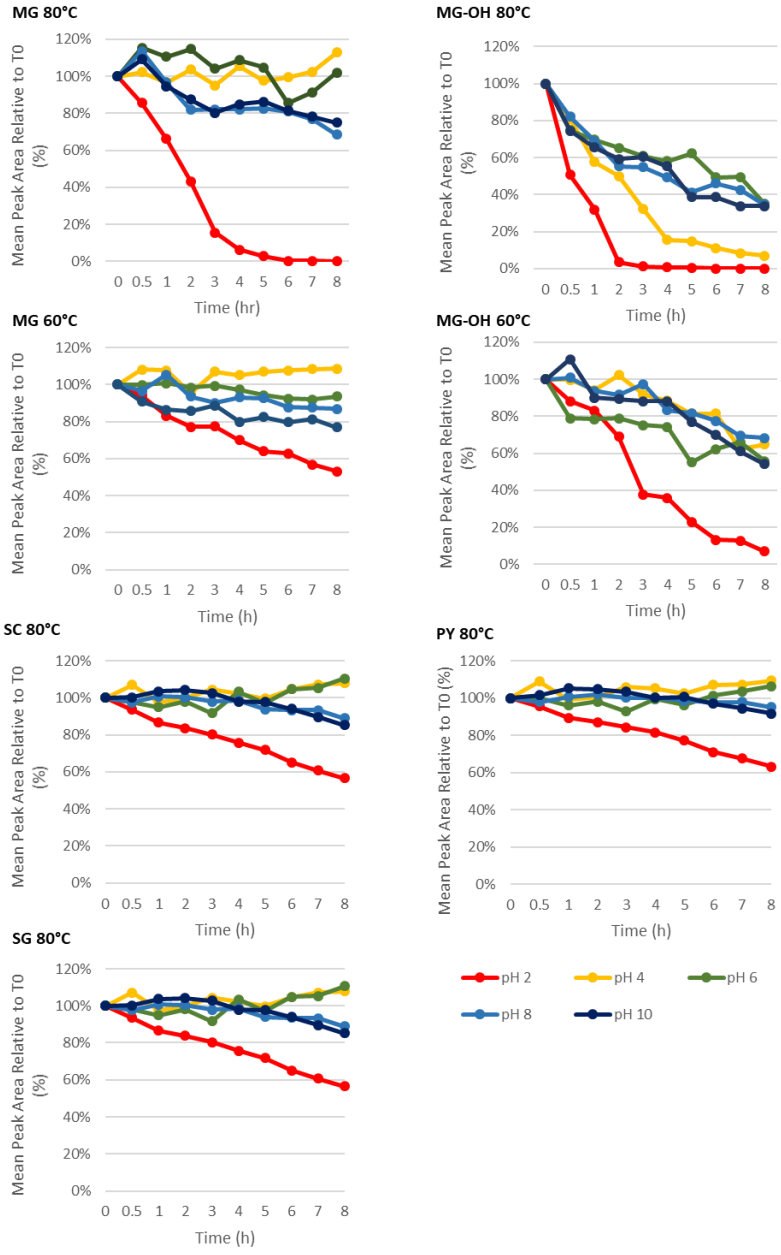
Results and discussion

Stability of Mitragyna alkaloids

The stability of MG, MG-OG, SC, SG and PY was evaluated under various conditions of pH and temperature. Changes in concentration over time are shown in **Figure 4.2**. **Table 4.2** summarizes the % loss at 8 hours for each condition tested. Significant losses (>20%) are highlighted in bold. Data represents the mean of duplicate measurements at each interval. CVs <20% were achieved for all compounds tested. As expected, both temperature and pH-dependent effects were observed. Mitragynine was completely stable for eight hours at pH 2-10 at 4, 20 and 40°C. In contrast, the drug was significantly acid labile at elevated temperature (60-80°C). At pH 2, 100% of the drug was lost within 6 hours at 80°C. Mitragynine was stable at moderate pH (4 and 6) for 8 hours, but the compound significantly degraded under strongly acidic conditions (pH 2) and moderate to strongly alkaline conditions (pH 8 and 10) at 80°C. At 60°C, significant losses were observed at the

most acidic and basic pH (2 and 10) (**Table 2.2**). These observations are consistent with previous studies that suggest *Mitragyna* alkaloids are acid labile (28).

Figure 4.2: Short-term stability of MG, MH-OH, SC, SG and PY.



In contrast to the rapid degradation of mitragynine under acidic conditions at elevated temperature, the drug was completely stable for eight hours at refrigerated and room temperature. This is consistent with studies that indicated the drug to be stable for at

least one month when refrigerated at 4°C (33, 34). However, it should be noted that mitragynine was not stable at pH 8 and 10 when placed in the autosampler for the duration of the run (~22 hours). When the T_0 samples were reinjected 24 hours after the initial injection, a >20% loss in abundance was observed at pH 8 and 10. In order to prevent degradation during analysis, these samples were diluted in acidic buffer prior to injection (described above).

Table 4.2: Mean % loss of mitragynine, 7-hydroxymitragynine, speciociliatine, speciogynine and paynantheine at 8 hours (n=2). Significant losses (>20%) are shown in bold.

Drug	pH	Mean % Loss at 8 hours				
		80°C	60°C	40°C	20°C	4°C
MG	2	**100**	**47**	0	1	0
	4	0	0	0	1	0
	6	0	7	0	8	0
	8	**31**	13	20	1	0
	10	**25**	**23**	12	18	0
7-MG-OH	2	**100**	**93**	**32**	4	0
	4	**93**	**35**	7	9	0
	6	**65**	**44**	19	0	0
	8	**57**	**32**	9	0	0
	10	**66**	**46**	8	7	0
SC	2	**58**	17	12	-	-
	4	0	0	0	-	-
	6	0	5	11	-	-
	8	20	2	11	-	-
	10	20	7	6	-	-
SG	2	**43**	5	6	-	-
	4	0	0	0	-	-
	6	0	6	11	-	-
	8	11	2	11	-	-
	10	15	3	4	-	-
PY	2	**37**	4	6	-	-
	4	0	0	0	-	-
	6	0	1	9	-	-
	8	5	0	7	-	-
	10	8	3	2	-	-

In contrast, 7-hydroxymitragynine was unstable at all pHs at temperatures of 60°C or above. Although all samples experienced a significant loss at the highest temperatures (80 and 60°C) regardless of pH, degradation rates were pH dependent. A loss of 100% was observed for pH 2 and a near complete loss (93% loss) was observed for pH 4 at 80°C. The findings are consistent with earlier studies by Manda, who reported that 7-hydroxymitragynine was unstable in simulated gastric fluid (pH 1.2, 37°C), but stable in simulated intestinal fluid (pH 6.8, 37°C) after approximately 2 hours (29). Like

mitragynine, 7-hydroxymitragynine was observed to be unstable at elevated temperatures in a pH-dependent manner. Both alkaloids were significantly acid labile. However, unlike mitragynine, 7-hydroxymitragynine also underwent significant degradation at moderate pH. However, no losses were observed for 7-hydroxymitragynine at ambient or refrigerated temperatures, regardless of pH (**Figure 4.2, Table 4.2**).

Speciociliatine, speciogynine and paynantheine demonstrated greater stability (**Figure 4.2, Table 4.2**). Significant losses at 8 hours were observed only under strongly acidic conditions (pH 2) at 80°C. Speciociliatine and speciogynine are both stereoisomers of mitragynine. SG and SC have different geometries at the C20 and C3 positions. Spectroscopic studies have confirmed their spatial configurations, which can greatly influence their chemical properties (38-40). Molecular conformation plays an important role in both chemical reactivity and bioactivity. Relatively small changes in spatial arrangements and conformational flexibility of the aliphatic regions of the rings themselves could impact stability. Rings A and B are aromatic and therefore planar in structure (**Figure 4.1**). More conformational flexibility is possible in rings C and D however. Mitragynine conformers are heavily influenced by the position of the nitrogen lone pair shared by rings C and D, theoretically allowing it to be *syn* or *anti* to the exocyclic ethyl group at C20 (Liu, 2010). As a result, this can place the nitrogen either above the plane of the aromatic rings, or below them. Interestingly, this is believed to play an important role in terms of its opioid receptor activity. Arrangement of the nitrogen above the rings is more stable, attributed to reduced steric hindrance at the CH2 group of ring C and ethyl group at C20 (41). Of the five alkaloids under investigation, only mitragynine and 7-hydroxymitragynine have the same spatial configuration at C3 and C20 (37). Ambient and refrigerated temperatures were

not evaluated for SC, SG or PY because these compounds did not experience any significant losses at 60 or 40°C.

Half-life determination

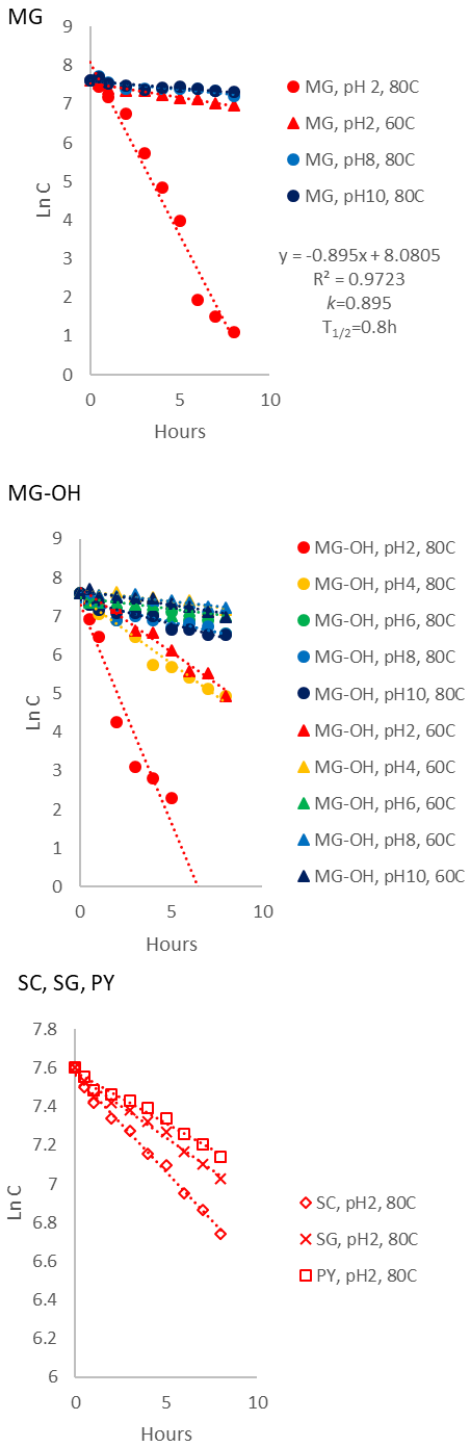
Half-lives ($T_{1/2}$) for each alkaloid were estimated from rate constants (k) assuming first order decay, based upon duplicate measurements at each time interval ($T_{1/2} = Ln2/k$) (**Table 4.3**). However, these estimates were performed only if there was a significant decrease (>20%) over at least three consecutive measurements, with suitable linearity. Rate plots for MG, 7-MG-OH, SC, SG and PY are shown in **Figure 4.3**. A representative calculation for $T_{1/2}$, equation of the line and coefficient of determination (R^2) is shown for MG. As expected, half-lives were both temperature and pH-dependent. The half-life for mitragynine under strongly acidic conditions (pH 2) ranged from 0.8h to 9.5h at 80 and 60°C, respectively. In contrast, in moderate to strongly basic conditions (pH 8 and 10), half-lives were >12h, even at extremely elevated temperature (80°C). However, at pH 4-6 half-lives could not be estimated due to overall stability.

Table 4.3: Estimated half-lives of mitragynine and 7-hydroxymitragynine, speciociliatine, speciogynine and paynantheine.

	pH	**Half-Life (h)**	
		80°C	**60°C**
MG	2	0.8	9.5
	8	15	-
	10	18	-
7-MG-OH	2	0.6	2.1
	4	2.0	12
	6	7.5	12
	8	6.8	14
	10	5.6	9.2
SC	2	6.9	-
SG	2	11	-
PY	2	13	-

7-Hydroxymitragynine was less stable than mitragynine and degraded significantly over the entire pH range at 60°C and above. However, like mitragynine, significant pH dependence was also observed. Half-lives ranged from 0.6 to 7.5h at pH 2 and 6 at 80°C. At 60°C, half-lives ranged from 2.1 to 14 hours. Although there was a significant loss of 7-MG-OH under strongly acidic conditions at 40°C, it was not possible to reliably estimate a half-life due to an insufficient number of measurements. Half-lives for SC, SG and PY were approximately 7-13h under strongly alkaline conditions at 80°C. Notably, the diastereoisomers of MG were notably more stable than the principal alkaloid itself. Half-lives under acidic conditions were more than ten-fold higher than mitragynine, and no short-term degradation was observed under alkaline conditions.

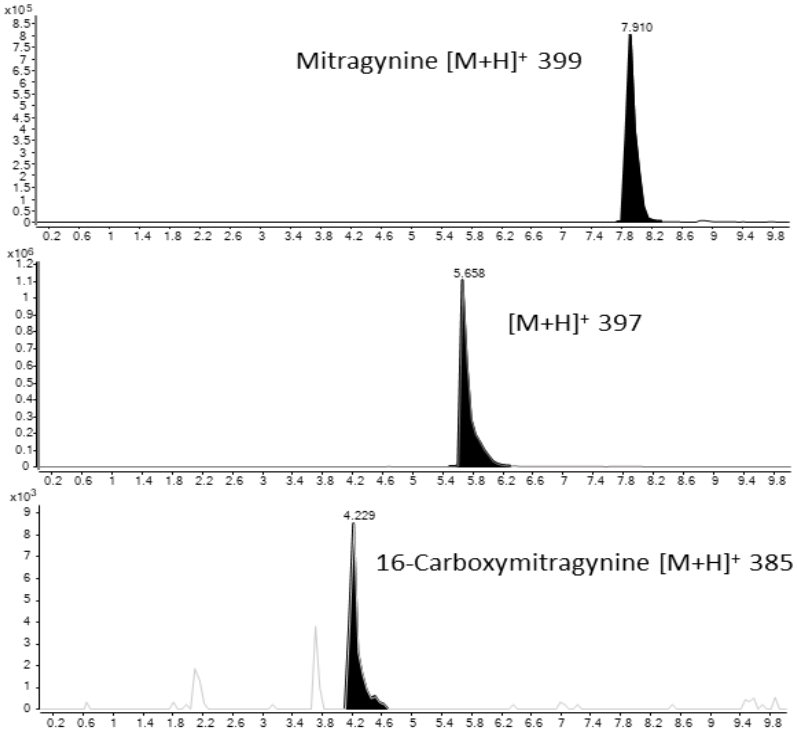
Figure 4.3: Rate plots and estimation of $T_{1/2}$ for MG, MH-OH, SC, SG and PY.



Identification of degradation products

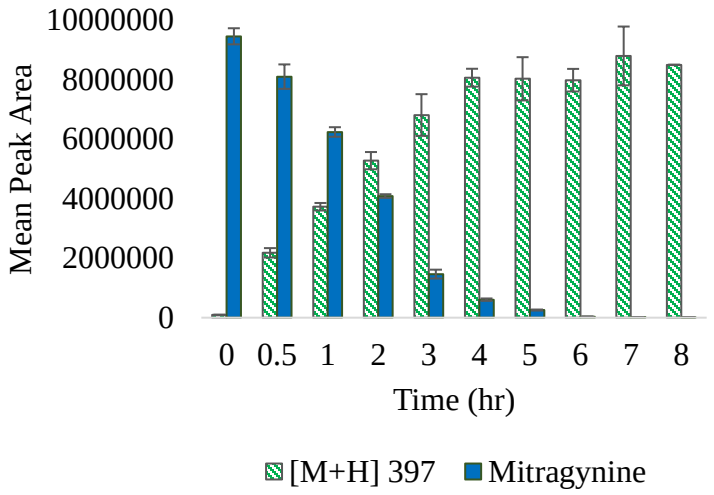
Samples were analyzed using auto scan mode to facilitate the identification of potential mitragynine degradation products. Two degradation products were observed for mitragynine (7.9 min) at 4.2 and 5.6 min, corresponding with $[M+H]^+$ *m/z* 385 and 397, respectively. Extracted ion chromatograms for the parent drug and degradation compounds are shown in **Figure 4.4**. The decrease in abundance of mitragynine over time was accompanied by an increase in the abundance of both species. **Figure 4.5** depicts this graphically for the *m/z* 397 breakdown product at pH 2 at 80°C. Although the *m/z* 385 species also increased in abundance over 8h, the shift was not as dramatic because the degradation rate of mitragynine under alkaline conditions occurs at a much slower rate.

Figure 4.4: Extracted ion chromatograms of mitragynine ([M+H]+ 399) and degradation products under acidic conditions ([M+H]+ 397, pH 2, 80°C, 8h) and alkaline conditions (16-carboxymitragynine ([M+H]+ 385, pH 10, 80°C, 8h).



MS/MS spectra and structural assignments for mitragynine and the two major degradation products are shown in **Figure 4.6**. The degradation product with *m/z* 385 was identified as 16-carboxymitragynine. This was produced only at pH 8 and 10, consistent with the alkaline hydrolysis of the methyl ester. In the absence of a commercially available reference standard, the MS/MS spectrum was used for identification purposes. The mass error of the molecular ion and its fragments was within 2 ppm for all structural assignments (**Table 4.4**). In addition to being present as a chemical breakdown product, 16-carboxymitragynine is also a known metabolite of mitragynine (42, 43).

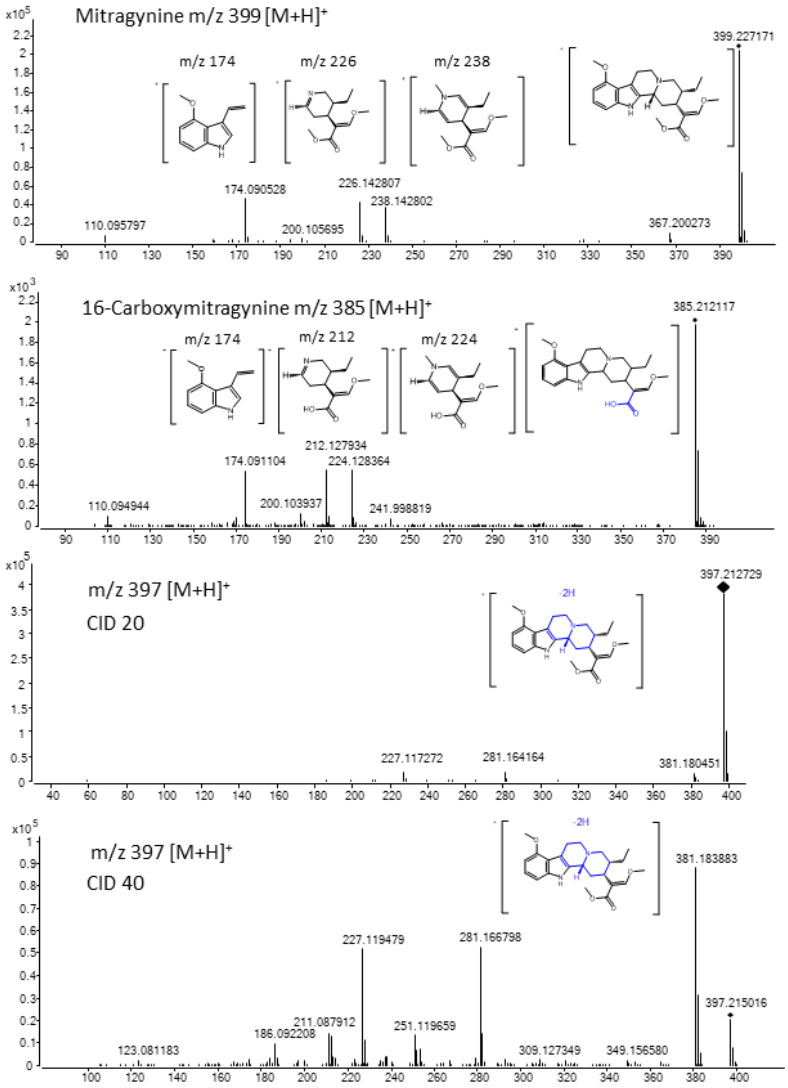
Figure 4.5: Formation of the acidic degradation product $[M+H]^+$ 397 at pH2 and 80°C.



