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The Identification of Prenatal Exposure to Mitragynine Using Umbilical Cord Tissue

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Abstract

Objective: Kratom is widely available and literature exploring the effects of prenatal kratom exposure is lacking. This study aims to report a validated method for the detection of mitragynine in the umbilical cord and report our observations for specimens received at a national commercial reference laboratory. **Study Design:** Assays were validated according to the recommendations of ANSI/ASB. A retrospective evaluation of records at a national reference laboratory was conducted to determine prevalence and co-exposure to other substances of abuse. **Result:** Mitragynine was detected in 19 of 4456 specimens (0.43%) with concentrations ranging from 4 to >50 ng/g. Thirteen (13) of these specimens were positive for only mitragynine while the other 6 were also positive for either marijuana or opiates. **Conclusion:** Umbilical cord is a suitable specimen type for the surveillance of maternal kratom use and can be used to identify exposed neonates for further investigations into short- or long-term health consequences.

Keywords

Kratom, Mitragyna Speciose, Mitragynine, Umbilical Cord, Prenatal Exposure, Maternal Drug Use

1. Introduction

Kratom comes from the Rubiaceae coffee plant family, specifically, *Mitragyna speciose* which is indigenous to Southeast Asia. Raw leaves of kratom, also known as ketum, kakuam, biak-biak, ithang, or thom, have been chewed or made into tea for multiple purposes for hundreds of years in Thailand, Malaysia and Myanmar due to its psychoactive properties [1]. In the mid-twentieth century, kratom was categorized as a banned substance in Malaysia and Thailand due to the po-

tential for dependence and addiction, with more recently being legal for medical reasons [2].

Kratom is widely available in the United States, with 3-5 million American consumers based on membership data from the American Kratom Association [3]. Kratom is legally sold in retail stores (gas stations, convenience stores, herbal stores) and through the internet as powder, capsules, raw leaves, and concentrated extracts. However, individual states in the United States have variable regulations on the legality of kratom. Consumers of kratom use it for pain relief, anxiety and depression, to reduce opioid withdrawal symptoms or as an opioid substitute [4]-[10]. Kratom is being evaluated for the potential future treatment for opioid addiction and chronic pain [11].

Kratom has both stimulant and opioid-like properties [12]. Over 40 alkaloids in varying levels have been documented in mitragyna speciose (Kratom), dependent on genetic variation and conditions of growth and processing [13]. Two of the most studied alkaloids are Mitragynine (MG) and 7-hydroxymitragynine (7OHMG) which act as partial agonists of the human μ-opioid receptor [13], with differing potency. The potency of 7OHMG is greater than MG and morphine due to a greater affinity to opioid receptor site [14] [15] [16].

Although reports of kratom use, abuse and overdose deaths are on the rise in western regions of the world since 2011 [17], identification of persons using kratom is inconsistent in clinical settings. Health care providers may not be aware of the properties, availability and lack of detection of kratom. Direct questions regarding kratom use may not be included during the substance use health history. Persons using kratom may have the impression that they do not need to report their use to their healthcare clinician due to the legal, wide availability of the substance. Internet sources aimed at the consumer of kratom, report little or no risks, which also adds to the sense that reporting kratom use is unnecessary.

Reports of the prevalence of kratom use in the general population have only recently been published due to the previous epidemiological reports focusing on the use trends of current kratom users [18]. Using responses from the 2019 National Survey on Drug Use and Health, an estimate of 0.7% (95% CI: 0.6, 0.8) of past-year users of kratom was reported [18]. Additionally, those participants identified with cannabinoid use disorder were 4.33 times more likely to also use kratom (95% CI: 2.61, 7.19; $p < 0.001$) [18]. Survey participants identified with prescription opioid use disorder were 3.2 times as likely to report past-year kratom use (95% CI: 1.38,7.41; $p < 0.01$) [18].

