KRA-01

Method Title: Quantitative and Qualitative Analysis of Mitragynine in 'Kratom" (Mitragyna Speciosa) by GC-MS, LC-MS/MS and UPLC-PDA

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LABORATORY INFORMATION BULLETIN

Quantitative and Qualitative Analysis of Mitragynine in 'Kratom" (Mitragyna Speciosa) by GC-MS, LC-MS/MS and UPLC-PDA

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In 2011, emergency rooms on the West Coast had patients showing up with opiate/heroin withdrawal symptoms from "Kratom." *Mitragyna speciosa*, or Korth (Thai name Kratom; Rubiaceae) is a medical plant native to Thailand and other Southeast Asian countries and is presently illegal in Thailand and other European countries [1]. In the United States, Kratom is readily available via the internet and local retail stores.

The leaves of *Mitragyna speciosa* consist of two primary active alkaloids: Mitragynine 66.2%, and 7 α -hydroxy-7H-mitragynine 2.0%, and three indole alkaloids: Paynantheine 8.6%, Speciogynine 6.6%, and Speciociliatine 0.8%. Since mitragynine is one of the major constituent of Kratom, mitragynine is used as the marker compound for the identification and quantitation of Kratom in a variety of products.

This Laboratory Information Bulletin describes methodology for the qualitative identification and quantitation of Kratom in different types of products such as but not limited to: powders, liquids, and spent-leaf materials. A quick methanolic based extraction procedure was used in combination with two instrument techniques: 1) GC/MS and/or LC-MS/MS for the initial screening and spectral confirmation of mitragynine in Kratom and quantitation via UPLC/PAD; 2) LC-MS/MS. Two different mass spectrometry systems were employed for confirmation/quantitation to permit flexibility within the regulatory laboratory for sample analysis.

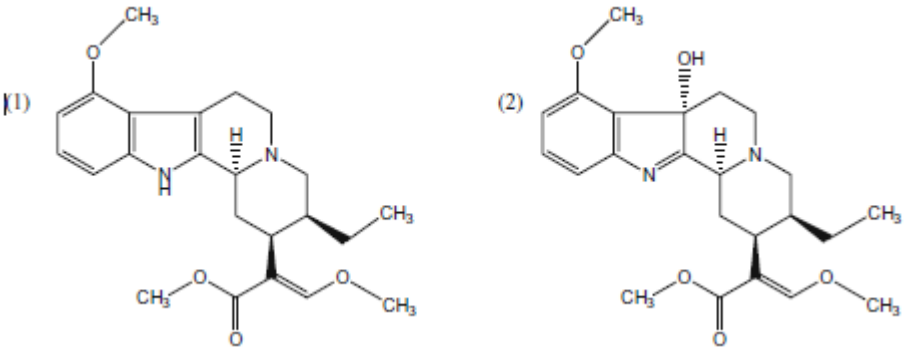
A mitragynine solvent standard was used for the comparative identification of Kratom and quantitation was reported based on the level of mitragynine in the product tested. Due to the low concentration of the mitragynine stock standard (100 µg/mL) and the high level of mitragynine in the products tested, traditional spiking of the standard via a wet/dry spike into a negative control was not feasible. Solvent based calibration curves were used for the quantitation of mitragynine in Kratom by UPLC/PDA and LC-MS/MS. Validation was performed by characterizing a Kratom product purchased via the internet. This positive control was extracted seven times over three days and analyzed by all three analytical techniques: GC/MS, LC-MS/MS and UPLC/PDA. The UPLC/PDA data demonstrated a mean value of 1.041% (n=21, 4.2%) and the LC-MS/MS 1.140% (n=14, 6.81%) for mitragynine in the positive control. This positive control was extracted and analyzed in duplicate with every analytical batch.