The degradation product corresponding with the *m/z* 397 precursor ion might be attributed to dehydrogenated mitragynine (-2 Da). Although the mass error associated with a dehydrogenated species ($C_{23}H_{29}N_2O_4$) was 7.1 ppm (**Table 4.4**), structural identification of this compound was not conclusive. Kamble reported a dehydrogenated species bearing an almost identical mass spectrum (44). They attributed the dehydrogenation to occur on the quinolizidine moiety of the mitragynine. Although the MS/MS spectrum of the acid degradation product was almost identical to that described by Kimble, a conclusive

identification was not possible. 3-Dehydromitragynine has been reported in the literature and is reported to be present in leaves of *M. speciosa* (11). Houghton was the first to identify it in plant material (45). MS/MS spectra were evaluated over a range of CID voltages (**Figure 4.6**) and *m/z* 397 was confirmed as the M+H molecular ion. The formation of the acidic breakdown product was investigated in the absence of oxygen. However, its abundance was just as intense when mitragynine was treated with acid under nitrogen.

Figure 4.6: MS/MS spectra of mitragynine ($[M+H]^+$ 399; CE 20), 16-carboxymitragynine ($[M+H]^+$ 385; CE 20) and acidic degradation product ($[M+H]^+$ 397; CE 20 and 40).



Mitragynine is a corynanthe-type alkaloid consisting of a conjugated pentacyclic skeleton with an open E ring. The mass fragmentation of *Mitragyna* alkaloids was described by Avula (46). Mitragynine and related alkaloids undergo characteristic loss of piperidine derivatives to form a methyl substituted indole fragment (*m/z* 174), cleavage at the C5 of the C ring to form *m/z* 238, and neutral loss of the methoxyindole and cleavage of the C5 and N to produce the *m/z* 226 piperidine species. The notable absence of the *m/z* 174 indole fragment suggests that either the structural modification occurs on this group,

or that the modification influences the formation of this stable fragment. The acidic degradation product produced prominent ions at *m/z* 381, 281 and 227 (**Figure 4.6**). The MS/MS spectrum suggests the M-16 to be attributed to loss of methane (7.9 ppm) rather than loss of oxygen (-89.6 ppm). Further, loss of $C_5H_8O_3$ (cleavage at C15) corresponds with the *m/z* 281 (7 ppm) and *m/z* 227 could be attributed to the piperidine fragment ($C_{14}H_{15}N_2O$), with an associated mass shift of 7 ppm. Although mass assignments for a dehydromitragynine derivative were within acceptable limits and consistent with previously reported literature (42), the chemistry of the *Mitragyna* alkaloids is complex and other species are possible.

Table 4.4: Chemical formula, retention time, exact mass, accurate mass, and mass error for mitragynine and its degradation products.

Compound	Retention Time	Molecular Formula $[M+H]^+$	Exact Mass $[M+H]^+$	Accurate Mass $[M+H]^+$	Mass Error (ppm)
Mitragynine $[M+H]^+$ 399	7.910	$C_{23}H_{31}N_2O_4$	399.227834	399.227171	-1.7
		$C_{11}H_{12}NO$	174.091340	174.090528	-4.7
		$C_{13}H_{20}NO_3$	238.143770	238.142802	-4.1
		$C_{12}H_{20}NO_3$	226.143770	226.142807	-4.3
16-Carboxymitragynine $[M+H]^+$ 385	4.229	$C_{22}H_{29}N_2O_4$	385.212184	385.212117	-0.2
		$C_{11}H_{12}NO$	174.091340	174.091104	-1.4
		$C_{12}H_{18}NO_3$	224.128120	224.128364	1.1
		$C_{11}H_{18}NO_3$	212.128120	212.127934	-0.9
$[M+H]^+$ 397	5.658	$C_{23}H_{29}N_2O_4$	397.212184	397.215016	7.1

Conclusion

Kratom is a drug of concern that has been associated with numerous intoxications, hospitalizations and fatalities. In this report, we describe the chemical stability of the principal *Mitragyna* alkaloids over a wide range of pH and temperature. Although stability in biological matrices is of concern in forensic toxicology investigations, stability studies in aqueous media lends itself to the investigation of extreme pH and is more conducive to the identification of chemical breakdown product because of the simplicity of the matrix. Furthermore, accelerated stability studies can help identify potential degradation pathways or products that may not be evident otherwise. Drugs can be exposed to a wide range of pH throughout the body, ranging from strongly acidic (in the stomach) to alkaline (in the intestines and in urine). Urinary pH can also change during long-term storage, especially when exposed to heat. Although toxicological specimens should not be exposed to the elevated temperatures used in this study, the results provide valuable insight regarding the stability of these alkaloids, and their potential use as biomarkers of kratom use. Exposure to elevated temperatures is relevant if kratom tea is ingested, and during toxicological analysis (particularly during derivatization, chemical hydrolysis of phase II metabolites, or evaporation steps following extraction).

While MG and 7-MG-OH both demonstrated chemical instability under acidic and basic conditions, both compounds were considerably more acid labile than base-labile. Under alkaline conditions, mitragynine undergoes hydrolysis to form 16-carboxymitragynine, a known metabolite. Degradation under acidic conditions produced a species with a 2Da loss, possibly corresponding with a dehydrogenation product. While mitragynine was more stable than 7-hydroxymitragynine, elevated temperature and

extreme pH should still be avoided. Although the drug was stable at 4, 20 and 40°C, only short-term stability was evaluated over 8 hours. The results of this study suggest that degradation in biological matrices should be considered, particularly for strongly acidic or basic matrices (e.g. stomach contents, or alkaline urine), particularly if it is exposed to elevated temperatures during the postmortem interval or during transport. An understanding of the chemical stability of these alkaloids also provides valuable information from the standpoint of method development and quantitative analytical procedures. Extreme pH (during solid phase extraction) and temperatures during analytical processing are unlikely to impact stability, with the exception of 7-hydroxymitragynine. Differences in stability between mitragynine and its diastereoisomers, speciogynine and speciociliatine, highlight the importance of stereochemistry on drug stability. Mitragynine is a basic drug with limited water solubility and poor gastrointestinal permeability (47). Ramanathan reported the oral bioavailability in rats was only 3% (28). The results if this study suggest that in addition to its relatively poor solubility, the low oral bioavailability might also be attributed to its tendency to be acid labile.

Acknowledgements

We gratefully acknowledge Dr. Ikhlas Khan and the National Center for Natural Products Research (NCNPR) at The University of Mississippi for providing a reference standard for speciogynine. This project was supported by Award No. 2016-DN-BX-0006 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect those of the Department of Justice.

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CHAPTER V

Identification of Metabolites and Potential Biomarkers of Kratom in Urine[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1]Basiliere, S., Kerrigan S. (2019). Journal of Chromatography B, [In Review].

Abstract

Mitragyna speciosa (kratom) is a drug that is increasingly used recreationally and "therapeutically", in the absence of medical supervision. Its use has been associated with a growing number of fatalities, and although its medicinal properties as an atypical opioid require further study, there are legitimate concerns regarding its unregulated use. Mitragynine is a prominent alkaloid within the plant. However, more than forty other alkaloids have been reported, including 7-hydroxymitragynine which is a potent opioid receptor agonist with increased abuse liability. In this report, biomarkers for mitragynine were investigated using liquid chromatography-quadrupole/time of flight mass spectrometry (LC-Q/TOF-MS). Speciociliatine and speciogynine were identified as alternative biomarkers, often exceeding the concentration of mitragynine in unhydrolyzed urine. 9-O-Demethylmitragynine and 7-hydroxymitragynine were identified in unhydrolyzed urine in 75% and 63% of the cases. Deconjugation of phase II metabolites using chemical hydrolysis was not suitable due to degradation of the *Mitragyna* alkaloids. Enzymatic hydrolysis was evaluated using three traditional glucuronidases, four sulfatases and four recombinant enzymes. Although enzymatic hydrolysis increased the concentration of 16-carboxymitragynine, it had nominal benefit for other metabolites. Deconjugation of urine was not necessary due to the abundance of parent drug (mitragynine), its diastereoisomers (speciociliatine and speciogynine) or metabolites (9-O-demethylmitragynine and 7-hydroxymitragynine).

Keywords: Mitragynine, 7-Hydroxymitragynine, Kratom, Deconjugation, Biomarkers

Identification of Metabolites and Potential Biomarkers of Kratom in Urine

Introduction

Mitragynine is an "atypical opioid" that is present in the leaves of *Mitragyna speciosa* (kratom). Although it was initially encountered as a recreational drug in the United States, more recently there has been considerable interest in its potential therapeutic use (1). Kratom's effects are mediated by both opioid and non-opioid receptor interactions, which can produce stimulant and opiate-like effects in a dose-dependent fashion (2). Most recently, it has attracted attention due to its use for the non-medically supervised treatment of opioid abstinence syndrome (3). Kratom is legal in many states and is not currently scheduled at the federal level, which leads to potential misuse as opioid users seek out kratom as a legal opiate replacement (4). Even in states where regulation is present, kratom is relatively easy to obtain through retail outlets and online vendors (3). Drug Enforcement Administration (DEA) surveillance data from the National Forensic Laboratory Information System (NFLIS) indicates that seizures involving mitragynine or kratom have increased since 2010 (5-7). There are also several published case reports detailing adverse side effects including seizures, coma and hepatotoxicity (8-12). In addition, several fatalities associated with kratom use have also been reported (13-22). Recently, a report from the Centers for Disease Control and Protection (CDC) detailed 152 fatalities involving kratom from 27 states (23). In addition to concerns regarding increased use, together with a common perception and vendor labeling that indicate the drug is "safe", the report also confirmed that kratom is frequently encountered among the opioid-abusing population. Among reported fatalities, fentanyl (or its analogs) (65%) and heroin (33%) were the most commonly encountered drugs in kratom-positive cases. Kratom use may be

underreported due to the scope of toxicological screening in biological matrices (23). Although confirmatory methods to identify *Mitragyna* alkaloids have been reported, many analytical methods used to identify kratom use target mitragynine alone (**Table 5.1**). Since kratom's primary route of administration is oral consumption (24), it is possible that other kratom alkaloids could also serve as biomarkers of kratom use.

Table 5.2: Summary of published analytical methods for *Mitragyna* alkaloids in biological matrices.

Matrix	Sample Volume	Extraction Method	LOD (ng/mL)	LOQ (ng/mL)	Linear Range (ng/mL)	Compounds Detected	Internal Standard(s)	Analytical Technique	Reference
Urine	1 mL	SPE	0.25-1	0.5-1	2-500	MG, 7-MG-OH, SC, SG, PY	MG-D_3, 7-MG-OH-D_3	LC-Q/TOF-MS	(32)
Urine	0.2 mL	LLE	-	1	1-500	MG, 7-MG-OH	Mitraphylline	LC-MS/MS	(38)
Urine	3 mL	SPE	100	-	-	MG, PY, SC, SG	-	GC-MS	(33)
Urine	1 mL	BAμE	0.1	0.3	0.6-24	MG	-	LC-DAD	(39)

Matrix	Sample Volume	Extraction Method	LOD (ng/mL)	LOQ (ng/mL)	Linear Range (ng/mL)	Compounds Detected	Internal Standard(s)	Analytical Technique	Reference
Blood, Tissue, Urine, Bile, Vitreous humor	1 mL or 1 g	LLE	0.25	1	1-10	MG	Proadifen	LC-MS/MS	(15)
Urine	2 mL	LLE	0.02	0.1	0.01-5	MG	Ajmalicine	LC-MS/MS	(40)
Plasma	0.1 mL	LLE	0.2	1	1-5000	MG	Amitriptyline	LC-MS	(41)
Blood, Tissue, Urine	1 mL	SPE	30	50	50-1000	MG	$MG\text{-}D_3$	GC-MS	(16)
Plasma	0.1 mL	LLE	2	10	10-4000	7-MG-OH	Tryptoline	LC-MS	(42)

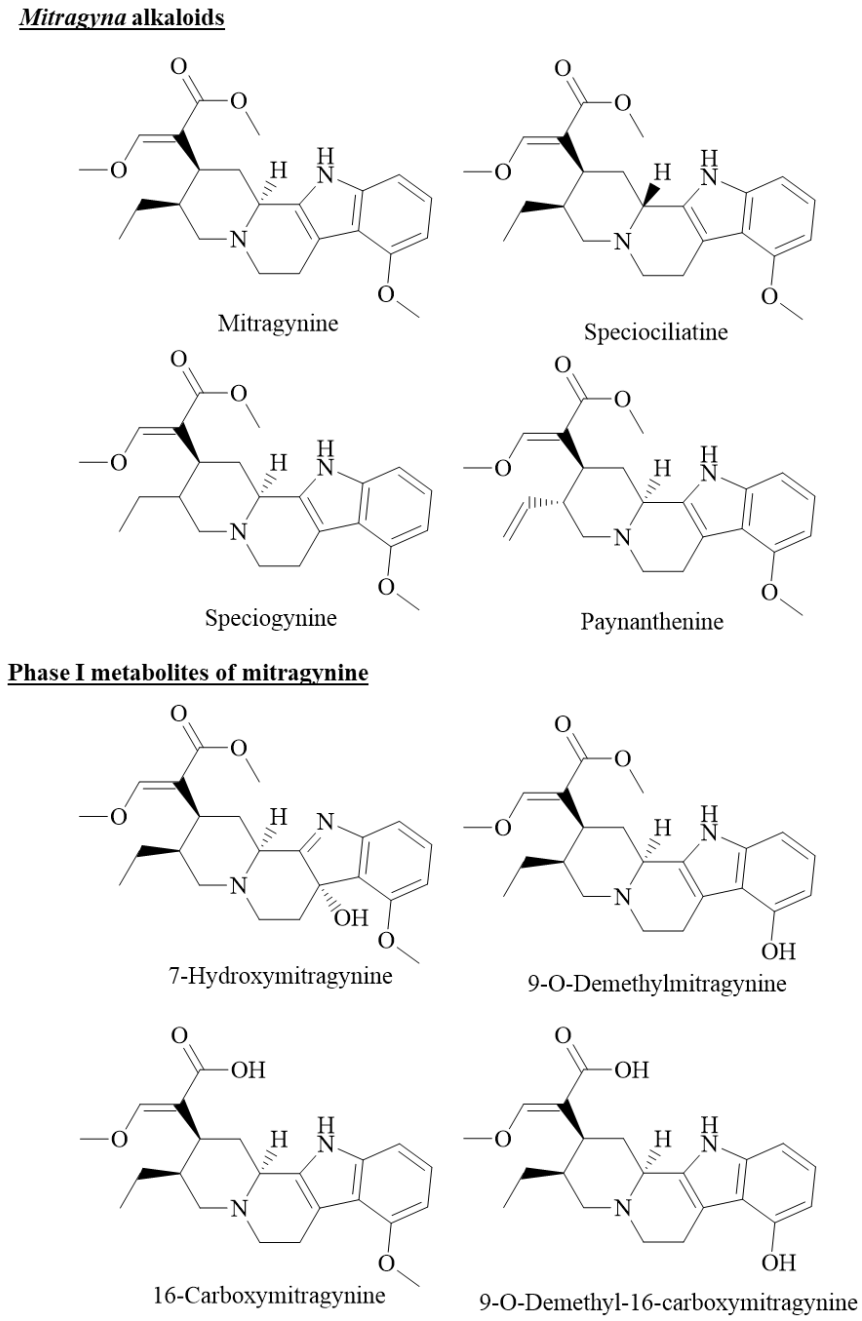
BAμE, bar adsorptive microextraction; GC-MS, gas chromatography-mass spectrometry; LC-DAD, liquid chromatography-diode array detector; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LC-Q/TOF-MS, liquid chromatography-quadrupole time-of-flight-mass spectrometry; LLE, liquid-liquid extraction; SPE, solid phase extraction.

The *Mitragyna* genus includes four species native to Africa and six species native to South and Southeast Asia (25). *Mitragyna speciosa* is distributed throughout the Malesian floristic region, including Thailand, Malay Peninsula, Sumatra, Borneo, the Philippines, and New Guinea. It is also cultivated in Southern Vietnam and Burma (Myanmar). More than forty kratom alkaloids have been reported in the literature and account for approximately 0.5 to 1.5% of the dried leaf material by weight, although there are variations based on location, age, and season (3). The two most commonly targeted *Mitragyna* alkaloids in published methods, mitragynine (MG) and 7-hydroxymitragynine (7-MG-OH), are reported to account for >60 and 2% of the overall alkaloid composition, respectively (1, 26, 27). Three minor *Mitragyna* alkaloids (speciociliatine, speciogynine, and paynantheine) have been reported to account for 9, 7, and 10% of the total alkaloid content, respectively (1, 26). However, US-grown *M. speciosa* has a different chemotype from Asian-African plants and is reported to be richer in oxindole alkaloids (25). In addition to these geographic and age-related variants, alkaloid content also varies within the plant. Speciogynine was identified as the principal alkaloid in leaves from young Thai plants, while speciociliatine was the major alkaloid in the fruits of Malaysian plants (25, 28). These findings suggest that additional biomarkers of kratom use should be investigated.

Chemical structures of these alkaloids are shown in **Figure 5.1**. Although studies detailing the metabolism of paynantheine (PY), speciogynine (SG), and speciociliatine (SC) have been reported (29-31), very few analytical methods to date have targeted these alkaloids or attempted to identify their prevalence following kratom use (32). One study by Philipp *et al*. detailed the qualitative analysis of mitragynine, speciociliatine,

speciogynine, and paynantheine metabolites in human urine following suspected kratom or Krypton (kratom and O-desmethyltramadol) use (33). Although speciociliatine, speciogynine, and paynantheine were not routinely identified, the limit of detection of the gas chromatograph-mass spectrometry (GC-MS) assay was very high (100 ng/mL). Following enzymatic deconjugation and chemical derivatization, metabolites of these species were identified. Speciociliatine and speciogynine are diastereoisomers of mitragynine. Although they can be separated chromatographically, there are considerable challenges associated with their detection. We previously reported the use of liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS) for the simultaneous identification of MG, 7-MG-OH, PY, SC and SG in urine (32). Arndt *et al*. identified paynantheine, speciociliatine, speciogynine and mitraciliatine in a living subject receiving treatment for opioid withdrawal (34). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for analysis and the limit of detection was 2 ng/mL. Notably, speciociliatine was reported to be the most prevalent alkaloid present. Although these other compounds might not be psychoactive (2, 3), they could be valuable biomarkers of kratom use, especially given recent research that suggests that they are more stable than mitragynine and 7-hydroxymitragynine (35).

Figure 5.1: Selected indole Alkaloids in *Mitragyna speciosa* associated with kratom use.



The metabolism of mitragynine was first described by Philipp *et al*. (36, 37). Glucuronidation and sulfation of hydroxylated and carboxylated phase I metabolites was reported. The primary locations for conjugation were the 16, 17, and 9 positions (**Figure 5.1**). The parent drug however does not undergo such reactions due to its functionality.

The hydrolysis of conjugated species is typically performed either chemically or enzymatically. Strong acids and bases (usually combined with high temperatures) and enzymatic reagents (β-glucuronidase and sulfatase) can be used for this purpose. Chemical hydrolysis is less costly, faster, and more efficient but can degrade unstable species (38). Enzymatic hydrolysis, which is performed using moderate conditions of temperature and pH is often preferred. However, the efficiency varies significantly based on the type of enzyme and the drug itself (39). In addition, longer incubation times are often necessary, bioreagents have shorter shelf-lives, and there are increased costs (40, 41); factors that are often undesirable for high throughput laboratories.

To date, two studies have addressed the necessity for deconjugation of phase II metabolites of mitragynine. The first study by Le *et al*, evaluated the efficiency of *E. coli* β-glucuronidase for the deconjugation of urine samples (n=12) from kratom users. Ultimately, they determined that deconjugation did not improve urine analysis and was not necessary for interpretation (42). The other study by Lee *et al*. compared the efficiency of *E. coli* β-glucuronidase and *H. pomatia* β-glucuronidase/sulfatase deconjugation using human urine (n=10). They reported that while they were able to successfully hydrolyze glucuronidated metabolites in order to improve analysis of the phase I metabolites, they were not able to hydrolyze the sulfated conjugates (43). Given increased vigilance regarding kratom use, and the widespread use of urine as a toxicological specimen, further investigation is warranted.

In this report we describe the efficiency of chemical and enzymatic deconjugation techniques and discus potential biomarkers of kratom use. Chemical hydrolysis using acidic and basic conditions, and eleven enzymatic reagents, including both traditional and

recombinant preparations (IMCSzyme™, BGTurbo™, BGS™, ASPC™, *E. coli* β-glucuronidase, *H. pomatia* β-glucuronidase, *P. vulgata* β-glucuronidase, *A. aerogenes* sulfatase, abalone entrails, *H. pomatia* sulfatase and *P. vulgata* sulfatase) are described. Using postmortem urine samples (n=16) from kratom users, the relative merits of hydrolysis are discussed within the overall context of drug detection.