Kratom overdoses and complications have been reported. The Food and Drug Administration (FDA) released a report of 36 kratom-related overdose deaths between 2010 and 2018 [19] [20]. The Drug Enforcement Administration (DEA), U.S. Department of Justice has listed kratom as a drug and chemical of concern [21].

A paucity of information and research exists regarding the perinatal considerations of prenatal kratom exposure. Use of kratom to mitigate opioid withdrawal

or as an opioid substitute may be perceived as a safer alternative for pregnant women with opioid abuse issues. A recent systematic review of the effects of prenatal kratom use on mothers and infants included six case reports, of which five of the infants required pharmacological wean due to signs of withdrawal [22]. Two of the infants were reported to be exposed prenatally to kratom only, while the remaining three were reported to be exposed prenatally to multiple substances, including kratom. Maternal withdrawal symptoms were reported as severe with either a return to kratom use or medicine-assisted therapy to alleviate the discomfort of withdrawal [22]-[28]. The case reports underscore the importance of the identification of prenatal kratom exposure for both the mother and infant outcomes. Case studies identifying Neonatal Abstinence Syndrome (NAS) indicate more information is needed about the effects of kratom in mothers and newborns during pregnancy [22]-[28]. In addition, use may be under-reported due to many reasons such as its legal status and lack of rapid toxicology testing.

Routine toxicology does not identify kratom, which adds to the under identification of kratom use reported in clinical settings. Currently, adequately sensitive analytical platforms (mass spectrometry coupled with either gas chromatography or liquid chromatography) for MG analysis in umbilical cord tissue (UC) are available for definitive testing. However, these methods are more time-consuming chromatographic assays and therefore not ideal for high throughput initial testing (screening). Economical and rapid immunoassay platforms for MG with the required sensitivity for high throughput UC screening are not available at this time. Laser Diode Thermal Desorption (LDTD) technology is a technological development that allows for very rapid mass spectrometric detection of compounds in biological extracts in the toxicology screening laboratory. Umbilical cord is a useful specimen for toxicology testing as it is available immediately after birth and can identify drug use during approximately the third trimester of pregnancy [29] [30].

The specific aims of this project are three-fold: 1) describe for the first time a validated initial testing method for the detection of MG in human UC using LDTD interfaced with tandem mass spectrometry; 2) report for the first time a validated confirmation method for MG in human UC using Liquid Chromatography Tandem Mass Spectrometry (LCMSMS); 3) report the positivity rates of MG in a convenience sampling of UC received at a national reference laboratory for toxicological analysis.

2. Experimental

2.1. Chemical, Reagents, and Materials

Standards (MG and MG-d_3) were obtained from Cerilliant (Rock Round, TX, USA). Methanol, acetonitrile, and ammonium acetate were HPLC grade, and isopropanol and formic acid were ACS grade or higher and purchased from Fisher Scientific (Hanover Park, IL, USA). Ethylenediaminetetraacetic acid (EDTA) was

purchased from Acros Organics (Fair Lawn, NJ, USA). LazWell™ plates were purchased from Overbrook Scientific (Boston, MA, USA).

2.2. Initial Testing

2.2.1. Calibration, QC, and Specimen Preparation

A stock solution was prepared by diluting purchased standards to a concentration of 50 ng/mL with methanol. Internal standard stock solutions were prepared by diluting purchased standards with methanol to a concentration of 100 ng/mL. Two separate working standards were made with subsequent dilutions with methanol to produce a 3-point calibration curve (2.5, 5 and 10 ng/g) and controls (0, 2.5 and 10 ng/g). The internal standard working solution contained each analyte's respective deuterated analog at a concentration of 10 ng/g.