INTRODUCTION

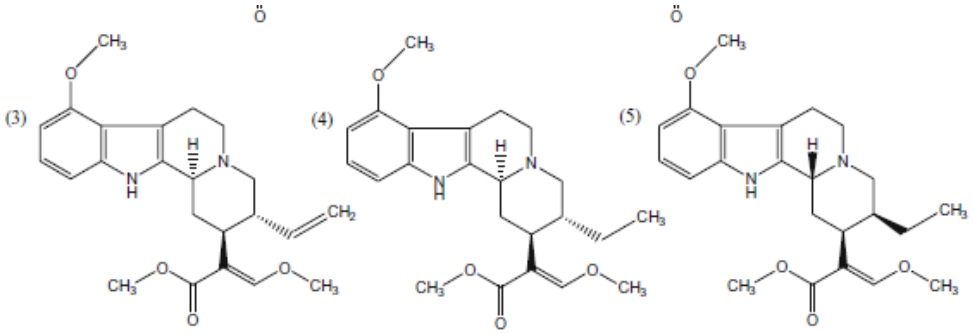
The leaves of the South Asian plant *Mitragyna speciosa*, also known as "Kratom" are described as having stimulating effects at low doses, and opiate-like analgesic and euphoric effects at high doses [2]. Kratom is not controlled in the United States, and the ready availability of kratom via the internet has led to its emergence as an herbal drug of abuse. With this growing popularity of Kratom, efficient procedures are needed to detect kratom in a variety of matrices. Kratom leaves are known to contain five alkaloid components in approximate proportion: mitragynine 66.2%, paynantheine 8.6%, speciogynine 6.6%, 7 α -hydroxy-7H-mitragynine 2.0 %, and speciociliatine 0.8%, refer to Figure 1[2].

Figure1. Chemical Structure of Mitragynine and 4 other Alkaloids

(1) Mitragynine (2) 7 α -hydroxy-7H-mitragynine



(3) Paynantheine (4) Speciogynine (5) Speciociliatine



The major constituent of Kratom is mitragynine and this alkaloid was used for qualitative identification of Kratom by two separate techniques GC/MS and LC-MS/MS. Sample extraction for the GC/MS used approximately 100 mg of sample extracted into 100% methanol, sonicated, and filtered for analysis. Extractions for the LC system were similar with the following variation: extraction solvent was 80% aqueous methanol and a secondary dilution followed by analysis on UPLC/PDA and/or LC–MS/MS. Presently, the only solvent standard available for mitragynine has a concentration of 100 µg/mL in methanol. This concentration of solution was too low to perform spikes at the levels of interest, which is at the percent level. The Denver Laboratory purchased different products online and one of these products was used as a positive control. The positive control is a ground Kratom Thai Leaf which was extracted twice per

analytical batch and served as the spike and spike duplicate The samples submitted to the Denver Laboratory ranged from small packets of drinks, capsules, tea leaves, powdered leaves and spent leaves from a manufacturing processing facility.

The objectives of this LIB were to develop a method to qualitatively confirm the identity and quantitate the amount of mitragyanine in products containing Kratom (*Mitragyna speciosa).*

METHODS AND MATERIALS

Equipment

a) GC/MS instrument. –
   a. Agilent (Wilmington, DE); 6890N GC with a 5973 MS (single quad) detector using Chemstation G17010A data acquisition and analysis software, or equivalent.
   b. Agilent (Wilmington, DE); 7890A GC with a 5975C MS (single quad) detector using MSD Enhanced Chemstation data acquisition and analysis software, or equivalent
b) LC-MS instrument. – A Thermo Finnegan TSQ Quantum triple quadrupole mass spectrometer with Surveyor MS pump and autosampler. An ESI ion source was used in positive ion mode along with LCQuant 2.5.6, Xcalibur 2.0.7 SP1 data acquisition and analysis software, or equivalent.
c) UPLC/PDA Instrument –
   a. Acquity H-Class Flow Through Needle Instrument – Empower Software
   b. Acquity Sample Manager Instrument –Empower Software
d) GC column. – Agilent (Wilmington, DE); DB-5 MS, 30 m x 0.250 mm, 0.25 µm column, or equivalent.
e) LC columns. –
   a. UPLC/PDA - 100 x 2.1 mm, 1.7 µm Acquity BEH C18 Waters (Milford, MA).
   b. LC-MS/MS – 100 mm x 2.1mm, 3.5µm XBridge BEH C18 Waters (Milford, MA).
f) Vortex Mixer – Vortex Genie 2, (Scientific Industries, Bohemia, NY).
g) Eppendorf Pipettes – Variable (5 µL to 1000 µL $\pm$ 0.8%) volume (Brinkman Instruments, Inc., Westbury, NY), or equivalent.
h) Volumetric Glassware – 10.0 mL $\pm$ 0.08mL volumetric flask Class A or equivalent: 1.00 mL glassware or equivalent.
i) Sonicator – Branson 8510 (Danbury, CT)
j) Centrifuge - refrigerated to 4 ºC, capable of accelerating 15 and 50 mL tubes to 6,000 rpm.
k) Syringe filters – Acrodisc® CR 13 mm syringe filter with 0.2 µm PTFE membrane (P/N 4542, Pall Life Sciences) with 1 mL disposable syringe (P/N 309602, Becton Dickinson, Franklin Lakes, NJ).
l) Centrifuge tubes. –15 mL and 50 mL disposable, conical, graduated polypropylene tubes with cap (Falcon® Blue Max™, P/N 50 mL tubes 352070, 15 mL tubes 352097, Becton Dickinson, Franklin Lakes, NJ).
m) Glassware and LC vials –disposable Pasteur pipettes; 2 mL LC vials with snap top, or equivalent.