Experimental

Chemicals and reagents

Mitragynine and mitragynine-D_3 were purchased in methanolic solution from Cayman Chemical Company (Ann Arbor, MI, USA). Reference standards for 7-hydroxymitragynine and 7-hydroxymitragynine-D_3 were purchased from Cerilliant Corp. (Round Rock, TX USA) in an ammoniated solution (1% concentrated ammonium hydroxide in methanol, v/v) and stored at -80^{o}C as per the manufacturer's recommendations. Paynantheine and speciociliatine reference standards were purchased from Chromadex (Irvine, CA, USA) in powdered form, and speciogynine (powder) was kindly provided by the National Center for Natural Products Research (NCNPR) at the University of Mississippi (University, MS, USA). All solvents and inorganic reagents were LC or ACS grade, unless stated otherwise. Concentrated ammonium hydroxide was purchased from Macron Fine Chemicals (Center Valley, MA, USA), and ethyl acetate, hexane, concentrated hydrochloric acid, and methanol were obtained from J.T. Baker (Center Valley, MA, USA). Acetonitrile and sodium hydroxide (solid) was obtained from Fisher Scientific (Fair Lawn, NJ, USA) and glacial acetic acid was obtained from Mallinckrodt Chemicals (St. Louis, MO, USA).

Ammonium acetate, potassium phosphate (monobasic and dibasic), ammonium acetate, β-glucuronidase from *Escherichia coli* (Type IX-A, lyophilized powder, 1,000,000-5,000,000 units/g), β-glucuronidase from (Type H-1, partially purified powder, ≥300,000 units/g), β-glucuronidase from *Patella vulgata* (Type L-II, lyophilized powder, 1,000,000-3,000,000 units/g), sulfatase from *Helix pomatia* (Type H-1, sulfatase ≥10,000 units/g), sulfatase from abalone entrails (Type VIII, lyophilized powder, 20-40 units/mg), sulfatase from *Aerobacter aerogenes* (Type VI, buffered aqueous glycerol solution, 2-5 units/mg protein, 10-20 units/mL) and sulfatase from *Patella vulgata* (Type IV, essentially salt-free, lyophilized powder, ≥10 units/mg solid) were obtained from Sigma-Aldrich (St. Louis, MO, USA). IMCSzyme™ (genetically modified β-glucuronidase, purified solution, >50,000 units/mL) and its proprietary Rapid Hydrolysis Buffer were obtained from Integrated Micro-Chromatography Systems, LLC (Irma, South Carolina, USA). BGTurbo™ (genetically enhanced β-glucuronidase solution, ~1 mg/mL, >90% purity, >200,000 units/mL), BGS™ (genetically enhanced β-glucuronidase and sulfatase solution, ~1 mg/mL (β-glucuronidase), ≥ 200,000 units/ml (β-glucuronidase), ~4.0 mg/ml (sulfatase), ≥ 37,000 units/ml (sulfatase)), ASPC™ (genetically enhanced sulfatase solution, ~1 mg/mL, ≥ 200,000 units/ml), and Proprietary Buffer 1 and 2 were obtained from Kura Biotec® (La Piedra Biotecnología, Puerto-Varas, Chile). Recombinant human cytochrome P450 (rCYP) isoenzymes expressed in *E. coli* (bactosomes) were obtained from Xenotech, LLC (Kansas City, Kansas) and reduced nicotinamide adenosine di-phosphate (NADPH) regenerating system solution A (40 Ug/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate), solution B (26 mM NADP+, 66 mM glucose-6-

phosphate and 66 mM magnesium chloride in aqueous solution) were obtained from Corning (Glendale, Arizona).

All other chemicals and reagents were obtained from VWR International (Radnor, Pennsylvania). Deionized water was purified in house using a Millipore Direct-Q®UV Water Purification system (Billerica, MA, USA). Pooled drug-free urine preserved with 1% sodium fluoride (w/v) was used for the preparation of calibrators and controls. Samples were extracted using PolyChrom ClinII 3 cc (35 mg) solid phase extraction (SPE) columns that were obtained from SPEware (Baldwin Park, CA, USA).

Postmortem specimens

Unhydrolyzed postmortem specimens (n=16) were analyzed to determine the presence of *Mitragyna* alkaloids (speciociliatine, speciogynine, paynantheine, mitragynine, and 7-hydroxymitragynine) and phase I metabolites in accordance with an IRB-approved study. Concentrations of MG, MH-OH, SC, SG and PY were quantitatively determined using a previously validated method (32). 7-Hydroxymitragynine, and the metabolites identified during a previous *in-vitro* study were investigated qualitatively (36). Internal standard solution containing mitragynine-D_3 and 7-hydroxymitragynine-D_3 was added to 1.0 mL of urine to achieve a final concentration of 100 ng/mL. Samples were then acidified with 2 mL of 0.1 M hydrochloric acid and transferred to PolyChrom Clin II SPE cartridges. Deionized water and 1 M acetic acid was then added to each SPE cartridge in 1 mL aliquots. The columns were then dried for five minutes at full vacuum, followed by additional washes of hexane, ethyl acetate, and methanol (1 mL). The compounds were then eluted with 1 mL of ethyl acetate/concentrated ammonium hydroxide (98:2) and evaporated to dryness under nitrogen at 50°C. The samples were then reconstituted in 25

µL of 1:1 mobile phase A/B prior to injection (1 µL) on the LC-Q/TOF-MS. The limit of quantitation (LOQ) for mitragynine, speciociliatine and speciogynine was 0.5 ng/mL. The LOQ for paynantheine and 7-hydroxymitragynine was 1 ng/mL, and the typical calibration range of the assay was 2-500 ng/mL (32). Due to the high concentrations of alkaloids in human urine, dilution integrity was also evaluated. *Mitragyna* alkaloids were quantitatively determined using 2, 10 and 100-fold dilutions of urine in 0.1 M HCl. Mitragynine-D_3 was used as the internal standards for MG, SC, SH and PY. 7-Hydroxymitragynine-D_3 was used as the internal standard for 7-OH-MG in accordance with **Table 5.1**.

In addition to the five targeted alkaloids (MG, MH-OH, SC, SG and PY), qualitative identification of metabolites in urine (1 mL) was also performed using untargeted acquisition and a modified mobile phase gradient (36). In the absence of commercially available reference standards, rCYPs (3A4, 2D6, 2C19, and 2C18) were used to prepare 9-O-demethylmitragynine, 16-carboxymitragynine, and 9-O-demethyl-16-carboxymitragynine *in-situ* for qualitative identification purposes. Retention times, MS/MS spectra and mass accuracy were used for identification purposes.

Chemical hydrolysis

Pooled postmortem urine (n=5) from kratom-positive fatalities was used to evaluate chemical hydrolysis. In order to evaluate multiple reaction conditions and accommodate duplicate analysis, the specimen volume was decreased to 100 µL. Pooled human urine (100 µL) was added to deionized water (400 µL) to achieve a final volume of 500 µL. An equal volume (500 µL) of concentrated hydrochloric acid (12M) or sodium hydroxide (1M) was added, and samples were incubated at 20, 60, and 80°C for 60 minutes (n=2). Upon

cooling, samples were extracted with the SPE protocol described above. Extracts were then reconstituted in 25 µL of 1:1 mobile phase A/B and 2 µL was injected onto the LC-Q/TOF-MS for analysis. A blank (n=2) consisting of pooled drug-free urine (100 µL) diluted with deionized water (900 µL) was extracted contemporaneously in each run.

Enzyme hydrolysis

The same pooled postmortem urine (n=5) was also used to evaluate enzymatic hydrolysis. Each hydrolysis reaction was performed in duplicate. Recombinant enzymes and traditional sulfatase and glucuronidase preparations were also evaluated. Enzymatic stock solutions (where applicable) and buffers were prepared in accordance with manufacturer's recommendations, when provided. Aqueous internal standard (2 µg/mL) was added to urine (100 µL) before sufficient deionized water and/or buffer was used to bring the total reaction volume to 1000 µL. The final concentration of IS in each sample was 100 ng/mL. Following hydrolysis, all samples were extracted as described above. Samples were then reconstituted in 25 µL of 1:1 mobile phase A/B and 2 µL was injected onto the LC-Q/TOF-MS for analysis. Hydrolysis conditions using traditional and recombinant enzymes are summarized in **Table 5.2**.

Table 5.3: Optimal and challenging conditions for enzymatic hydrolysis using pooled urine (n=5).

Enzyme	Total Units per mL	Buffer	Urine Volume (µL)	Incubation Temperature (°C)	Incubation Time (min)
IMCSzyme™	5,000	Proprietary	100	53-55	30
BGTurbo™	8,000	Proprietary			10
BGS™	20,000	Proprietary			30
ASPC™	20,000	Proprietary			30
E. coli	2,000	0.1 M phosphate (pH 6.8)	100	37	180
H. pomatia	2,000	0.1 M acetate (pH 5.0)		37	
P. vulgata	2,000	0.1 M acetate (pH 5.0)		55	
A. aerogenes	1	0.1 M acetate (pH 5.0)	100	37	180
Abalone Entrails	20			37	
H. pomatia	200			37	
P. vulgata	20			55	

LC-Q/TOF-MS analysis

An Agilent Technologies 6530 LC-Q/TOF-MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 1290 Infinity autosampler was used for analysis. Nitrogen was generated using a Genius 3040 nitrogen generator (Peak Scientific, Billerica, MA, USA). Separation was achieved using an Agilent Technologies Series 1200 LC system with a thermostatically controlled column compartment (35^{o}C), an Agilent Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 µm) and an Agilent Poroshell 120 EC-C18 guard column (2.1 × 5 mm, 2.7 µm). The mobile phases used for analysis consisted of 5 mM ammonium acetate solution in deionized water (A) and acetonitrile (B).

Mitragyna alkaloids (MG, 7-MG-OH, SC, SG, and PY) were identified using a previously published method that was validated in accordance with published recommendations (32, 44). Briefly, in addition to the LC-Q/TOF-MS configuration described above, a flow rate of 0.4 mL/min was maintained using the gradient elution profile as follows: 47% B (0–0.5 min), 47-90% B (0.5–10 min), followed by re-equilibration (2 min). The total acquisition time was 12 min and the target compounds eluted between 1.9 mins and 5 mins. The LC-Q/TOF-MS was equipped with an electrospray ionization source (positive mode) under the following conditions: nebulizer, 45 psi; sheath gas temperature, 400^{o}C; nitrogen sheath gas flow, 12 L/min; drying gas (N_2), 13 L/min; drying gas temperature, 350^{o}C; capillary voltage, 4000 V; nozzle voltage, 0 V; fragmentor, 150 V; and skimmer, 65 V. Data was acquired in targeted mode using a mass range of 100–1000 Da, with a MS scan rate of 8 spectra/s and a MS/MS Scan rate of 3 spectra/s. Agilent MassHunter software was used for acquisition, qualitative and

quantitative analysis. Precursor and product ions, collision energies, retention times and the internal standard for each alkaloid are summarized in **Table 5.3**.

Table 5.4: Product and precursor ions, collision energies (CE), retention time (RT), and internal standard (IS) selection for the targeted analysis of *Mitragyna* alkaloids and selected phase I metabolites, including 9-O-demethylmitragynine (9-ODM-MG) and 16-carboxy-mitragynine (16-COOH-MG). Quantitation ions are underlined.

Alkaloid	Precursor Ion (*m/z*)	Product Ions (*m/z*)	CE (V)	RT (min)	IS
MG	399.2278	<u>174.0913</u> 226.1436 238.1438	30	4.61	MG-D_3
SC	399.2278	<u>174.0913</u> 226.1436 238.1438	30	2.00	MG-D_3
SG	399.2278	<u>174.0913</u> 226.1436 238.1438	30	3.32	MG-D_3
7-MG-OH	415.2227	<u>190.0863</u> 238.1438 226.1436	27	2.54	MG-OH-D_3
PY	397.2122	<u>174.0913</u> 236.1281 224.1281	26	3.79	MG-D_3
9-O-DM-MG	385.2122	<u>160.0757</u> 226.1438 224.1281	25	4.6	MG-D_3
16-COOH-MG	385.2122	<u>174.0913</u> 212.1281 238.1438	25	3.00	MG-D_3
MG-D_3 (IS)	402.2467	<u>177.1102</u> 226.1436 238.1438	30	4.54	-
7-MG-OH-D_3 (IS)	418.2416	<u>193.1051</u> 226.1436 238.1438	28	2.49	-

For the identification of metabolites in case samples and following deconjugation, the same LC-Q/TOF-MS configuration, source conditions, flow rate and scan rates were utilized. However, the mobile phase gradient was modified as follows to accommodate

more polar species: 10% B (0–0.5 min), 10-90% B (0.5–10 min), followed by re-equilibration (2 min). The total acquisition time was 12 min and the metabolites eluted between 1.2 min and 8.0 min. Data was acquired in auto MS/MS (full scan) mode using a preferred list of suspected metabolites based upon a previously published study (36).

Results and discussion

Analysis of postmortem specimens

Mitragynine, 7-hydroxymitragynine, speciociliatine, speciogynine and paynantheine were identified in urine samples from kratom users. The time of use, quantity, and form of the drug (*e.g.* tea, capsules, extract, powder) were not known. The results are summarized in **Table 5.4**. Despite acceptable precision and bias during method validation, 7-hydroxymitragynine should be considered semi-quantitative due to higher than acceptable matrix effects being observed for this analyte (and the deuterated IS) during the validation (32). Appropriate dilutions of specimen were performed to achieve quantitative results within the calibration range (2-500 ng/mL). Using this procedure, dilution integrity was established up to100-fold in urine (36).

Table 5.4: Concentrations of mitragynine, speciociliatine, speciogynine, paynantheine and 7-hydroxymitragynine in unhydrolyzed urine (n=16).

Case Sample	MG (ng/mL)	SC (ng/mL)	SG (ng/mL)	PY (ng/mL)	7-MG-OH (ng/mL)
A	1,987	3,309	1,684	317	ND
B	26	161	59	2	ND
C	461	2,161	910	103	204
D	176	23	96	15	14
E	1,271	150	388	65	83
F	677	97	278	48	ND
G	642	1,293	517	88	295
H	402	283	338	32	63
I	540	1,138	850	59	192
J	938	1,396	1,499	144	ND
K	258	1,197	272	23	129
L	54	306	99	6	ND
M	254	916	411	49	119
N	249	1,103	332	34	60
O	980	1,516	1,159	88	123
P	520	1,637	877	72	ND

ND, Not detected;

Concentrations of *Mitragyna* alkaloids in urine ranged from 2 ng/mL to >3,000 ng/mL, highlighting the wide range of forensic interest. Interestingly, the concentration of speciociliatine (23-3,309 ng/mL) and speciogynine (59-1,684 ng/mL) exceeded that of mitragynine (26-1,987 ng/mL) in 75% and 63% of cases, respectively. This finding could

be attributed to the increased stability of these isomers compared to mitragynine (35) or the use of kratom products with significant SC or SG alkaloid content as described earlier. Nevertheless, it suggests that both SC and SG could serve as useful biomarkers for kratom use. In contrast, paynantheine (2-317 ng/mL) was always present at a lower concentration than mitragynine, although it was detectable in all sixteen cases. Although literature regarding these other alkaloids is limited, our findings our consistent with those of Arndt *et al.* who reported that speciociliatine was the most prominent alkaloid in an single antemortem urine specimen obtained from an individual undergoing treatment for opioid withdrawal (34). Finally, 7-hydroxymitragynine (60-295 ng/mL) was detected in only 9 of the 16 cases (56%), albeit at much lower concentrations than mitragynine. This might be explained by its decreased stability relative to mitragynine or extensive phase II conjugation. Mean, median and concentration ranges for all five targeted alkaloids are summarized in **Table 5.5** and **Figure 5.2** depicts extracted ion chromatograms for a representative extract (Sample G). Appropriate dilutions were performed to achieve quantitative values within the calibration range.

Figure 5.2: Extracted ion chromatograms for a representative urine extract (Case Sample G) containing speciociliatine (1,293 ng/mL), speciogynine (517 ng/mL), paynantheine (88 ng/mL), mitragynine (642 ng/mL) and 7-hydroxymitragynine (295 ng/mL). Appropriate dilutions were performed to achieve quantitative results within the calibration range (2–500 ng/mL).

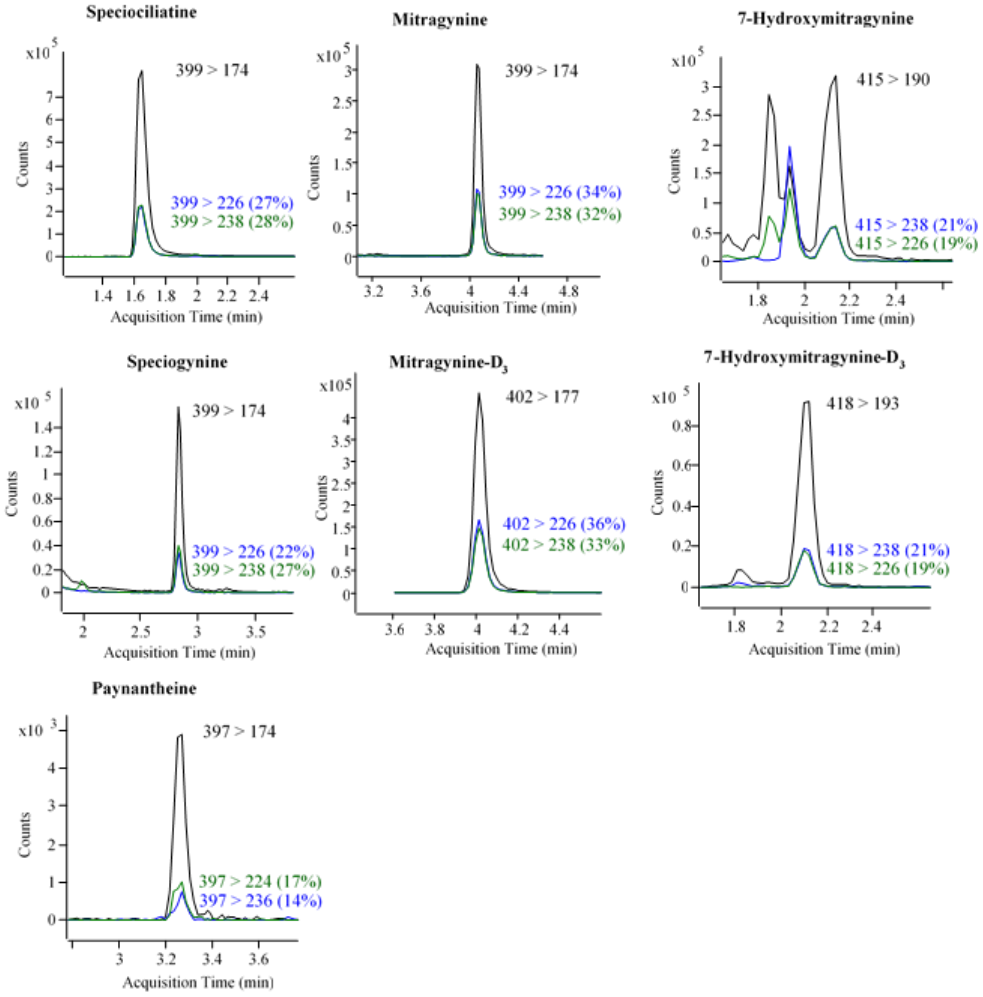


Table 5.5: *Mitragyna* alkaloids and phase I metabolites of mitragynine in urine case specimens (n=16).

Compound	Presence in Case Specimens (%)	Mean Concentration (ng/mL)	Median Concentration (ng/mL)	Concentration Range (ng/mL)
MG	100	590 (n=16)	491	26-1,987
7-MG-OH	63	128 (n=10)	121	14-295
SC	100	1,043 (n=16)	1,121	23-3,309
SG	100	611 (n=16)	400	59-1,684
PY	100	72 (n=16)	54	2-317

Metabolites of mitragynine were identified in 14 (88%) of the 16 samples tested. The most prevalent metabolites detected in unhydrolyzed samples were 9-O-demethylmitragynine (n=12, 75%) and 7-hydroxymitragynine (n=10, 63%). 16-Carboxymitragyine was only identified in two urine samples. Due to the absence of reference materials, the presence of these metabolites was confirmed using rCYP-generated controls analyzed contemporaneously within the run. The abundance of these metabolites in hydrolyzed urine is discussed later. Extracted ion chromatograms for mitragynine, 7-hdroxymitragynine, 9-O-demethylmitragynine and 16-carboxymitragynine in a representative extract (Case K) are shown in **Figure 5.3**. Associated MS/MS spectra for these metabolites are found in **Figure 5.4**.

Figure 5.3: Extracted ion chromatograms for mitragynine, *m/z* 399 (7.2 min), 7-hydroxymitragynine, *m/z* 415 (5.4 min), 9-O-demethylmitragynine, *m/z* 385 (4.5 min) and 16-carboxymitragynine *m/z* 385 (3.0 min) from unhydrolyzed urine (Case Sample K).