Umbilical Cord specimens (0.5 g) were accurately weighed into 5 mL polypropylene tubes. Following the addition of 50 mL of internal standard working solution, aliquots were homogenized in 3 mL of acetonitrile by adding 3 stainless steel wood screws into each tube and placing the tubes in a Bullet Blender® (Next Advance, Troy, NY, USA) at setting 7 for 5 minutes. The homogenates were filtered through fritted reservoir columns (United Chemical Technologies, Bristol, PA, USA) and evaporated under a stream of nitrogen at 40˚C. The dried residues were reconstituted in 200 µL of 75:25 methanol: DI water with 100 µg/mL EDTA solution. The extracts were spotted (8 µL) onto 96 LazWell™ Plate, dried at 35˚C in a recirculating oven, and forwarded for LDTD-MS/MS analysis.

2.2.2. Instrument Parameters

Extracts were initially tested using a Phytronix LDTD-960 with a Sciex 6500+ Mass Spectrometer. The LDTD laser pattern started at zero and was increased to 35% over 3 seconds, held for 2 seconds, and returned to zero for 2 seconds for a total analysis time of 7 seconds. Mass spectrometry parameters are outlined in Table 1.

2.2.3. Identification Criteria

A three-point curve was used which included a low calibrator (0.5× cutoff), a cutoff calibrator and a high calibrator (2× cutoff). Controls consisted of an above threshold at 2× the cutoff, a below threshold at 0.5× the cutoff, and a negative control. All calibrators and controls were prepared in homogenized negative

Table 1. MSMS parameters for the LDTD-MSMS initial test.

Analyte	Screening Parameters					
	Precursor Ion (m/z)	Product Ion (m/z)	Declustering Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)	Entrance Potential (V)
MG-d_3	402.1	177.3	171.00	41.00	14.00	10.00
MG*	399.1	174.2	106.00	41.00	12.00	10.00

*Quantization transition.

umbilical cord specimen. For a batch of specimen results to be considered acceptable, the negative and below threshold controls must have an analyte to internal standard ratio less than the cutoff calibrator while the above threshold control must have an analyte to internal standard ratio greater than the cutoff calibrator. Specimens with an analyte to internal standard ratio greater than the cutoff calibrator were considered to be presumptive positive and were forwarded to confirmation testing. Specimens with an analyte to internal standard ratio less than the cutoff calibrator were considered negative. Because there was no chromatographic separation, there were no criteria on the peak appearance of the analyte or internal standard.

2.2.4. Method Validation

The method was validated according to the recommendations of the 2019 ANSI/ASB Standard 036. The criteria analyzed were the precision and accuracy, the statistical limit of detection, the stability of the extract, the stability of the dried extract in the LazWell™ plate, potential interferences, and matrix effect.

2.3. Confirmation Testing

2.3.1. Calibration, QC, and Specimen Preparation

A stock solution was prepared by diluting purchased standards with methanol to a concentration of 100 ng/mL. Two separate working standards were prepared with subsequent dilutions with methanol to produce a matrix matched calibrator with a final concentration of 5 ng/g of each analyte, and matrix matched controls with concentrations of 2, 6.25 and 50 ng/g respectively for the low, mid and high levels. The internal standard used each analyte's respective deuterated analog and at a concentration of 5 ng/g for each analyte.

Umbilical Cord specimens (0.5 g) were weighed and placed into 5 mL polypropylene tubes. Following the addition of 50 mL of internal standard working solution, the aliquots were homogenized in 3 mL of acetonitrile by adding 3 stainless steel wood screws and placing the tubes in a Bullet Blender® (Next Advance, Troy, NY) at setting 7 for 5 minutes. The homogenates were decanted into salinized test tubes and centrifuged at 560 *g* for 5 minutes. The supernatant was decanted through a fritted reservoir column and evaporated under a stream of nitrogen at 40˚C. The residues were reconstituted in 200 µL of 10 mM ammonium acetate with 0.1% formic acid.

2.3.2. LCMSMS Conditions

Separation was achieved with an Agilent 1200 HPLC system with a Phenomenex Polar RP 4 × 2 mm Security Guard Cartridge and a Phenomenex 50 × 2 mm Polar-RP column with 2.0 µm particle size. The column compartment was held at 50˚C. The mobile phase A was 10 mM ammonium acetate with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid with a flow rate of 500 µL/min. Mobile phase B was ramped from 10% to 90% from 0 to 2 minutes, held for 0.5 minutes, then decreased to 10% between 2.5 to 3 minutes and held until 5 minutes. The autosampler injection volume was 0.5 µL, with a draw and

ejection speed of 200 µL/min.