Reagents and Standards

a) Solvents. –
    a. Ammonium formate – Acros Organics (Fisher Scientific, Pittsburgh, PA, USA).
    b. Acetonitrile – Chromatographic Grade for GC/MS and Optima Grade for UPLC/PDA and LC-MS/MS methods (Fisher Scientific, Pittsburgh, PA, USA)
    c. Methanol – Chromatographic Grade for GC/MS and Optima Grade for UPLC/PDA and LC-MS/MS methods (Fisher Scientific, Pittsburgh, PA, USA)
    d. Water – Optima Grade (Fisher Scientific, Pittsburgh, PA, USA)
b) Gases – Helium and Nitrogen was Ultra High Purity grade, General Air or equivalent
c) Extraction solution. –
    a. GC/MS – 100% methanol
    b. UPLC/PDA and LC-MS/MS - 80% aqueous methanol solution
d) Diluting solution. –
    a. UPLC/PDA and LC-MS/MS - 90% /10%, 0.1% formic acid:acetonitrile (v/v).
e) LC Systems mobile phases. – Acetonitrile, water, and formic acid used for LC-MS mobile phase preparation. Mobile phase A was prepared by diluting 1.00 mL of formic acid in 1000 mL of water. Mobile Phase B – 1000mL acetonitriile
f) Analytical standards. –
    a. Mitragynine (CAS 4098-40-2, MW: 398.50), 100 µg/mL in Methanol, Cerilliant (Round Rock, TX, US).
    b. Mitragynine was also purchased from Toronto Research Chemicals (North York, ON, Canada).
    c. 7 α -hydroxy-7H-mitragynine (CAS 174418-82-7, MW: 414.49) 100 µg/mL in 1N Ammonia in methanol, Cerilliant (Round Rock, TX, US).
g) Negative Control - herbal products previously tested and found to be free of kratom by the Denver Laboratory
h) Positive Control - Ground Kratom Thai Leaf

Standard Preparation

a) GC/MS – 10 µg/mL or 20 µg/mL, 100 µL or 200 µL to a final volume of 1.00 mL in methanol. Standard is prepared directly in the GC vial and vortexed.
a) Positive Control Sample –Kratom Private Reserve Thai Leaf
b) Working standard solutions –.
    a. UPLC solvent calibration standard. – Prepare according to Table 1. All solutions are prepared in the HPLC vials and vortexed. An ICV standard is prepared at the 5.0 µg/mL level.

Table 1. Preparation of uPLC Solvent Standard Calibrants

Calibration Curve	Volume of 100 µg/mL mitragynine Std	Amount of diluent
1 µg/mL	10 µL	990
2.5 µg/mL	25 µL	975
5.0 µg/mL	50 µL	950
7.5 µg/mL	75 µL	925
10 µg/mL	100 uL	900

b. LC-MS/MS solvent calibration curve – Prepared according to Table 2. An initial dilution of the 100 µg/mL mitragynine solution is prepared as follows: 100 µL of stock standard diluted to a final volume of 10.0 mL methanol. This working standard is equivalent to 1000 ng/mL. All solutions are prepared in the HPLC vials and vortex. An ICV standard is prepared at the 50 ng/mL level.

Table 2. Preparation of LC-MS/MS Solvent Standard Calibrants

Calibration Curve	Volume of 1000 ng/mL mitragynine Std	Amount of diluent
10 ng/mL	10 uL	990
25 ng/mL	25 uL	975
50 ng/mL	50 uL	950
75 ng/mL	75 uL	925
100 ng/mL	100 uL	900

Sample Preparation

For all extraction techniques: reagent blank, negative control and the positive control in duplicate analyzed are per analytical batch. The extraction for all the different techniques was based on the method developed by CHAN et al [3] and Kikura-Hanajiri et al [2].