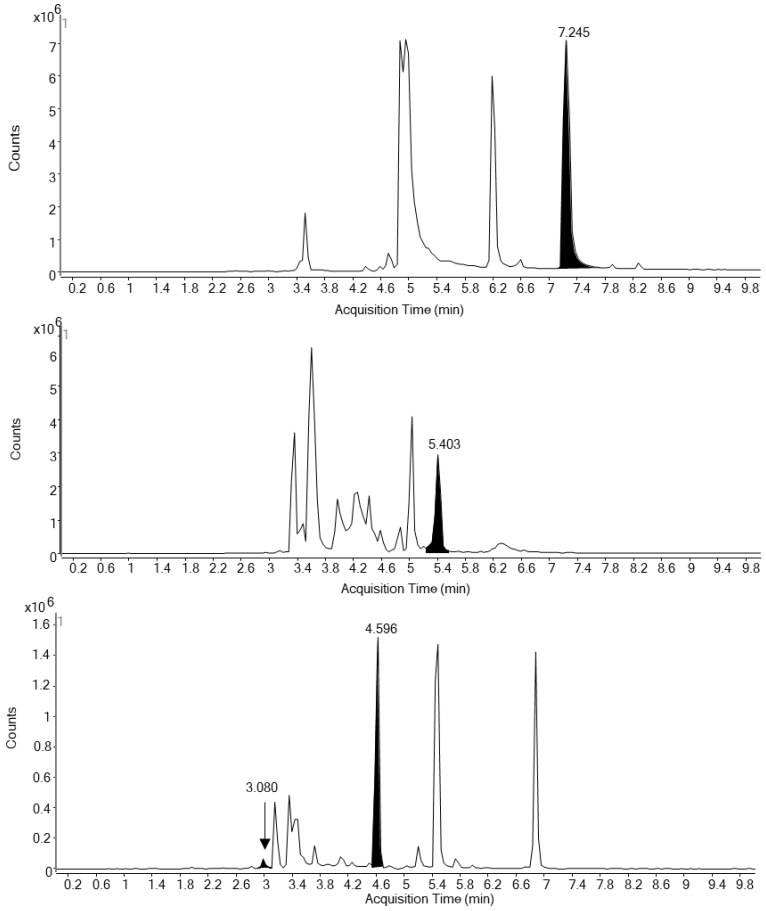
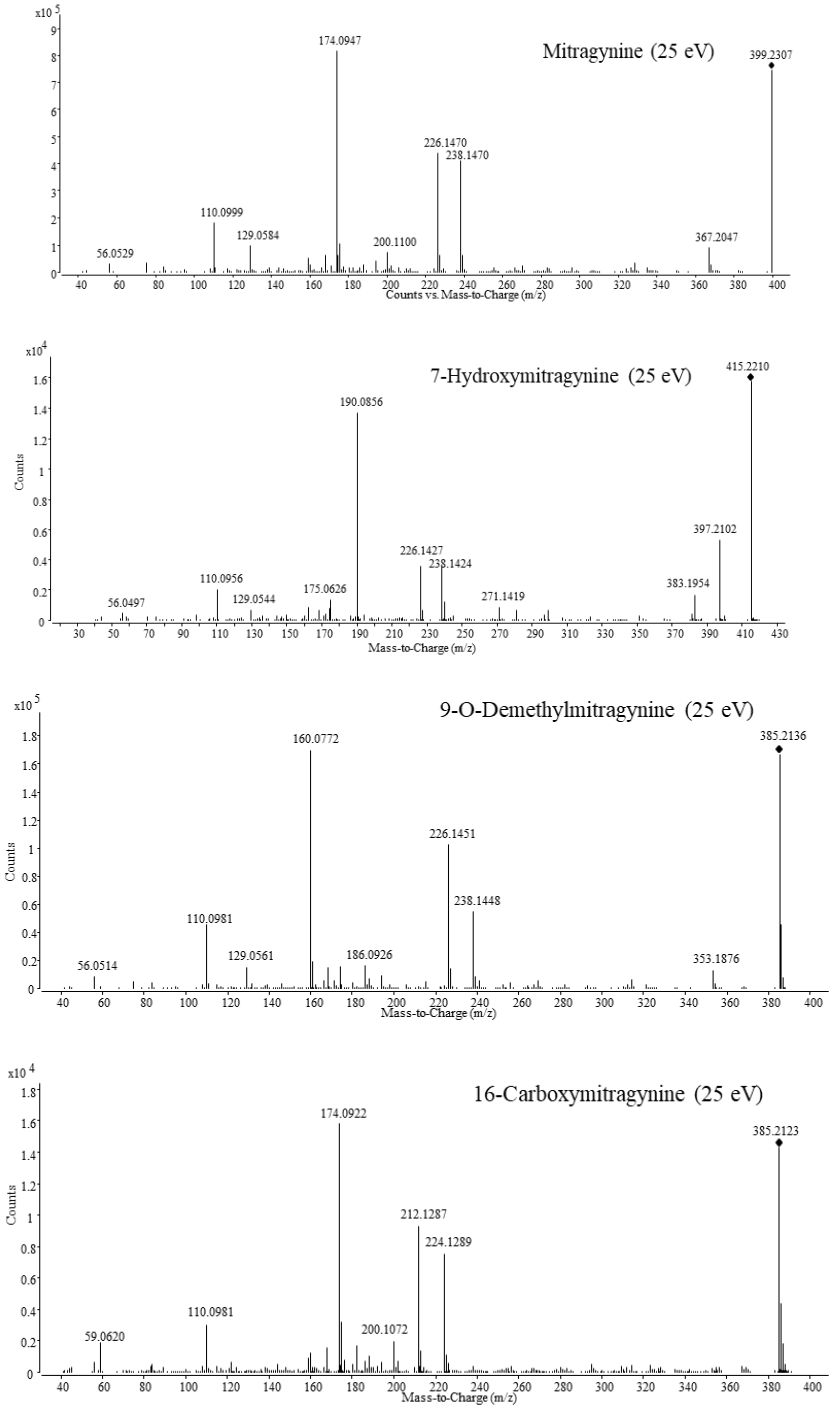


Figure 5.4: MS/MS spectra of mitragynine, 7-hydroxymitragynine, 9-O-demethylmitragynine and 16-carboxymitragynine (Case Sample K).



Deconjugation efficiency

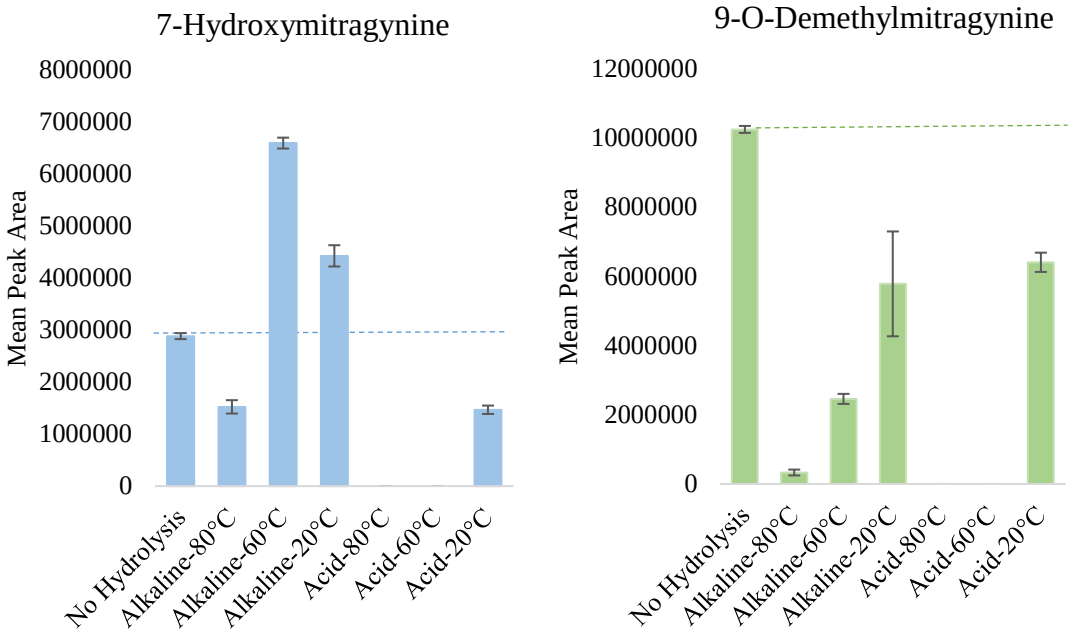
The hydrolysis of conjugated species can be accomplished by non-specific chemical cleavage, or enzymatic hydrolysis (39). β-Glucurionidases sourced from *E. coli*, *H. pomatia*, and *P. vulgata* are commonly used for this purpose in forensic toxicology. Sulfatases, although less widely used, were also investigated here. More recently, recombinant sulfatase and glucuronidase reagents have become available, which are capable of hydrolyzing phase II metabolites within minutes, rather than hours (45). Since hydrolysis methods for conjugated mitragynine metabolites have not been fully explored, three traditional β-glucuronidase systems (*E. coli*, *H. pomatia*, and *P. vulgata*), four traditional sulfatases (*A. aerogenes*, abalone entrails, *H. pomatia*, and *P. vulgata*) and four recombinant enzymes (IMCSzyme™, BGTurbo™, BGS™, and ASPC™) were evaluated. Deconjugation efficiency was evaluated by comparing the mean peak area of hydrolyzed (n=2) and unhydrolyzed (n=2) species.

Chemical hydrolysis

Acid and alkaline hydrolysis was unsuitable for the deconjugation of phase II metabolites of mitragynine. Changes in metabolite abundance (relative to no hydrolysis) for 7-hydroxymitragynine and 9-demethylmitragynine are shown in **Figure 5.5**. Under most conditions, the abundance of metabolite actually decreased. This indicated that chemical hydrolysis was not only ineffective, but it also degraded free (unconjugated) metabolite in the urine. This is consistent with previously published literature that suggests that mitragynine is acid labile, and to a lesser extent alkaline-labile under some conditions (35, 46, 47). Alkaline hydrolysis at 60°C and 20°C were the only conditions that increased the abundance of 7-OH-MG (229% and 154%, respectively). However, under these same

conditions 9-O-demethylmitragynine decreased significantly (degraded). **Figure 5.5** also highlights the acid lability of both metabolites. Interestingly, no 16-carboxymitragynine was identified in the pooled urine (n=5), even after hydrolysis. This is consistent with our previous results which suggested that this was a relatively minor metabolite (36). Although chemical hydrolysis is clearly unsuitable for *Mitragyna* alkaloids, it highlights important considerations regarding the stability of metabolites during sample preparation and isolation from biological matrices in general.

Figure 5.5: Chemical hydrolysis of 7-hydroxymitragynine and 9-O-demethylmitragynine in pooled urine (n=5).

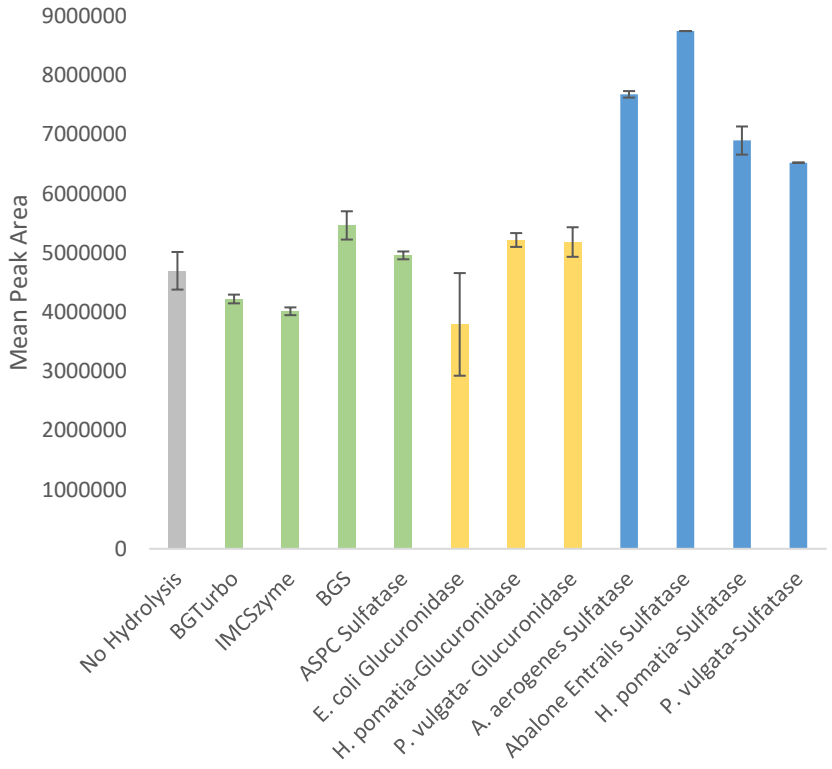


Enzymatic hydrolysis

7-Hydroxymitragynine and 9-O-demethylmitragynine were the major metabolites identified in either non-hydrolyzed or hydrolyzed pooled urine (n=5). **Figure 5.6** depicts changes in abundance for 7-hydroxymitragynine following enzymatic treatment. Significant increases in abundance were observed for all four sulfatases, but not the

glucuronidases. A statistically significant but only nominal increase was observed for BGS™, one of the recombinant enzymes that contained both sulfatase and glucuronidase. However, increases in abundance following hydrolysis were nominal at best. No significant increases were observed for 9-O-demethylmitragynine. Small decreases in abundance were observed, again suggesting that this metabolite actually decreased in concentration during the sample preparation step. Since no 16-carboxymitragynine was observed in the pooled urine (n=5), it was necessary to investigate the individual specimens. For this evaluation a larger volume of urine (500 µL) was utilized. **Figure 5.7** depicts the increase in 16-carboxymitragynine concentration following hydrolysis with a traditional glucuronidase (*H. pomatia*) and dual (glucuronidase/sulfatase) recombinant enzyme (BGS™) as described in **Table 5.2**. 16-Carboxymitragynine was identified in 12 additional specimens following hydrolysis. Ten of the twelve specimens had no detectable free 16-carboxymitragynine, suggesting that this metabolite was extensively conjugated. Both *H. pomatia* and the recombinant glucuronidase/sulfatase were effective for the deconjugation of 16-carboxymitragynine. **Figure 5.8** depicts a side-by-side comparison of both recombinant enzymes (BGS™ and BGTurbo™) for Case Sample P (n=2). Significant increases in abundance were observed using both recombinant enzymes. However, differences between BGS™ and BGTurbo™ were not significant (α=0.05), suggesting that no sulfates were present and the metabolite is principally glucuronidated. More importantly however, increases in 7-hydroxymitragynine and 9-O-demethylmitragynine were not observed, suggesting that deconjugation was not beneficial for these metabolites.

Figure 5.6: Enzymatic hydrolysis of 7-hydroxymitragynine in pooled urine (n=5).



Although *E. coli* and *H. pomatia* systems have been utilized in previously published methods, the efficiency of the hydrolysis was not reported (42, 43). Philipp identified both sulfate and glucuronide conjugates of 9-O-demethylmitragynine using linear ion trap mass spectrometry (37). 16-Carboxymitragynine-glucuronide was identified, but no associated sulfate metabolite. This is consistent with our results, which suggest that use of sulfatase did not increase the abundance of this metabolite. Other phase II metabolites were identified at low abundance, but notably no 7-hydroxymitragynine was reported. Although enzymatic deconjugation has been employed for the determination of mitragynine metabolites in urine (33, 37), our results show that it has nominal benefit in terms of overall drug detection.

Figure 5.7: Enzymatic hydrolysis of 16-carboxymitragynine in human urine (n=16) using *H. pomatia* and BGS™.

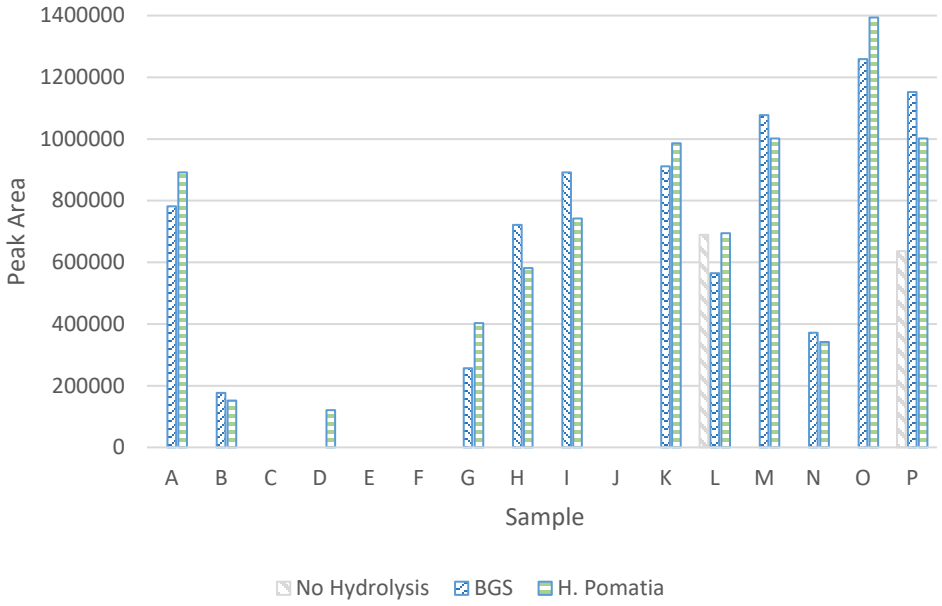
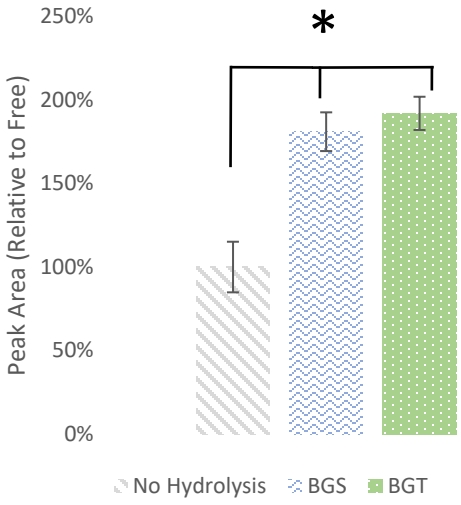


Figure 5.8: Enzymatic hydrolysis of 16-carboxymitragynine (Case Sample P) using BGS™ and BGTurbo™ (n=2).



Conclusions

Using a series of postmortem urine specimens from known drug users, speciociliatine and speciogynine were identified as important biomarkers of kratom in urine. Although mitragynine is reported to be the principal alkaloid in many reports, both

speciociliatine and speciogynine are prominent alkaloids in young plants and fruits from some geographical regions. Parent drug was identified in all samples tested, ranging in concentration from approximately 20 to almost 2,000 ng/mL. 9-O-demethylmitragynine and 7-hydroxymitragynine were the most prevalent metabolites, detected in 75% and 63% of urine samples without hydrolysis. 16-Carboxymitragynine was infrequently detected in untreated urine, but hydrolysis increased its prevalence from 13% to 75%. Due to significant quantities of free mitragynine, its diastereoisomers (speciociliatine and speciogynine), and phase I metabolites (9-O-demethylmitragynine and 7-hydroxymitragynine) in untreated urine, enzymatic hydrolysis was not necessary.

Acknowledgements

We gratefully acknowledge the North Carolina Office of the Medical Examiner (NCOME) for providing the authenticated urine samples. We gratefully acknowledge Dr. Ikhlas Khan and the National Center for Natural Products Research School of Pharmacy at The University of Mississippi for providing speciogynine.

This project was supported by Award No. 2016-DN-BX-0006, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of the Department of Justice.

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CHAPTER VI

Identification of Five *Mitragyna* Alkaloids in Blood and Tissues using Liquid Chromatography-Quadrupole/Time of Flight Mass Spectrometry[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1]Basiliere, S., Brower, J., Winecker, R., Kerrigan S. (2019). Forensic Toxicology, [In Review].

Abstract

Purpose: Kratom is a botanical drug with psychoactive properties that is increasingly being used recreationally and "therapeutically" in a non-medically supervised setting. Analytical methods for the detection of kratom use in biological matrices are limited in scope. Prevalence of these alkaloids and their metabolites in forensic specimens is not well understood. The purpose of this study develop and validate a procedure to identify five *Mitragyna* alkaloids in blood and tissues using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q/TOF-MS).

Methods: Mitragynine (MG), speciociliatine (SC), paynantheine (PY), speciogynine (SG) and 7-hydroxymitragynine (7-MG-OH) were identified in postmortem blood (n=40) and liver specimens (n=20). *Mitragyna* alkaloids were determined quantitatively using targeted acquisition and metabolites were identified qualitatively using full scan (untargeted) acquisition.

Results and conclusions: The analytical procedure was validated in accordance with published recommendations. Limits of quantitation were 0.5-2 ng/ml for the five targeted alkaloids. Precision, bias, and matrix effects were all within acceptable thresholds. Concentrations of mitragynine in central and peripheral blood were 1-422 ng/mL and 1-412 ng/mL. 7-Hydroxymitragynine was confirmed in blood in at least 95% of the cases. Liver concentrations ranged from <4 to 1,454 ng/g. In addition to 7-hydroxymitragynine, 9-O-demethylmitragynine and 16-carboxymitragynine were also identified in postmortem blood. Notably however, speciociliatine was frequently identified at concentration in excess of mitragynine, therefore serving as an alternative biomarker of kratom use.