Detection of analytes was accomplished using a Sciex Triple Quad™ 5500 mass spectrometer with ESI source in MRM mode with positive ionization. To aid the evaporation of mobile phase in the ESI source and keep the background at a minimum a post-column infusion of acetonitrile was used with a flow rate of 100 µL/min [31]. Mass spectrometry parameters appear in **Table 2**. Data were analyzed using Sciex Analyst® software version 1.6.3.

2.3.3. Identification Criteria

Identification criteria of each analyte were a relative retention time within 2.5% of the calibrator, symmetrical peak shape, chromatographic resolution of greater than 85% return to baseline, and the signal to noise greater than a ratio of 3:1 for all mass transitions. The transition ratios of the control and patients were within 30% of the corresponding transition ratios of the calibrator.

2.3.4. Method Validation

The analysis performance and criteria were developed following the guidelines published by ANSI/ASB Standard 036, First Edition, 2019. The following criteria were evaluated: calibration model, precision, accuracy, limit of detection, limit of quantitation, carryover, interferences, matrix effects, and extract stability.

2.4. Authentic Specimen Challenge

To further challenge the assays, a sampling (n = 56) of UC were selected that were received over a two-day period in the laboratory (May 10 & 11, 2021) where we received multiple kratom positive specimens. All 56 specimens were analyzed using the above LDTD-MSMS and LCMSMS procedures. The outcomes of the 2 assays were evaluated for agreement. All specimens used for development and validation of the assays were de-identified remnants and were exempt from institutional review board approval.

2.5. Survey of Specimens

Deidentified toxicology records for UC received at a national commercial laboratory (USDTL, Des Plaines, IL, USA) between May 2020 and March 2021 for analysis that included a request for analysis of MG were reviewed to provide insight with

Table 2. MSMS parameters for the LCMSMS confirmation.

Analyte	Confirmation Parameters							
	Precursor Ion (m/z)	Product Ion (m/z)	Declustering Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)	Dwell (msec)	CUR (V)	Entrance Potential (V)
MG-d_3	402.3	177.0	171.00	41.00	14.00	100.00	30.00	10.00
MG*	399.3	174.0	106.00	41.00	12.00	100.00	30.00	10.00
MG**	399.3	238.0	106.00	31.00	24.00	100.00	30.00	10.00

*Quantization Transition; **Qualifying Transition.

regard to the prevalence of maternal kratom use in the demographic routinely tested by our laboratory. UC were obtained for cases that fit local hospital testing criteria and collected following established procedures (https://www.usdtl.com/). Specimens were refrigerated following collection and shipped ambient overnight to the laboratory. Once received at the laboratory, specimens were stored refrigerated until testing was complete. Secondary analysis of de-identified results did not require institutional review board approval. The positivity rates, medians, and interquartile ranges were calculated using Excel. Additionally, polysubstance use patterns were evaluated.

3. Results

3.1. Initial Test Validation Results

The precision and accuracy for the assay were acceptable. At the cutoff, the coefficient of variation was less than 20% and the mean of the replicates at each concentration tested was within 15% of target value. There were no observed interferences when challenged with the selected potentially interfering substances listed in Table 3. We did not observe a significant matrix effect. Both the dried plate and the extracts were stable up to 48 hours at room temperature. The limit of detection for MG was 2.5 ng/g.