Keywords: Postmortem toxicology, Kratom, Mitragynine, 7-Hydroxymitragynine, Blood, Liver

Identification of Five *Mitragyna* Alkaloids in Blood and Tissues using Liquid Chromatography-Quadrupole/Time of Flight Mass Spectrometry

Introduction

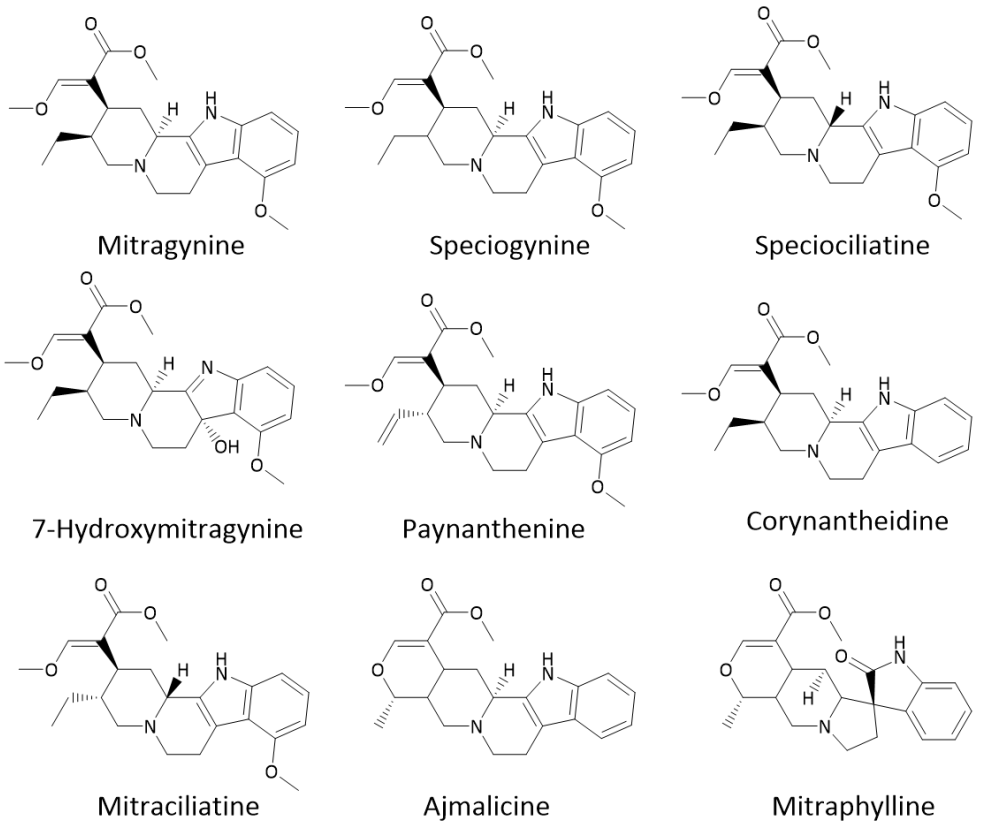
Kratom (*Mitragyna* speciosa) is a psychoactive botanical substance that presents a variety of analytical challenges (1). *Mitragyna* alkaloids can produce dose-dependent effects. Stimulant-like effects predominate at low dose, while higher dosages produce opiate-like effects (2). In the United States, the drug is used recreationally and for non-medically approved purposes as an "atypical opioid" (3). Mitragynine, one of the principal alkaloids in the plant, mediates its effects via mu opioid receptors, in addition to serotonergic and adrenergic receptors (4, 5). Kratom is often labeled by users as a "safe" alternative to opioids and as an effective way to treat afflictions such as chronic pain, opioid addiction, depression, diarrhea, insomnia, diabetes and impotence (2, 6-9). However, the drug is not currently approved for medical use in the United States. Multiple reports describing the unintended effects associated with the drug, including fatalities, have raised concerns regarding its unregulated use (2, 10-26).

Kratom is legal throughout most of the United States and is not scheduled federally (27). The drug is readily available via the Internet and in retail outlets (28). Kratom's pharmacodynamic profile, lack of regulation, and wide availability have led to an increase in its use. The National Forensic Laboratory Information System (NFLIS) reports an increase in mitragynine seizures since 2010 (29-31) and the Drug Enforcement Agency (DEA) has labeled kratom as a "drug of concern" (32). Recently, the Center for Disease Control and Protection (CDC) released a report detailing 152 deaths involving kratom from 27 states that occurred between July 2016 and December 2017 (27). This report has further

raised concerns regarding the increased use of a drug that is considered by many to be "natural" or "safe".

The 2019 CDC report also suggested that kratom use may be underreported due to difficulties with analytical detection. More than forty compounds have been isolated from the leaves of *Mitragyna speciosa* (33). Chemical structures for selected alkaloids are shown in **Figure 6.1**. The major psychoactive constituent is reported to be mitragynine which, according to one study can account for 60-66% of the total alkaloid content in plant material (34). However, the alkaloid content varies considerably based on age, species, and environmental factors (35). For example, Takayama *et al*. reported that mitragynine content in a Thai specimen was 66% higher than a Malaysian specimen (36). Interestingly, some specimens grown in other locations have been reported to contain different alkaloid contents, including specimens where the predominant alkaloid was not mitragynine (35).

Figure 6.1: Indole alkaloids in *Mitragyna speciosa*.



A limited number of analytical methods for blood and plasma have been published in the literature, but they have almost exclusively focused on the detection of mitragynine (**Table 6.1**). Analysis is challenging due to the large number of related compounds, including structural isomers and diastereoisomers of mitragynine, some of which share identical molecular weights and in some cases mass spectral fragmentation (1). In this study, five *Mitragyna* alkaloids were targeted in whole blood using liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS). Target compounds include the two mu mu agonists mitragynine (MG) and 7-hydroxymitragynine (7-MG-OH), two diastereoisomers of MG (speciociliatine (SC) and speciogynine (SG)), and paynantheine (PY).

Although there are several case reports detailing fatalities associated with kratom use (20, 21, 27, 37), there is limited information regarding its overall prevalence in forensic casework. Philipp *et al*. described the alkaloid content in the urine of 120 suspected kratom users, but attempts to identify paynantheine, speciociliatine and speciogynine consistently in specimens were hampered by detection limits of 100 ng/mL (38). Another study by Arndt *et al*. described the analysis of mitragynine, speciociliatine, speciogynine, and paynantheine in an antemortem urine sample using liquid chromatography tandem mass spectrometry (LC-MS/MS) (39). While this was only a qualitative method and had a small sample size (n=1), they reported that speciociliatine was the most prevalent alkaloid. In a series of sixteen postmortem cases MG, 7-MG-OH, SC, SG and PY were identified in urine using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q/TOF-MS) (40). Speciociliatine and speciogynine were present in all of the mitragynine-positive case samples, frequently at concentrations that exceeded MG, consistent with Arndt *et al.* (39). 7-Hydroxymitragnyine and 9-O-demethylmitragynine were also reported in the majority of cases without hydrolysis.

Table 6.5: Overview of published analytical methods for *Mitragyna* alkaloids in blood and plasma.

Matrix	Sample Volume	Extraction Method	LOD (ng/mL)	LOQ (ng/mL)	Calibration (ng/mL)	Compounds Detected	Internal Standard	Analytical Technique	Reference
Blood	1 mL	LLE	0.25	1	1-10	MG	Proadifen	LC-MS/MS	(26)
Plasma	0.1 mL	LLE	0.2	1	1-5000	MG	Amitriptyline	LC-MS	(41)
Blood	1 mL	SPE	30	50	50-1000	MG	MG-D_3	GC-MS	(20)
Plasma	0.1 mL	LLE	2	10	10-4000	7-MG-OH	Tryptoline	LC-MS	(42)
Blood	0.5 mL	LLE	0.16	-	5-500	MG	MG-D_3	LC-MS/MS	(1)
Plasma	0.5 mL	LLE	0.2	0.2	0.2-1000	MG	Amitriptyline	LC-MS/MS	(43)
Plasma	0.1 mL	SPE	25	50	50-10,000	MG	Mefloquine	LC-UV-DAD	(44)

GC-MS, gas chromatography-mass spectrometry; LC-UV-DAD, liquid chromatography-UV-VIS -diode array detector; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLE, liquid-liquid extraction; SPE, solid phase extraction.

In this study, we describe a validated analytical method using LC-Q/TOF-MS that was used to identify five *Mitragyna* alkaloids (MG, 7-MG-OH, SC, SG, and PY) in blood and tissues. We also describe the prevalence of these five alkaloids and the phase I metabolites of mitragynine in 60 postmortem specimens.

Materials and methods

Chemicals and reagents

All solvents and inorganic reagents were LC or ACS grade, unless stated otherwise. Mitragynine and mitragynine-D_3 were purchased in methanolic solutions from Cayman Chemical Company (Ann Arbor, MI, USA). Reference standards for 7-hydroxymitragynine and 7-hydroxymitragynine-D_3 were purchased from Cerilliant Corp. in ammoniated methanol (1% concentrated ammonium hydroxide in methanol) (Round Rock, TX USA). Drugs used in the interference study were also purchased from Cerilliant Corp. Paynantheine (solid) and speciociliatine (solid) reference standards were purchased from Chromadex (Irvine, CA, USA) and Speciogynine (solid) was provided by the National Center for Natural Products Research (NCNPR) at the University of Mississippi, (University, MS, USA). Paynantheine, speciogynine, and speciociliatine were reconstituted and stored as methanolic solutions. Ethyl acetate, hexane, concentrated hydrochloric acid, and methanol were obtained from J.T. Baker (Center Valley, MA, USA). Concentrated ammonium hydroxide was purchased from Macron Fine Chemicals (Center Valley, MA, USA) and glacial acetic acid was obtained from Mallinckrodt Chemicals (St. Louis, MO, USA). Acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate (solid) and potassium phosphate (monobasic and dibasic) (solid) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant

human cytochrome P450 (rCYP) isoenzymes expressed in *E. coli* (bactosomes) were obtained from Xenotech, LLC (Kansas City, Kansas) and reduced nicotinamide adenosine diphosphate (NADPH) regenerating system solution A (40 Ug/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate), solution B (26 mM NADP+, 66 mM glucose-6-phosphate and 66 mM magnesium chloride in aqueous solution) were obtained from Corning (Glendale, Arizona). All other miscellaneous chemicals and reagents were obtained from VWR International (Radnor, Pennsylvania).

Deionized water was purified in house using a Millipore Direct-Q®UV Water Purification system (Billerica, MA, USA). Samples were extracted using PolyChrom ClinII 3 cc (35 mg) solid phase extraction (SPE) columns that were obtained from SPEware (Baldwin Park, CA, USA). Bovine blood containing 1% sodium fluoride and 0.2% potassium oxalate was purchased from Quad Five (Ryegate, MT, USA). Drug-free human liver was provided by the Applied Anatomical Research Center at Sam Houston State University. Postmortem blood (n=40) and liver (n=20) samples were received from the North Carolina Office of the Chief Medical Examiner in accordance with an Institutional Review Board (IRB)-approved study.

Instrumentation

An Agilent Technologies 6530 LC-Q/TOF-MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 1290 Infinity autosampler was used to analyze processed samples. Nitrogen was generated using a Genius 3040 nitrogen generator (Peak Scientific, Billerica, MA, USA). Separation was achieved using an Agilent Technologies Series 1200 LC system, an Agilent Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 µm) and an Agilent Poroshell 120 EC-C18 guard column (2.1 × 5 mm, 2.7 µm). The column

was stored in a thermostatically controlled column compartment (35ºC) and the autosampler was maintained at 4ºC. The mobile phase systems consisted of 5 mM ammonium acetate solution in deionized water (A) and acetonitrile (B).

LC-Q/TOF-MS analysis was performed using a flow rate of 0.4 mL/min with a gradient elution profile was as follows: 47% B (0–0.5 min), to 90% B (0.5–10 min). The LC-Q/TOF-MS was equipped with an electrospray ionization (ESI) source operated in the positive mode under the following conditions: drying gas (N_2), 13 L/min; drying gas temperature, 350ºC; nebulizer, 45 psi; sheath gas temperature, 400ºC; nitrogen sheath gas flow, 12 L/min; capillary voltage, 4000 V; nozzle voltage, 0 V; fragmentor, 150 V; skimmer, 65 V. Agilent MassHunter software was used for qualitative and quantitative analysis. Precursor ions were selected using a 1 Da window and a minimum of two transition ions for each alkaloid were selected for targeted MS/MS acquisition. Data was acquired using a mass range of 100–1000 Da, with a MS scan rate of 8 spectra/s and a MS/MS scan rate of 3 spectra/s.

For the identification of metabolites in case samples, the same LC-Q/TOF-MS configuration, source conditions, flow rate and scan rates were used as described above. The mobile phase elution gradient was modified as follows: 10% B (0–0.5 min), 10-90% B (0.5–10 min). Data was acquired in auto MS/MS (full scan) mode using a preferred list of identified metabolites based upon a previously published study (45). Agilent MassHunter software was used for qualitative analysis. Retention times, MS/MS spectra and mass accuracy were used for identification of these metabolites. Precursor and product ions, collision energies, acquisition mode, retention times and the internal standard for each drug and metabolite are summarized in **Table 6.2**.

Table 6.6: Molecular formula**,** product and precursor ions, collision energies (CE), retention time (RT), acquisition mode, and internal standard (IS) selection for *Mitragyna* alkaloids of interest. Quantitation ions are italicized.

Alkaloid	Molecular Formula [M+H$^+$]	Precursor Ion (*m/z*)	Product Ions (*m/z*)	CE (V)	RT (min)	Acquisition Mode	IS
MG	$C_{23}H_{31}N_2O_4$	399.2278	*174.0913* 226.1436 238.1438	30	4.6	Targeted	MG-D_3
7-MG-OH	$C_{23}H_{31}N_2O_5$	399.2278	*190.0863* 238.1438 226.1436	27	2.5	Targeted	7-MG-OH-D_3
SC	$C_{23}H_{31}N_2O_4$	399.2278	*174.0913* 226.1436 238.1438	30	2.0	Targeted	MG-D_3
SG	$C_{23}H_{31}N_2O_4$	415.2227	*174.0913* 226.1436 238.1438	30	3.3	Targeted	MG-D_3
PY	$C_{23}H_{29}N_2O_4$	397.2122	*174.0913* 236.1281 224.1281	26	3.8	Targeted	MG-D_3
9-O-DM-MG	$C_{22}H_{29}N_2O_4$	385.2122	*160.0757* 226.1438 238.1438	25	4.6	Full Scan	MG-D_3
16-COOH-MG	$C_{22}H_{29}N_2O_4$	385.2122	*174.0913* 212.1281 224.1281	28	3.0	Full Scan	MG-D_3
9-O-DM-16-COOH-MG	$C_{21}H_{27}N_2O_4$	371.1965	*160.0757* 212.1281 224.1281	25	1.3	Full Scan	MG-D_3

16-COOH-MG, 16-carboxymitragynine; 9-O-DM-16-COOH-MG, 9-O-demethyl-16-carboxymitragynine; 9-O-DM-MG, 9-O-demethylmitragynine; MG, mitragynine; MG-D_3, mitragynine-D_3; 7-MG-OH, 7-hydroxymitragynine; 7-MG-OH-D_3, 7-hydroxymitragynine-D_3; PY, paynantheine; SC, speciociliatine; SG; speciogynine.

Preparation of standards and reagents

Working standards containing SC, SG, PY, MG-D_3, and MG were prepared in methanol whereas standards containing 7-MG-OH and 7-MG-OH-D_3 were prepared in ammoniated methanol (described earlier). Working standards for the five target

compounds were prepared at 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 µg/mL for calibration and validation purposes. Internal standard working solutions were prepared at 2 µg/mL. In the absence of commercially available reference standards, rCYPs (3A4, 2D6, 2C19, and 2C18) were used to prepare 9-O-demethylmitragynine, 16-carboxymitragynine, and 9-O-demethyl-16-carboxymitragynine *in-situ* for qualitative identification purposes as described previously (40). The SPE elution solvent which was prepared before each extraction, consisted of concentrated ammonium hydroxide in ethyl acetate (2%, v/v).

Blood extraction

Bovine blood (1.0 mL) was fortified with the targeted compounds at various concentrations using the working standard solutions. Internal standard solution (50 µL) was added to achieve a final concentration of 100 ng/mL for MG-D_3 and 7-MG-OH-D_3. All targeted analytes and internal standards were added to the blood while continuously vortexing. Proteins were precipitated with 2 mL of cold acetonitrile while vortex mixing. Samples were then centrifuged at 4000 RPM for 10 minutes, and the supernatant was decanted into new disposable culture tubes. Blood was then acidified with 2 mL of 0.1 M hydrochloric acid and briefly vortexed before being transferred to SPE columns and allowed to flow through under gravity or sufficient vacuum in order to maintain a constant flow of approximately 1 mL/min. Columns were rinsed with 1 mL deionized water followed by 1 mL of 1 M acetic acid before being allowed to dry at full vacuum for five minutes. After drying, samples were washed with 1 mL aliquots of hexane, ethyl acetate and methanol. All compounds were eluted using 2 mL of elution solvent and then were evaporated to dryness under nitrogen at 50ºC. Extracts were reconstituted in 25 µL of a

50:50 mixture of Mobile Phase A/B, briefly vortexed, and then centrifuged at 2500 rpm for 10 mins before ultimately being injected (1 µL) onto the LC-Q/TOF-MS for analysis.

Assay validation

Assay performance was evaluated in terms of extraction efficiency, calibration model, precision, bias, limit of detection (LOD), limit of quantification (LOQ), ion suppression, interference, dilution integrity, processed sample stability, and carryover in accordance with published recommendations (46). Extraction efficiency in blood was determined at 250 ng/mL by direct comparison of extracted and non-extracted samples. Samples containing internal standard were extracted in the presence and absence of the target compounds. Samples extracted without target compounds were fortified post-extraction (prior to evaporation and reconstitution) with 250 ng analyte. Analytical recovery was calculated by comparing the relative peak area (drug/IS) for extracted samples (n=4) with the mean relative peak area for the non-extracted samples (n=4).

Limits of detection and quantitation were established using drug-free bovine blood fortified with target analytes. Three independent sources of blood were analyzed in duplicate over three independent runs. The LOD was the lowest concentration of drug that produced a reportable result (signal to noise (S/N) ratio of 3:1 or more; retention time ± 2% of the standard; ion ratios ± 20%). The LOQ was determined contemporaneously and was defined as the lowest concentration of drug to produce a quantitative value within 20% of the expected value, a S/N ratio of 10:1 or more, retention time ± 2 % of the standard, ion ratios within 20%, and a concentration within 20% of the expected value.

Precision and bias was evaluated at 5, 200 and 400 ng/mL using pooled fortified matrix (in triplicate), at three concentrations (low, medium, high), over five runs. Within-

run precision was calculated for each concentration (n=3) over each of the five assays. Between-run precision was calculated for each concentration over all five days (n=15). Bias was evaluated contemporaneously with precision using the same concentrations over five days. The acceptable range for bias and precision was ± 20%.

The calibration model was established using nine non-zero calibrators (2, 5, 10, 25, 100, 200, 300, 400 and 500 ng/mL) over five independent runs. Calibration models were evaluated using the coefficient of determination (R^2) and standardized residual plots. Interferences associated with the biological matrix, isotopically labeled internal standards, common drugs and structurally related compounds were systematically evaluated. Matrix interferences and ion suppression/emhancement were evaluated using ten different blood samples that were extracted in the absence of internal standard and the targeted compounds. Ion contributions arising from the use of stable isotope internal standards were evaluated by fortifying blood with internal standard (100 ng/mL) and monitoring the signal of the target analytes.

Interferences from the matrix, isotopocally labeleld internal standards and common drugs were assessed in accordance with published recommendatons (46). Drug interferences were evaluated using more then seventy common drugs and other therapeutic drugs of significance using a 100-fold excess of interferent (relative to the target drug) in triplicate. Ion suppression and enhancement were quantitatively assessed using post-extraction addition at two concentrations (20 ng/mL and 400 ng/mL). Samples from 10 drug-free matrices were extracted (n=2) in the absence of drug and fortified with drug post extraction. Ion suppression/enhancement was calculated by comparing the mean peak areas of drug in matrix with the drug in mobile phase (no matrix). Carryover was assessed by

analyzing a negative control immediately following the injection of the highest calibrator (500 ng/mL). Carryover was present if the reporting criteria for the targeted analytes was achieved (signal to noise ratio of 3:1 or more, retention time ± 2% and ion ratios within 20% of expected).