3.2. Confirmation Test Validation Results

The calibration model demonstrated linearity between 1 - 50 ng/g and carryover was not observed up to 500 ng/g. The precision was <11% for within-run and <14% for between-run. The bias was within 15% of expected values. Endogenous and exogenous interferences (Table 3) were not observed and extracts were stable up to 7 days at room temperature. Matrix effect experiments demonstrated significant enhancement for MG (>100%). Due to the matrix effect being greater than ±25%, a minimum of 10 negative lots were included in the study for matrix effect and the limit of detection. The use of isotopically labeled internal standard (MG-d_3) behaved similar to the analyte and thus minimized the effect on the quantitation [32]. The statistical limit of detection was 0.5 ng/g. The calibration

Table 3. List of potentially interfering substances.

Drug Class	Compounds Analyzed
Amphetamines	Amphetamine, Methamphetamine, MDA, MDMA, MDEA
Cocaines	Benzoylecgonine
Opiates	Morphine, Codeine, Hydrocodone, Hydromorphone
Benzodiazepines	7-Aminonitrazepam, 7-Aminoclonazepam, 7-Aminoflunitrazepam, *α*-Hydroxymidazloam, *α*-Hydroxytriazolam, *α*-Hydroxyalprazolam, 2-Hydroxyethylflurazepam, Lorazepam, Oxazepam, Temazepam, Nordiazepam
Cannabinoids	CBC, exo-THC, CBDV, THCV, CBL, CBN, CBG, Δ8-THC
Other	Ibuprofen, Naproxen, Ketoprofen, Lidocaine, Ephedrine, Pseudoephedrine, Phentermine, Dihydrocodeine, Phenylpropanolamine, Dextromethorphan

model used an administrative LOD of 1 ng/g.

Chromatograms for a calibrator, negative control, and an authentic patient specimen containing 23.1 ng/g of MG are depicted in Figure 1. The chromatograms for all three internal standards, the calibrator and patient specimen satisfied the identification criteria requirements listed previously. The signals observed in the negative control were less than 3:1 signal to noise and the calculated values was less than the limit of detection. The de-identified patient specimen depicted in Figure 1 was also positive for carboxy-THC (71 pg/g; marijuana metabolite).

3.3. Authentic Specimen Challenge

Agreement was observed with the result outcomes for the 56-specimen convenience sampling. Of the 56 specimens, 54 specimens were negative by both methods and there were 2 specimens that were positive by both LDTD-MSMS and LCMSMS.