The influence of sample dilution was evaluated using fortified blood diluted ten-fold in deionized water prior to extraction. Quantitative results were evaluated and concentrations within ±20% of the expected concentration were deemed acceptable. The stability of processed samples was evaluated by extracting samples (100 ng/mL) in triplicate and analyzing them over a period of up to 48 hours. The samples were considered stabled if the bias remained within ± 20% of the expected quantitative value.

Tissue extraction

Liver homogenates were prepared using a Bead Ruptor 12 (OMNI International, Kennesaw, GA, USA) and a reinforced sample tube (7 mL) containing one part tissue (1 g) with 3 parts deionized water. Full homogenization was achieved using three 30-s pulses at the highest speed setting. The samples vials were pre-filled with five 2.8 mm and ten 1.4 mm ceramic beads (OMNI International). Liver homogenate (0.5 mL) was transferred to a culture tube, and internal standard solution was added while vortex mixing. If the liver homogenate was being used as a control, then targeted compounds were also added. The samples were then precipitated and extracted using the SPE procedure for blood described above. Quantitative liver determinations were performed using whole blood calibrators and matrix-matched (liver) controls. Blank liver homogenate (0.5 mL) was fortified in triplicate with 25 ng drug, thus reflecting final liver concentrations of 200 ng/g. If the targeted

compounds met the acceptance criteria for bias (± 20%), then the quantitative value was reported.

Postmortem specimens

A total of 20 kratom-positive cases were identified and a total of 60 specimens were included in this study. These specimens comprised of liver (n=20), central blood (n=21), and peripheral blood (n=19). Central blood was identified as aortic blood (n=18) and vena cava blood (n=3), Peripheral blood was identified as femoral blood (n=9) and iliac blood (n=10). Due to the long-term storage of specimens prior to quantification, inferences regarding postmortem redistribution and central to peripheral drug concentrations ratios were not deemed appropriate. In addition to the five targeted *Mitragyna* alkaloids, blood specimens (n=32) were also reanalyzed qualitatively to determine the presence of metabolites known metabolites (*e.g.,* 9-O-demethylmitragynine, 16-carboxymitragynine, 9-O-demethyl-16-carboxymitragynine) (47).

Results and discussion

Blood validation

Significant emphasis was placed on chromatographic separation during method development due to the structural and stereoisomers involved. **Figure 6.2** presents an overlaid extracted ion chromatogram (EIC) that depicts the chromatographic separation of all five *Mitragyna* alkaloids in blood at 100 ng/mL. These alkaloids are known to produce characteristic fragmentation patterns when exposed to ESI resulting in near identical MS/MS spectra (48, 49) (**Figure 6.3**). Briefly, *Mitragyna* alkaloids undergo the cleavage of the quinolizine ring to yield both indole (*m/z* 174 and 190) and piperidine derivative fragments (*m/z* 238, 236, 226, 224). Ultimately, complete chromatographic resolution for

the five *Mitragyna* alkaloids was achieved (**Figure 6.2**) using the previously published LC-Q/TOF-MS conditions (49).

Figure 6.2: Chromatographic separation of *Mitragyna* alkaloids in a representative blood extract (100 ng/mL). Speciociliatine, 2.06 min; 7-hydroxymitragynine, 2.53 min; speciogynine, 3.34 min; paynantheine, 3.82 min; and mitragynine, 4.62 min.

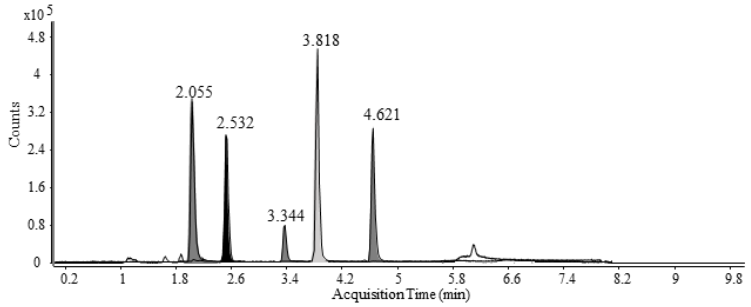
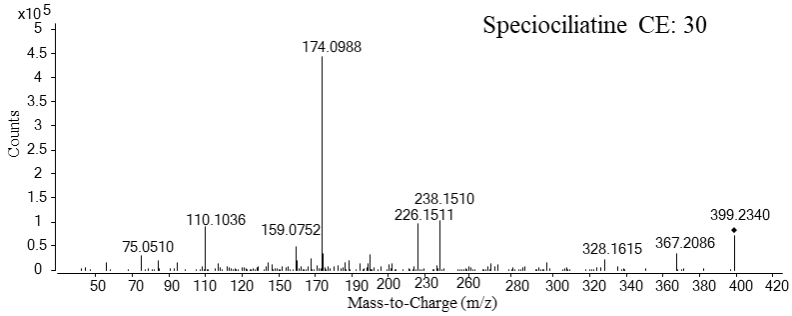
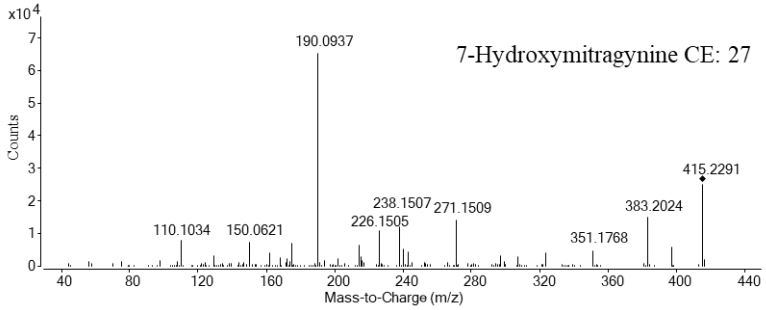
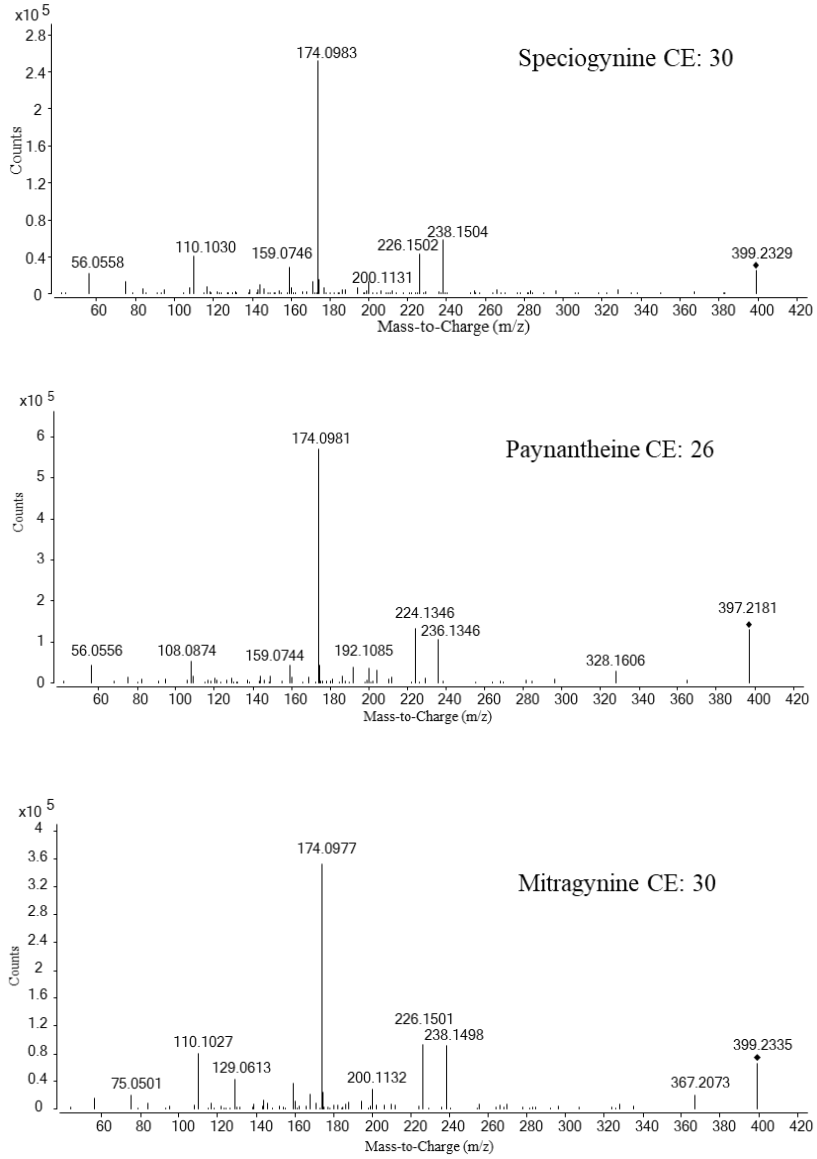


Figure 6.3: MS-MS spectra of speciociliatine, 7-hydroxymitragynine, speciogynine, paynantheine, and mitragynine from a representative extract (100 ng/mL).







The extraction efficiency of mitragynine using the extraction protocol described above, was 80%, with 82-87% for speciociliatine, speciogynine, and paynantheine (**Table 6.3**). Despite the very low extraction efficiency of 7-hydroxymitragynine, the limit of quantitation was still sufficiently low (2 ng/mL), with quantitative bias of -2% and a CV of 10.6% (n=18). Precision and bias for all analytes at the LOQ is summarized in **Table 6.4**.

Table 6.3: Extraction efficiencies for *Mitragyna* alkaloids in blood (250 ng/mL, n=4).

Alkaloid	Extraction Efficiency (%) Mean ± SD (n=4)
MG	80 ± 7
SC	85 ± 8
SC	87 ± 11
7-MG-OH	16 ± 1
PY	82 ± 7

Table 6.4: Limits of detection (LOD), limits of quantitation (LOQ), and assay performance at the limit of quantitation (n=18). Signal to noise ratio (S/N) is presented as the mean value for the quantitation ion at the limit of detection.

Targeted Alkaloid	LOD (ng/mL)	LOQ (ng/mL)	Mean ± SD (ng/mL)	Mean S/N Ratio	Bias (%)	CV (%)
MG	0.5	0.5	0.47 ± 0.04	551:1	-6	10
7-MG-OH	2	2	1.97 ± 0.21	108:1	-2	11
SC	1	2	2.18 ± 0.25	2612:1	6	12
SG	2	2	2.14 ± 0.17	564:1	9	8
PY	1	1	0.86 ± 0.08	2548:1	-14	9

Following visual, analytical, and statistical evaluation of various calibration models, a weighted (1/x) quadratic model was selected for speciociliatine, speciogynine, mitragynine, and 7-hydroxymitragynine to minimize heteroscedasticity. Paynantheine produced homoscedastic data using an unweighted quadratic model. The coefficients of determination were above 0.99 for all models and when residual plots underwent visual assessment, the data appeared to be randomly dispersed for each model. Statistical evaluation also indicated that calibration curve selection for these compounds was optimal. Extracted ion chromatograms for all targeted *Mitragyna* alkaloids at the LOQ are shown in **Figure 6.4**. The LOD and LOQ for MG, 7-MG-OH, SC, SG and PY ranged from 0.5-2 ng/mL and was within the range of forensic interest. At the limit of quantitation, bias and precision ranged from -9-14% and 8-12%, respectively. Precision and bias were also

evaluated at low, medium, and high concentrations in triplicate over five days (**Table 6.5**). Intra-assay CVs were 1-18% (5 ng/mL); 1-16% (200 ng/mL); 1-19% (400 ng/mL) (n = 3) and inter-assay CVs (over the same concentration range) were 2-6%, 1-6% and 1-3% (n = 15). Bias for all three concentrations (5, 200, and 400 ng/mL) ranged from -3-6% for all five alkaloids. Overall, bias and precision were within acceptable ranges (±20%) for all compounds. In addition, there was also no carryover present after the injection of the highest calibrator (500 ng/mL) for any of the five compounds.

Figure 6.4: Extracted ion chromatograms at the limit of quantitation for targeted alkaloids and the internal standards (underlined) (100 ng/mL).

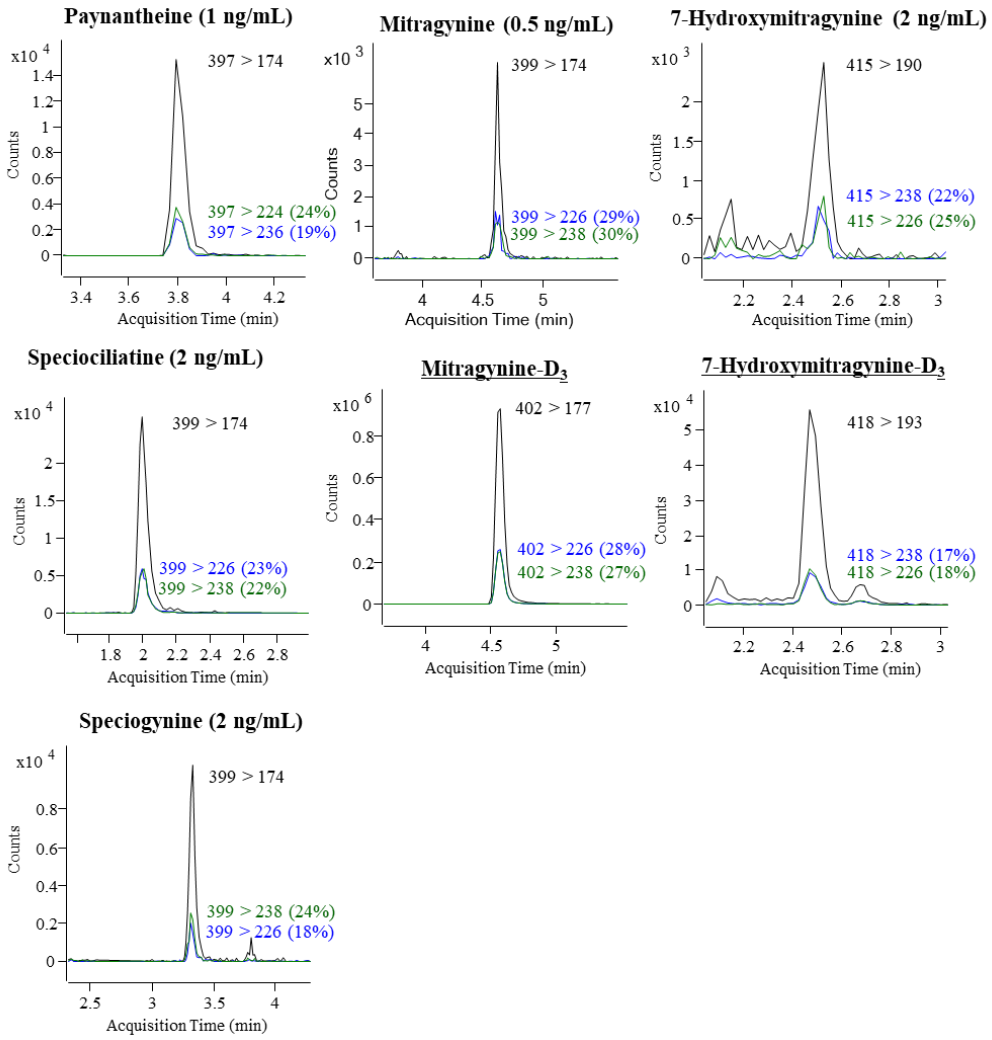


Table 6.5: Precision and bias (n=15) at low (5 ng/mL), medium (200 ng/mL), and high (400 ng/mL) concentrations.

Analyte	Intra-Assay CV (%) (n=3)			Inter-Assay CV (%) (n=15)			Bias (%) (n=15)		
	5 ng/mL	200 ng/mL	400 ng/mL	5 ng/mL	200 ng/mL	400 ng/mL	5 ng/mL	200 ng/mL	400 ng/mL
MG	2-7	1-8	1-12	2	1	2	6	0	3
7-MG-OH	1-12	2-14	1-8	2	2	3	4	3	0
SC	4-17	1-16	1-19	6	4	1	5	2	3
SG	3-18	2-13	2-18	4	2	3	5	5	3
PY	2-7	2-8	1-10	6	6	1	3	-3	2

No interferences were present from either matrix, isotopically labelled internal standards, and no qualitative interferences from other drugs were present for any of the target compounds. Ion suppression or enhancement was evaluated quantitatively using the post-extraction addition technique for all five analytes and two internal standards with ten independently sourced bovine blood samples. Matrix effects were measured at both low and high concentrations (20 and 400 ng/mL). Matrix effects for all five *Mitragyna* alkaloids ranged from -18 to 20% at 20 ng/mL, and -4 to 4% at 400 ng/mL. Corresponding CVs for all five compounds were 9-17% and 2-6%, respectively (**Table 6.6**). Dilution integrity was evaluated using ten-fold dilutions of matrix at 400 ng/mL in triplicate. Bias ranged from -14.3% to 13.4% using diluted specimens, which was within acceptable tolerance. Finally, processed samples were stable in the refrigerated autosampler (4ºC) for 48 hours at 100 ng/mL. Quantitative measurements for all analytes produced concentrations within 7% of the expected value at 48h and absolute peak areas for target compounds or their internal standards did not change by more than 18% during that period.

Table 6.6: Matrix effect (%) and associated CVs (%) at 20 and 400 ng/mL.

Analyte	CV (%) n=10		Matrix Effect (%)	
	20 ng/mL	400 ng/mL	20 ng/mL	400 ng/mL
MG	-18	15	0	3
7-MG-OH	-17	16	-2	3
PY	9	15	-2	2
SC	10	17	0	3
SG	20	17	-4	3
MG-D_3	12	11	0	4
7-MG-OH-D_3	-8	9	4	6

Identification of Mitragyna alkaloids and metabolites in postmortem specimens

A total of 60 specimens (liver (n=20); central blood (n=21); peripheral blood (n=19)) from 20 kratom-positive cases were analyzed using the validated method described above. Postmortem findings for all specimens are summarized in **Table 6.7** and extracted ion chromatograms of the *Mitragyna* alkaloids from a representative case specimen are shown in **Figure 6.5.** Concentrations in blood ranged from 1-422 ng/mL (mitragynine), <2-1,574 ng/mL (speciociliatine), <2-386 ng/mL (speciogynine), <1-67 ng/mL (paynantheine), and 3-434 ng/mL (7-hydroxymitragynine). In order to quantitatively assess the liver samples, precision and bias were measured using liver controls (0.5 mL of homogenate) fortified with drug at 200 ng/g (n=3). Liver controls were analyzed concurrently with blood calibrators and controls. All compounds with the exception of speciociliatine, were within ± 20% of the expected value using 0.5 mL of liver homogenate. Negative bias for speciociliatine in liver was attributed to matrix effects; attempts to reduce the quantity of matrix (0.1 mL liver homogenate) did not resolve the issue, so speciociliatine (the earliest eluting compound) was reported only qualitatively in liver. Concentrations in liver ranged from 7-1,454 ng/mL (mitragynine), 17-1,642 ng/mL

(speciogynine), <8-422 ng/mL (paynantheine), and <4-920 ng/mL (7-hydroxymitragynine).

Table 6.7: *Mitragyna* alkaloids in blood (n=40) and tissue (n=20) from twenty postmortem investigations.

Compound	Matrix	Prevalence in Case Specimens (%)	Mean Concentration (ng/mL or ng/g)	Median Concentration (ng/mL or ng/g)	Concentration Range (ng/mL or ng/g)
MG	Central Blood	90	87 (n=19)	54	1-422
	Peripheral Blood	79	59 (n=15)	21	1-412
	Liver	95	656 (n=19)	708	<4-1,454
7-MG-OH	Central Blood	95	135 (n=20)	104	4-434
	Peripheral Blood	100	94 (n=19)	78	3-360
	Liver	85	285 (n=17)	229	37-920
SC	Central Blood	100	362 (n=21)	232	<2-1,574
	Peripheral Blood	95	238 (n=18)	101	<2-944
	Liver	95	-	-	-
SG	Central Blood	86	86 (n=18)	42	<2-386
	Peripheral Blood	63	50 (n=12)	17	2-284
	Liver	85	748 (n=17)	488	17-1,642
PY	Central Blood	76	18 (n=16)	12	<1-67
	Peripheral Blood	58	11 (n=11)	3	2-46
	Liver	90	161 (n=18)	138	<8-422

In addition to the five *Mitragyna* alkaloids, blood samples (n=32) were also reanalyzed qualitatively to determine the presence of phase I metabolites (**Table 6.8**) as described previously (45). Metabolites of mitragynine were identified in 31 (97%) of the

32 blood samples tested. The most prevalent metabolite in all 32 case samples was 7-hydroxymitragnine (97%). However, because 7-hydroxymitragynine is an alkaloid present in the plant and also a metabolite, its presence cannot be attributed solely to biotransformation (50). The phase I metabolites identified in blood were 9-O-demethylmitragynine (28%) and 16-carboxymitragyine (25%). The minor phase I metabolite, 9-O-demethyl-16-carboxymitragynine, was not detected in any of the blood samples. Interestingly, three known metabolites with an *m/z* 387 were also identified in 28 (88%) of the 32 authentic specimens. As reported previously with the urine specimens from the same case samples (45) these compounds produce MS/MS spectra consistent with 17-O-demethyl-16,17-dihydromitragynine reported by Philipp *et al*. (47). However, because speciogynine and speciociliatine form analogous 17-O-demethyl-16, 17-dihydro metabolites (51, 52), the MS/MS spectra for all three compounds are indistinguishable. Due to the absence of reference materials for these compounds, further differentiation was not possible. Extracted ion chromatograms and MS/MS spectra for the phase I metabolites are shown in **Figures 6.6 and 6.7**.

Figure 6.5: Extracted ion chromatograms for a representative blood (aortic) extract containing speciociliatine (1,574 ng/mL), speciogynine (386 ng/mL), paynantheine (67 ng/mL), mitragynine (285 ng/mL) and 7-hydroxymitragynine (349 ng/mL). Appropriate dilutions were performed to achieve quantitative results within the calibration range (2–500 ng/mL).

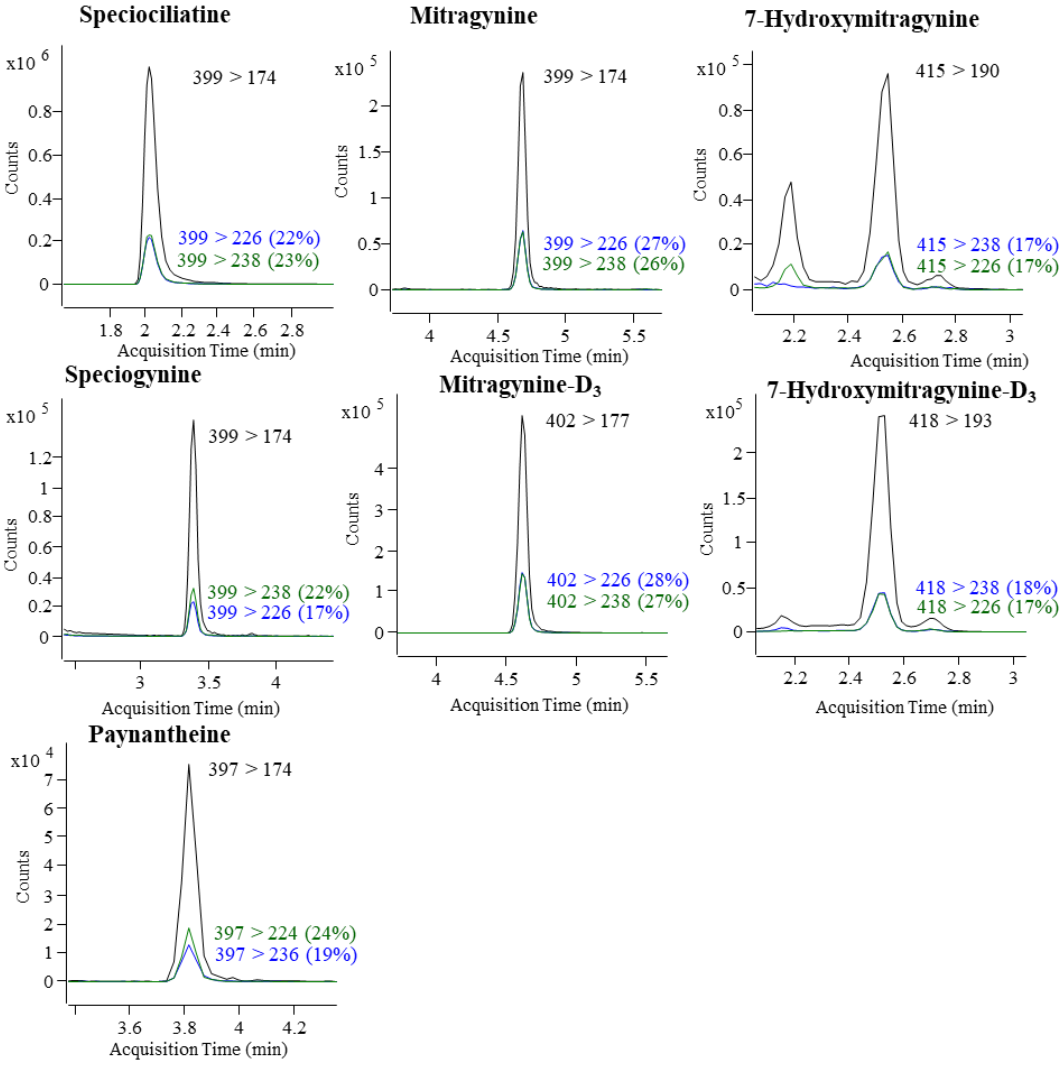


Table 6.8: Identification of mitragynine and other metabolites in blood (n=32). Identification of 17-O-demethyl-16, 17-dihydro (17-O-DM-16,17-DH) metabolites was tentative. ND indicates not detected.

Case Number	Matrix	Mitragynine Concentration (ng/mL)	9-O-DM	7-OH-MG	16-COOH-MG	17-O-DM-16,17-dihydro metabolites
1	Aortic Blood	2		+	+	+[1]
	Femoral Blood	ND		+	+	
2	Aortic Blood	ND		+		
	Femoral Blood	1		+		+[1]
3	Vena Cava Blood	ND				
	Iliac Blood	ND		+		
4	Aortic Blood	79		+		+[1]
	Femoral Blood	33	+	+		+[1,2]
5	Aortic Blood	27		+		+[1]
	Iliac Blood	ND		+		+[1]
6	Aortic Blood	65		+		+[1]
	Iliac Blood	2		+		+[1]
7	Aortic Blood	54		+	+	+[1,3]
	Iliac Blood	ND		+		+[1]
8	Aortic Blood	159		+		+[1]
	Femoral Blood	55	+	+	+	+[1,3]
9	Aortic Blood	1		+		+[1,3]
	Iliac Blood	1		+		+[1,3]
10	Vena Cava Blood	75	+	+		+[3]
	Iliac Blood	47	+	+		

Case Number	Matrix	Mitragynine Concentration (ng/mL)	9-O-DM	7-OH-MG	16-COOH-MG	17-O-DM-16,17-dihydro metabolites
11	Aortic Blood	422	+	+	+	+[1,3]
	Iliac Blood	4	+	+	+	+[1,3]
12	Aortic Blood	7		+		+[1,2,3]
	Iliac Blood	108	+	+	+	+[1,3]
13	Aortic Blood	1		+		+[1]
	Iliac Blood	1		+		+[1]
14	Femoral Blood	1		+	+	+[1,3]
15	Aortic Blood	1		+		+[1]
	Femoral Blood	21	+	+		+[1,3]
17	Aortic Blood	4		+		+[1,3]
19	Aortic Blood	1		+		+[1]
20	Aortic Blood	285	+	+		+[1,2]

[1] *m/z* 387, 4.1 min; [2] *m/z* 387, 5.1 min; [3] *m/z* 387, 5.9 min;

Figure 6.6: Extracted ion chromatograms depicting mitragynine metabolites in a representative postmortem blood extract: 7-hydroxymitragynine, *m/z* 415 (5.3 min), 9-O-demethylmitragynine, *m/z* 385 (4.5 min), 16-carboxymitragynine *m/z 385* (3.0 min) and 17-O-demethyl-16-17-dihydro metabolites, *m/z* 387 (4.1 and 5.9 min).

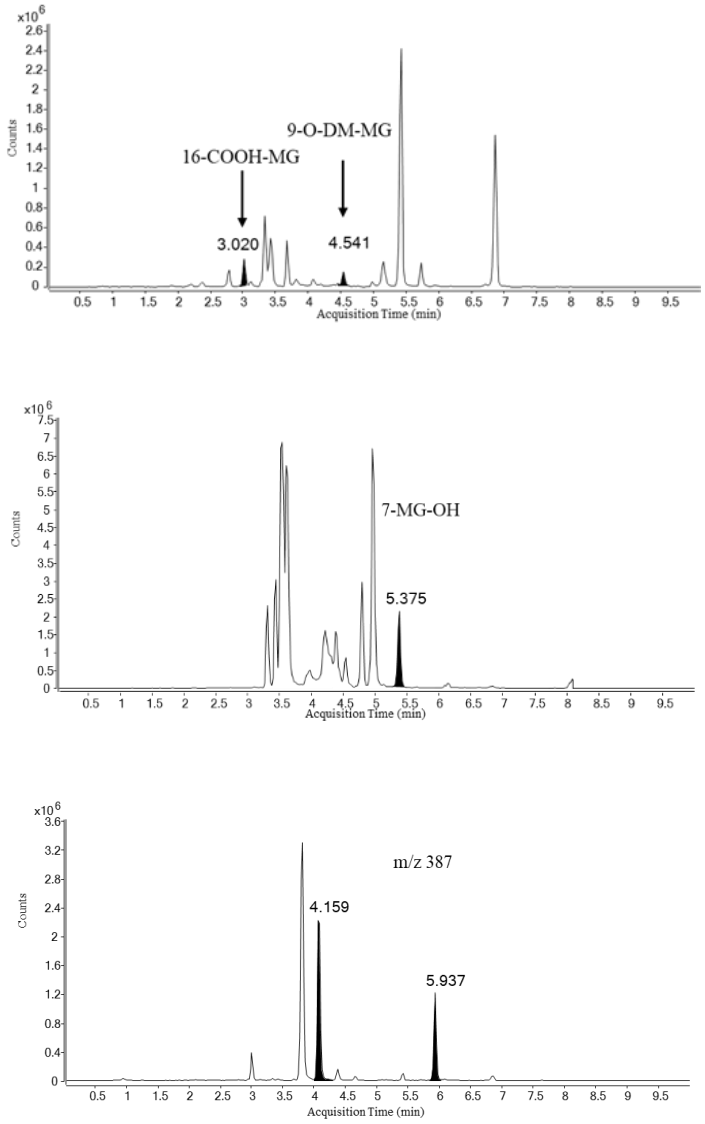
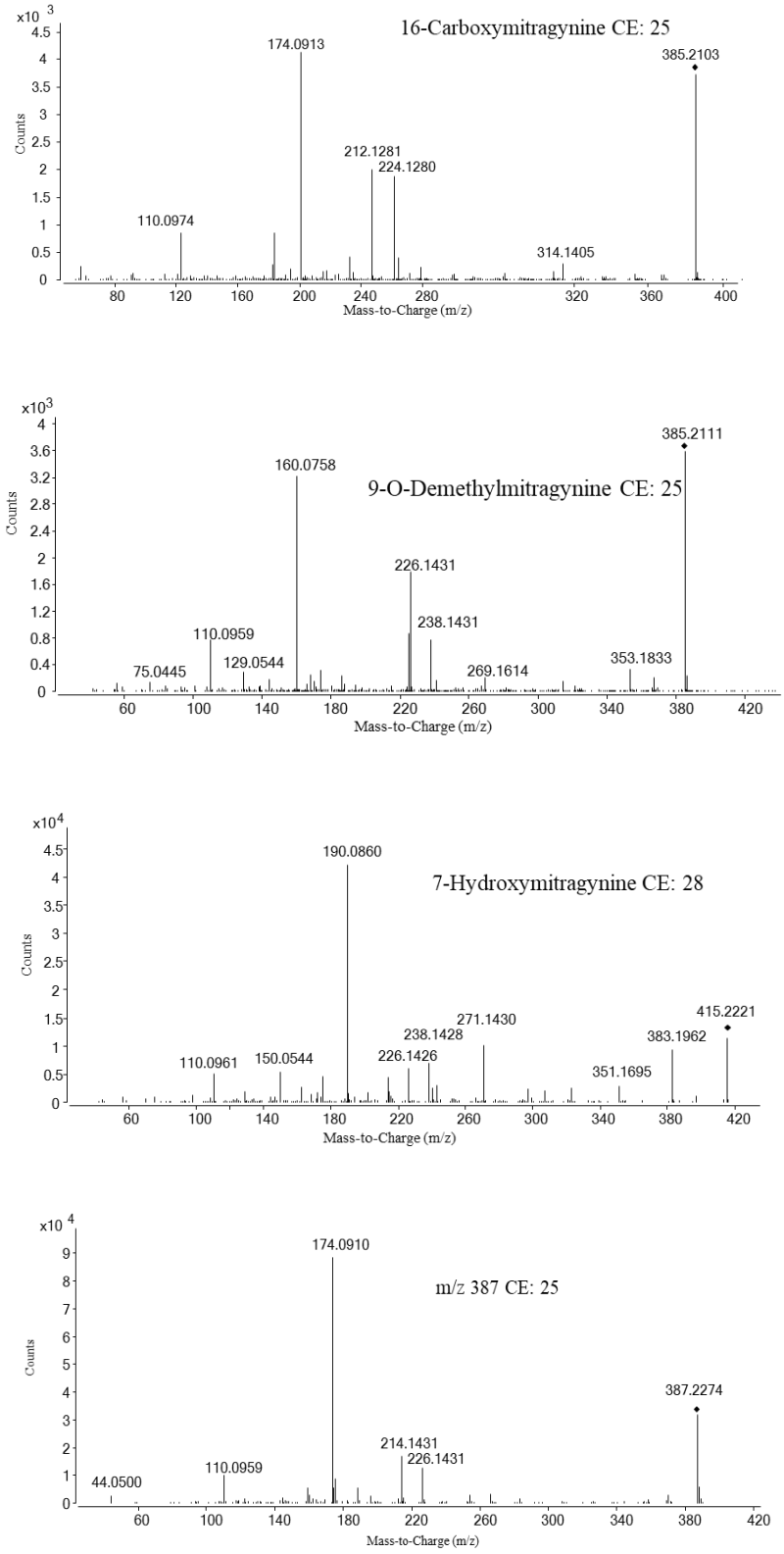


Figure 6.7: MS/MS spectra depicting mitragynine metabolites in a representative postmortem blood extract: 16-carboxymitragynine, 9-O-demethylmitragynine, 7-hydroxymitragynine and 17-O-demethyl-16-17-dihydro-metabolite $[M+H]^+$ *m/z* 387.



Conclusion

Kratom is a psychoactive substance that presents many challenges from the standpoint of toxicological analysis. In addition to structural isomers that must be chromatographically resolved, there are concerns regarding its stability, and its prevalence may be under-reported during routine investigations. In this report five *Mitragyna* alkaloids were identified in postmortem blood (n=40) and liver specimens (n=20). The results confirm earlier reports in urine that suggest that additional biomarkers of kratom use exist, particularly speciociliatine, which may exceed the concentration of mitragynine. It is not known if this observation is due to the increased overall stability of this alkaloid relative to mitragynine, or due to the use of kratom products with high speciociliatine content. Mitragynine metabolites were also identified in 32 unhydrolyzed blood samples (97%). In addition to 7-hydroxymitragynine (a potent mu opioid receptor agonist), 9-O-demethylmitragynine, 16-carboxymitragyine and 17-O-demethyl-16-17-dihydro-metabolites were also identified.

Acknowledgements

We gratefully acknowledge Dr. Ikhlas Khan and the National Center for Natural Products Research School of Pharmacy at The University of Mississippi for providing speciogynine.

This project was supported by Award No. 2016-DN-BX-0006, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect those of the Department of Justice.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures involving biological samples obtained from human decedents were in accordance with the ethical standards of the Sam Houston State University Institutional Review Board (Protection of Human Subjects Committee) in accordance with 45CFR46.101(b) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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CHAPTER VII

CONCLUSION

Significant advances were made in terms of the identification of *Mitragyna* alkaloids in biological matrices. LC-Q/TOF-MS was used to identify mitragynine, speciociliatine, speciogynine, paynantheine and 7-hydroxymitragynine in blood, urine and postmortem tissue. The deuterated compounds, mitragynine-D_3 and 7-hydroxymitragynine-D_3, were used as internal standards. Extraction efficiencies were 63-96% for urine and 16-87% for blood, which was an improvement on existing methods in the literature. Limits of detection and quantitation were 0.25-1 ng/mL in urine and 0.5-2 ng/mL in blood. Using weighted (1/x) and unweighted quadratic models, the routine calibration range for each assay was 2-500 ng/mL, which is well within the range of forensic interest. Precision (both intra- and inter-assay CV) and bias were well within accepted standards for both matrices (±20%) and no carryover was observed, except for extremely elevated concentrations of paynantheine in urine. No qualitative interferences were seen for any of the five targeted alkaloids. Ion suppression was within tolerable limits (<±20%) for all targeted compounds in both matrices with one exception. 7-Hydroxymitragynine experienced significant ion suppression (>40%) in urine. These validated methods for blood and urine are the first in published literature to simultaneously analyze the five *Mitragyna* alkaloids and quantify the diastereoisomers in specimens of forensic interest.

The metabolism of mitragynine was investigated using recombinant CYPs. Four phase I metabolites were identified in the initial investigation: 9-O-demethylmitragynine, 16-carboxymitragynine, 9-O-demethyl-16-carboxymitragynine, and 7-

hydroxymitragynine. 7-Hydroxymitragynine is a notable metabolite because of its pronounced pharmacological effect, relative to the parent drug. Four CYPs were found to contribute to mitragynine's metabolism (CYP3A4, CYP2D6, CYP2C18 and CYP2C19). 9-O-Demethylmitragynine was the most abundant metabolite in the *in vitro* study and was produced by three isoenzymes (2C19, 3A4 and 2D6). 16-Carboxymitragynine was produced by rCYPs 2C19, 2C18 and 2D6, while 7-hydroxymitragynine was produced exclusively by CYP 3A4. 9-O-Demethyl-16-carboxymitragynine, a very minor metabolite, was attributed to 2C19. Using a series of kratom-related fatalities, 9-O-demethylmitragynine was the most prominent phase I metabolite and other alkaloids were identified as potential biomarkers. The involvement of the various CYP450 isoforms provides valuable insight into potential adverse drug reactions and drug-drug interactions that may result from poly drug use.

The short-term stability of mitragynine, 7-hydroxymitragynine, paynantheine, speciogynine and speciociliatine was investigated under accelerated conditions. Five temperatures (4°C, 20°C, 40°C, 60°C, and 80°C) and pH (2, 4, 6, 8, and 10) were investigated over an 8 hour period. All *Mitragyna* alkaloids were significantly acid labile, exhibiting losses >20% loss at pH 2 when exposed to high temperatures. Mitragynine and 7-hydroxymitragynine were also base-labile, though to a lesser degree. Interestingly, speciociliatine, speciogynine and paynantheine were more stable than mitragynine and 7-hydroxymitragynine, even when exposed to highly unfavorable conditions. Degradation products were also investigated for mitragynine. Under alkaline conditions, mitragynine underwent hydrolysis of the methylester to form 16-carboxymitragynine. Only tentative identification of the acidic degradation product was possible. These findings highlight the

need to further investigate the short- and long-term stability of the *Mitragyna* alkaloids in biological samples.

Phase II metabolites of mitragynine were investigated using urine from kratom users. The efficiency of both chemical and enzymatic deconjugation methods were explored. Chemical hydrolysis was unsuitable due to stability issues and the extreme pH required for deconjugation. Enzymatic hydrolysis was explored using both traditional (β-glucuronidase: *Escherichia coli*, *Patella vulgata*, and *Helix pomatia*; sulfatase abalone entrails, *Aerobacter aerogenes*. *Patella vulgata*, and *Helix pomatia*) and recombinant systems (BGTurbo™, BGS™, ASPC™ , and IMCSzyme). Enzymatic hydrolysis was deemed unnecessary for 7-hydroxymitragynine and 9-O-demethylmitragynine, since these metabolites were present at sufficient quantity in free (unconjugated) form. However, enzymatic hydrolysis did improve the detection of 16-carboxymitragynine. This was the first study to explore the efficiency of the deconjugation, or the necessity to perform this additional sample preparation step, for kratom-positive specimens.

Finally, postmortem tissue, blood and urine specimens were investigated to determine the presence of *Mitragyna* alkaloids and phase I metabolites. Overall, 76 specimens (urine (n=16), blood (n=40) and liver (n=20)) from 20 fatalities were analyzed. Speciocilatine and speciogynine (diastereoisomers of mitragynine) were identified as potential biomarkers of kratom use. Concentration of these alkaloids, particularly speciociliatine often exceeded the concentration of mitragynine. These findings could be attributed to its enhanced stability, or the use of kratom products with high speciociliatine content. 9-O-Demethylmitragynine and 7-hydroxymitragynine were identified in the majority of blood and urine specimens. Since most analytical methods used to detect

kratom focus on mitragynine alone, identification of these biomarkers may encourage forensic laboratories to expand their scope of testing, therefore enhancing their ability to identify kratom-related cases in forensic investigations.