3.4. Survey of Specimens

Between May 2020 and March of 2021, 4456 UC specimens were received at

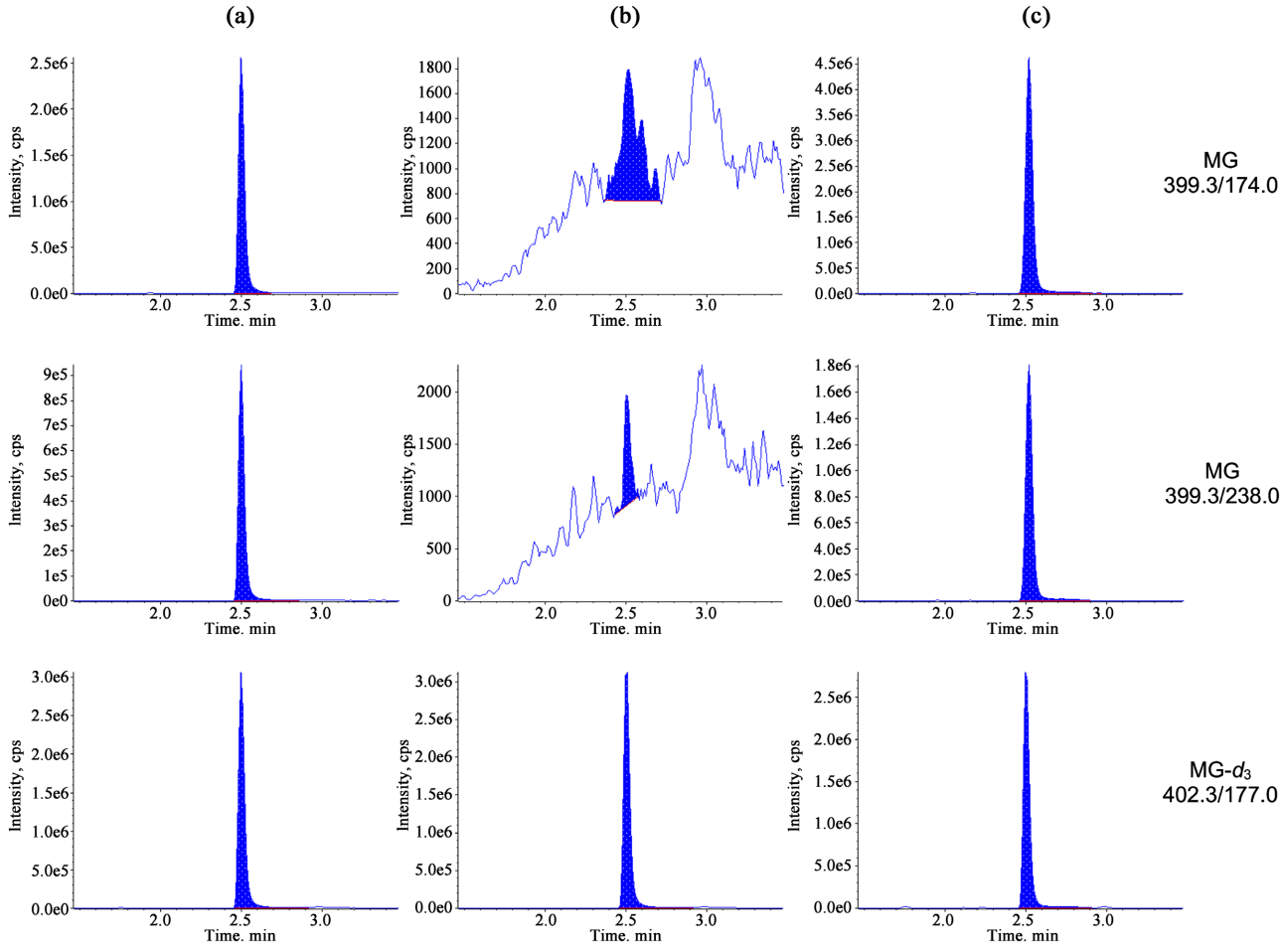

Figure 1. Representative LC-MS/MS chromatograms for the: (a) cutoff calibrator (5 ng/g), (b) negative control, and (c) authentic patient UC containing 23.1 ng/g MG.

USDTL for routine toxicological analysis involving LDTD-MSMS initial testing with presumptive positive specimens reflexing to LCMSMS for confirmation. Of the 4456 specimens analyzed, MG was detected in 19 specimens (0.43%). The results were not normally distributed (skew = 1.04; kurtosis = 0.17), primarily due to the small number of positive specimens. The concentration of MG ranged from 8 ng/g to >50 ng/g (median = 82 ng/g; IQR: 54 ng/g, >50 ng/g). The final calculated concentrations that were >50 ng/g for specimens whose instruments values were <50 ng/g due to using < 1g of tissue were used in the median and IQR calculation.

Of the 19 specimens where MG was found, 10 contained only MG. There were 3 specimens that contained MG and marijuana metabolite (carboxy-THC). One specimen contained MG, codeine, and morphine. One specimen contained MG, gabapentin, methadone, and methadone metabolite (EDDP). One specimen contained MG, gabapentin, tramadol, buprenorphine, and norbuprenorphine (buprenorphine metabolite).

4. Discussion

This study demonstrates the feasibility of a high throughput screening method for the presence of MG in UC specimens using LDTD-MSMS with a 7 second run time per specimen. This screening method was paired with LCMSMS confirmation to provide a robust method for screening and confirming MG in UC to identify *in utero* exposure. Both the screening and confirmation method were validated, met acceptable criteria, and demonstrated agreement in an authentic specimen challenge (n = 56).