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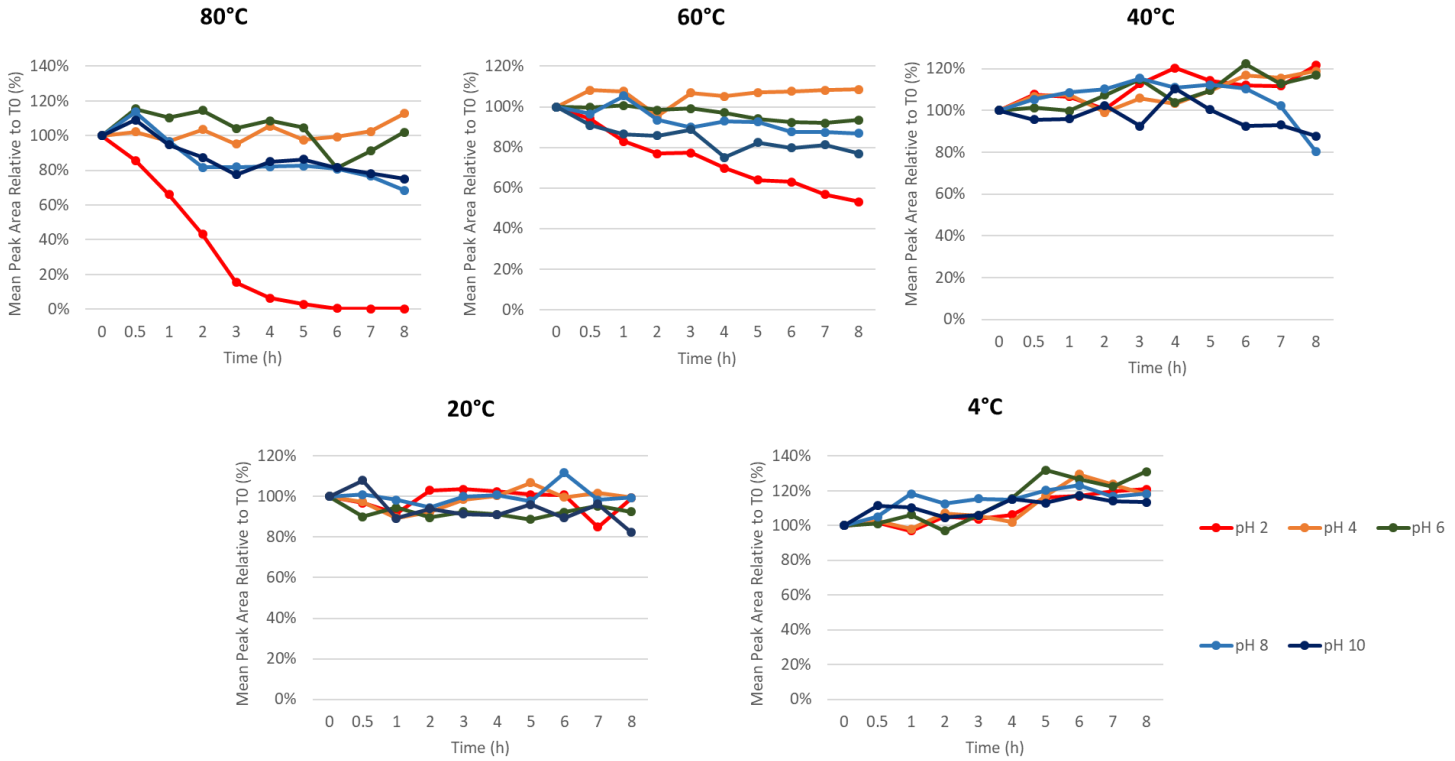
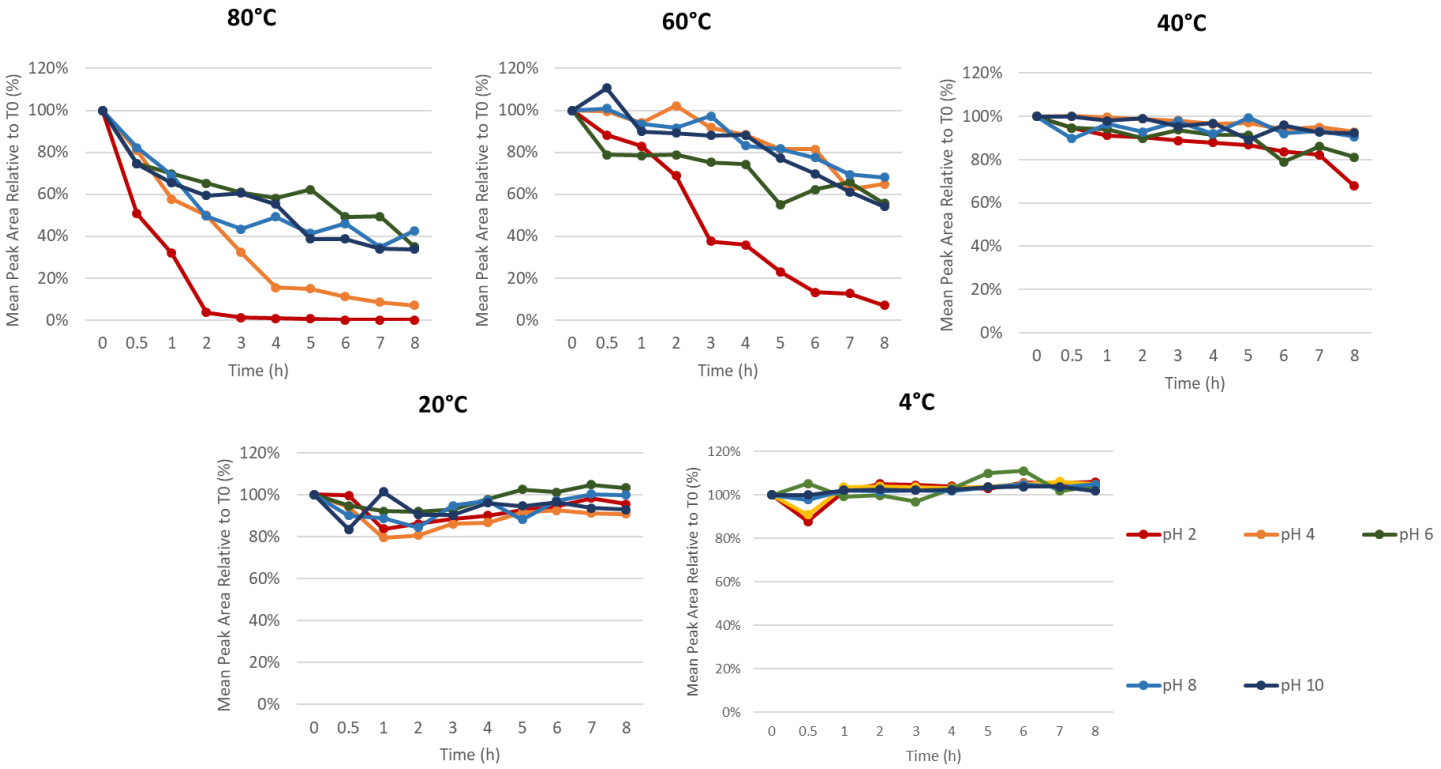
APPENDIX

Stability plots for Mitragynine, Speciociliatine, Speciogynine, Paynantheine, and 7-Hydroxymitragynine

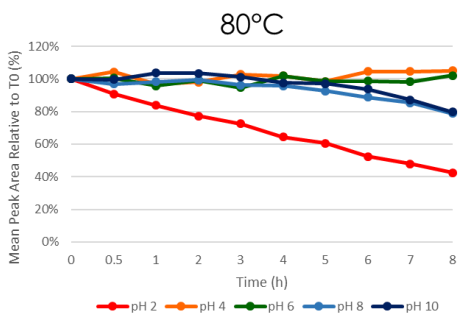
Mitragynine

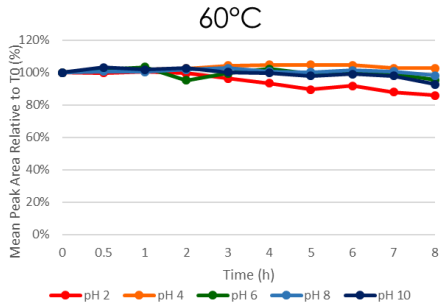


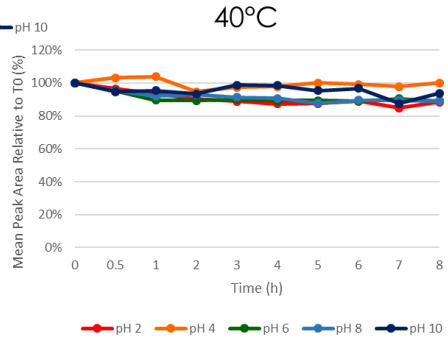
7-Hydroxymitragynine



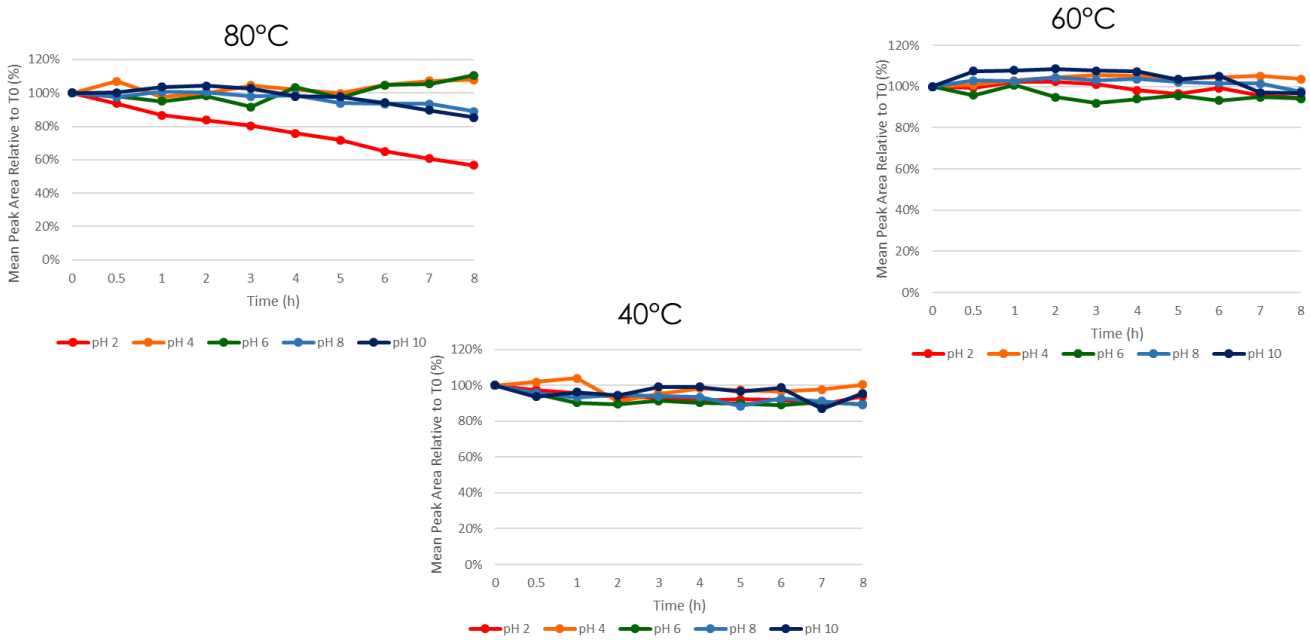
Speciociliatine



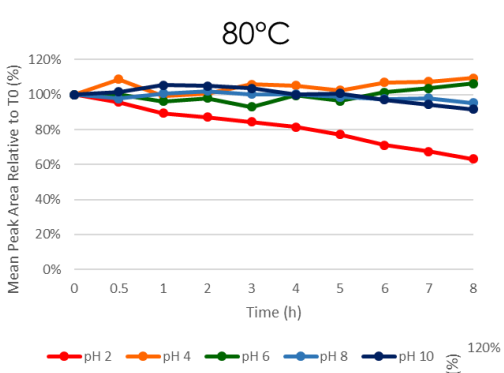


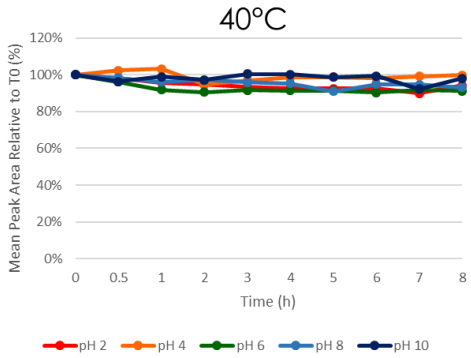


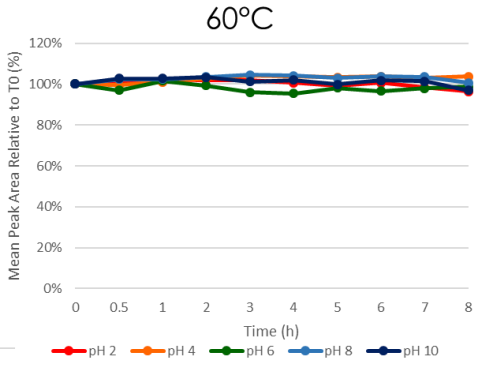
Speciogynine



Payanantheine







VITA

Stephanie Basiliere

Relevant Professional Experience

Sam Houston State University, Department of Forensic Science: August 2014 – Present

- Graduate Research Assistant
  - Aided in laboratory preparation, inventory, administrative duties, and troubleshooting instruments
  - Gas chromatography-mass spectrometry (GC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS), Chemstation software, MassHunter software

Forensic Drug Chemistry Lab at University of Massachusetts' Medical School: May 2014 – August 2015

- Student Intern (Drug Chemistry)
  - Aided in assisting analysts with evidence preparation and analysis, inventory, administrative duties (including laboratory information management system (LIMS) system), and instrument maintenance and troubleshooting,
  - Gas chromatography-mass spectrometry (GC-MS), ChemStation software, and Thin layer chromatography (TLC)

Hamilton Sundstrand: May 2012 – August 2012

- Student Intern
  - Aided in assisting advisor with daily tasks and engineering projects, attended meetings with clients, worked with the primary client, NASA, on projects and training.

Sam Houston State University, Department of Chemistry: February 2010 – July 2013

- Teaching Assistant
  - Aided in assisting professor by instructing some of their classes, provided tutoring for students, created assignments, and graded assignments

Education

Sam Houston State University: August 2013 – Present

- Pending Doctor of Philosophy in Forensic Science
- Graduation: December 2019 (Anticipated)

Sam Houston State University: August 2009 – May 2013

- B.S. in Chemistry for Professional Chemists (ACS certified) with minors in Forensic Chemistry and Biology
- Graduated with Honors in May 2013

Relevant Education Experience

Sam Houston State University (Selected Coursework)

- Forensic Instrumental Analysis, Advanced Instrumental Analysis, Advanced Forensic Chemistry, Forensic Medicine, Forensic Lab Management, Scientific Communications, Research Methods, Quality Assurance, Law and Forensic Science, Forensic Toxicology, Neuropsychopharmacology, Drug and Toxin Biochemistry, Chromatographic Separations, Forensic Statistics and Interpretation, Statistical Genetics, Controlled Substances Analysis, Pattern and Physical Evidence Concepts, Crime Scene Investigation, Trace Evidence and Microscopic Analysis, Biochemistry, Quantitative Analysis, Instrumental Analytical Chemistry, Forensic Chemistry

Skills and Qualifications

Screening and Sample Preparation

- Proficient with liquid-liquid extraction (LLE) (urine), solid phase extraction (SPE) (urine and blood), and thin layer chromatography (TLC)
- Experience using Enzyme linked immunosorbent assay (ELISA) and color tests

Instrumentation

- Proficient using liquid chromatography-quadrupole/time-of-flight-mass spectrometry (LC-Q/TOF-MS), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS)
  - Agilent Technologies gas chromatography-mass spectrometer
    - 5975B VL MSD
  - Agilent liquid chromatography-quadrupole/time of flight-mass spectrometer
    - 6530 Q/TOF-MS
- Experience using UV-Vis Spectrophotometer, Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy (ATR-FTIR), Ion Mobility Spectroscopy (IMS),

Headspace Gas Chromatography (HS-GC), and liquid chromatography tandem mass spectrometry (LC-MS/MS)

Software

- Proficient using Agilent MassHunter Acquisition, Qualitative Analysis, and Quantitative Analysis, ChemStation, and ACD/Spectrus Platform
- Experience using R Statistical Software

Research Grant Funding

- National Institute of Justice – Graduate Research Fellowship (2016-DN-BX-0006)
- *Improved Detection of Kratom Alkaloids in Forensic Toxicology*
- $147,160
- PI: Stephanie Basiliere, CO-PI: Sarah Kerrigan

Publications in Peer Reviewed Journals

1. Basiliere, S., Bryand, K., and Kerrigan, S. (2018) Identification of five *Mitragyna* alkaloids in urine using liquid chromatography-quadrupole/time of flight mass spectrometry. Journal of Chromatography B, 1080, 11-19

2. Basiliere, S., and Kerrigan, S., CYP450-Mediated Metabolism of Mitragynine and Investigation of Unhydrolyzed Phase I Metabolites in Human Urine. Journal of Analytical Toxicology [In Press].

3. Basiliere, S., and Kerrigan, S. Temperature and pH-Dependent Stability of *Mitragyna* Alkaloids. Journal of Analytical Toxicology [In Press].

4. Basiliere, S., and Kerrigan, S. Identification of Metabolites and Potential Biomarkers of Kratom in Urine. Journal of Chromatography B [In Review].

5. Basiliere, S., and Kerrigan, S. Identification of Five *Mitragyna* Alkaloids in Blood and Tissues using Liquid Chromatography-Quadrupole/Time of Flight Mass Spectrometry. Forensic Toxicology [In Review].

Peer-Reviewed Presentations/Posters

1. Basiliere, S., Bryand, K., and Kerrigan, S. Fragmentation Pathways and Structural Characterization of Mitragynine and its Metabolite Using Electrospray Ionization

and High Resolution Mass Spectrometry. Proceedings of the American Academy of Forensic Sciences (2016). Las Vegas, NV. (Poster Presentation)

2. Basiliere, S., Bryand, K., and Kerrigan, S. The Optimized Separation and Identification of Kratom Alkaloids using High-Resolution Mass Spectrometry. Proceedings of the American Academy of Forensic Sciences (2018), Seattle, WA. (Poster Presentation)

3. Basiliere, S., and Kerrigan, S. Phase I Metabolism of Mitragynine Using In Vitro Methods. Proceedings of the Society of Forensic Toxicology (2018), Minneapolis, MN. (Oral Presentation)

4. Basiliere, S., and Kerrigan, S. Identification of Mitragynine and Its Metabolites using High Resolution Mass Spectrometry. Proceedings of Pittcon (2019), Philidelphia, PA. (Oral Presentation)

5. Basiliere, S., and Kerrigan, S. Basiliere, S., and Kerrigan, S. Stability of Mitragynine and 7-Hydroxymitragynine. Proceedings of the Society of Forensic Toxicology (2019), San Antonio, TX. (Poster Presentation)

6. Basiliere, S., and Kerrigan, S. Identification of *Mitragyna* Alkaloids and Metabolites as Biomarkers of Kratom Use in Postmortem Urine Samples. Proceedings of the American Academy of Forensic Sciences (2020), Anaheim, CA. (Oral Presentation)

Awards

- Institute for Forensic Research Training and Innovation Scholarship- Summer 2017
- Forensic Science Scholarship (SHSU)- Fall 2013 – Fall 2019

Professional Affiliations

- Institute for Forensic Research Training and Innovation Scholarship- Summer 2017
- Forensic Science Scholarship (SHSU)- Fall 2013 – Fall 2019

Continuing Education

- Bloodborne and Airborne Pathogens, National Safety Council (NSC) (2013)
- Blood Bourne Pathogens and Laboratory Standards, Occupational Safety and Health Administration (OSHA) certification (2013)

- Answering the NAS: The Ethics of Leadership and the Leadership of Ethics, Forensic Technology Center of Excellence (FTCoE) (2014)
- Introduction to Uncertainty in Forensic Chemistry and Toxicology, FTCoE (2014)
- Standard Operating Procedure (SOP) Writing for ISO 17025 Accreditation, FTCoE (2014)
- To Hell and Back: The Ethics of Stewardship and the Stewardship of Ethics, FTCoE (2014)
- Applications of Higher Resolution Mass Spectrometry in Drug Testing, FTCoE (2015)
- Fundamentals of Chromatography used in Toxicology, FTCoE (2015)
- SWGTOX Standard Practices for Method Validation in Forensic Toxicology/Advanced Excel, Society of Forensic Toxicology (SOFT) continuing education workshop, Milwaukee, WI (2016)
- High Resolution Mass Spectrometry for Qualitative and Quantitative Analysis: An Introduction, American Society for Mass Spectrometry Annual Meeting San Antonio, TX, (2015)