In this study, 19 of the 4456 (0.43%) specimens analyzed were positive for MG which is similar to the prevalence rate of 0.7% reported by Palamar [18]. Three of the specimens were positive for more than one substance, predominately opioids and marijuana. This observation was also aligned with the observations of Palamar [18].

Our assay did include monitoring for 7-hydroxymitragynine (7OHMG) but we encountered issues perhaps due to stability and/or purity of purchased drug standard. During our study we only observed 3 specimens where both MG and 7OHMG were detected with 7OHMG concentrations between 6% - 11% of MG. This observation is similar to those of Le, Goggin, and Janis [33]. In a urine-based study, they reported 7OHMG/MG ratios of between 0.1% and 2.4%. We did not observe any specimens where 7OHMG was detected in the absence of MG. Therefore, inclusion of 7OHMG provided no additional value to the primary purpose of this assay which is to identify prenatal exposure to kratom.

Although the positivity rate was low, MG were detected in specimens received at this laboratory, indicating a potential benefit to adding MG to toxicology testing for cases of suspected NAS. While the detection rate of kratom in UC for this demographic was not as high, for example, as cannabinoids which generally exceed a positivity rate of >20% (USDTL unpublished data), the positivity rate was very similar to the quarterly positivity rates for meperidine (0.3%), tramadol (0.4%) and alprazolam (0.4%; USDTL unpublished data) which are components

of popular testing panels offered by our laboratory. In addition, MG testing was requested in only 8% of specimens received at the laboratory which may introduce significant selection bias. This fact also reflects on the current lack of physician awareness and toxicology for kratom in the field. Additional studies in this population would be beneficial to better understand the prevalence of maternal kratom use and implications of use during pregnancy.

There are limitations of this study that limit the generalizability of our findings. The specimens used in this study were a convenience sampling received at a national reference laboratory and therefore was not a true prevalence study. The laboratory was blind to the medical record and the hospital's selection protocol. Future studies that capture self-report, demographics and geography would be helpful to determine the true prevalence of prenatal kratom exposure.

At this time, we are not able to determine the detection window, specific time of use, the size of the dose when used, or frequency of use. Therefore, we are unable to determine if the co-exposures reported here were from simultaneous use, intermittent use, or use throughout the third trimester. Due to ethical reasons, prospective random controlled studies are not feasible in the field of newborn toxicology, and this presents the limitation of the appropriateness of the sensitivity of the method used in this study. Future studies are needed to challenge the methods presented here using kratom specific self-report elements as well as analysis of paired biological specimens.

Specimens used in this study were obtained through routine workflow which did not include freezing for storing or shipping specimens. This may have negatively affected the positivity rate reported here due to the known stability issues of kratom-related analytes. Future prevalence studies should be designed to mitigate this issue.

Another limitation of this study was the lack of chromatographic separation of the diastereomers of mitragynine. These diastereomers are present in and unique to the kratom plant. The presence of any unique kratom alkaloid in UC is indicative of kratom exposure which is the primary purpose of this assay. However, future directions of study may include diastereomer separation, as those pure standards are made available, to evaluate specific negative consequences of these compounds.

Due to the paucity of literature on the effects of prenatal kratom exposure and the widespread availability of kratom, further study of prenatal kratom exposure is warranted. Surveillance measures such as the inclusion of kratom-based items on maternal intake questionnaires and increased testing of newborn specimens for kratom, would be reasonable next steps. Future studies are needed to follow prenatal kratom exposure cases to document any negative short- or long-term health consequences.

5. Conclusion

This study has demonstrated the utility of combining LDTD-MSMS with LCMSMS

as a screen and confirms strategy for the purpose of identifying prenatal exposure to MG. Kratom is widely available and presents a potential public health issue. More research is needed to understand the short- and long-term implications associated with prenatal exposure to kratom use.

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Author Contribution Statement

MEW and JJ developed and conceptualized the study. DC, AS, AR, and MJ developed and validated the assays described in the study. All authors contributed to the preparation and revisions of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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