GC/MS System

Sample Extraction for Solid Samples

1. Weigh out appropriate amount of solid sample into centrifuge tube. Typically 100 mg of solid sample is used although the analysis of spent herbs may require approximatley1g of solid sample in either a 15 mL or 50 mL graduated polypropylene tubes.
2. Add 10 mL of methanol.
3. Sonicate for 30 minutes.
4. If necessary let sample settle or centrifuge before filtering.
5. Filter through 0.2 um PTFE filter into GC vial.
6. If further dilution is needed dilute sample filtrate with methanol.
7. Analyze on the GC/MS

Sample Extraction for Liquid Samples

1. Dilute liquid sample with methanol in volumetric flask (typically 1 mL to final volume of 10 mL) and mix.
2. If necessary let sample settle or centrifuge before filtering.
3. Filter through 0.2 um PTFE filter into GC vial.
4. Analyze on the GC/MS

UPLC/PDA and LC-MS/MS Systems

Sample Extraction for Solid Samples

1. Weigh 100 mg (± 0.1g) of sample into 50 mL centrifuge tube.
   Note: Sample weight may be adjusted due to varying levels of Kratom in the sample
2. Add 10 mL of extraction solution to sample.
3. Vortex or shake by hand briefly and then mix on a Geno grinder at 1X 500 rpm for 5 min.
4. Sonicate for 20 min.
5. Centrifuge sample 6,000 rpm for 10 min, 4 °C.
6. Decant the supernatant into a 10.0 mL volumetric flask and dilute to a final volume with methanol.
   (Note: Sample is brought to final volume of 10.00 mL to ensure accurate final volume.)
7. Filter sample with 0.2micron PTFE syringe filter and use dilution solution to perform appropriate dilutions.
8. Initial sample extract is 20X (50 µL into a final volume 1.00 mL) of diluent; dilution is performed directly into HPLC vials.
9. If the amount of mitragynine is above the highest point of the curve, further dilute the sample into the range of the calibration curve.

If confirmation and or quantitation are performed on the LC-MS/MS further dilution of the samples is required.

1. Follow the above extraction procedure
2. Initial dilution is 50X, (20 µL into a final volume of 1.00 mL), dilution is performed directly into HPLC vials.
3. Analyze on the LC-MS/MS
4. If the amount of mitragynine is above the highest point of the curve, further dilute sample into the range of the calibration curve

Sample Extraction for Liquid Samples

1. Aliquot 1.00 mL of sample into a 10.00 mL volumetric flask and fill with extraction solution.
2. Sonicate for 20 min.
3. Centrifuge sample 6,000 rpm for 10 min, 4 °C.
4. Filter through 0.2 um PTFE filter into GC vial.

Instrumental Methods

For all instrument techniques: reagent blank, negative control and the positive control in duplicate analyzed are per analytical batch. The extraction for all the different techniques was based on the method developed by CHAN et al [3] and Kikura-Hanajiri et al [4].

GC/MS

Oven Temperature:

Initial Temperature: 200°C; hold at initial temperature 2 minutes
Ramp: 10°C/min; Final Temperature: 325°C; hold at final temperature 20 minutes
Total Run Time: 34.5 minutes

Column Parameters:

Gas Flow: 1.0 mL/min Mode: Constant Flow
Average Velocity: 39 cm/sec

Inlet Parameters:

Inlet Mode: Splitless Inlet Temperature: 250°C
Purge Flow: 50 mL/minute Purge Time: 0.5 minute

Injection Volume: 2 µl

MS Parameters:

Tune: Autotune (to maximize sensitivity across mass range)
Voltage is increased - 100
MSD Transfer Line Heater: 280°C
MS Quad: 150°C MS Source: 230°C
Low Mass: 50 High Mass: 650
Filament Delay: 3 min

GC/MS Single Quadrapole – Full Scan Spectra

Analyte	Q Ion (m/z)	Confirmation Ions (m/z)
Mitragynine	397	383, 269, 199

UPLC/PDA

The quantitative method was performed using a Waters Acquity UPLC/PDA. A Waters BEH C18 UPLC column was used with a gradient of 0.1% aqueous formic acid in channel A and acetonitrile in channel B. The flow rate was 0.50 mL/min and the column is kept at 40º C. The spectrum was acquired via photodiode array detection (PDA) and the 254 nm wavelength was extracted for quantitation. Injection volume was equal to 2 µL. The mobile phase gradient was as follows:

Time (min)	%A	%B
0	95	5
6.0	10	90
6.5	10	90
7.0	95	5

Acquity H-Class Flow Through Needle Instrument Setup

Wash Solvent	50:50 Acetonitrile/Milli-Q 18.2 Ω Water
Pre-Inject Wash Time	6.0 seconds
Post-Inject Wash Time	6.0 seconds
Purge Solvent	10:90 Milli-Q 18.2 Ω Water /Acetonitrile
Strong Wash Volume	200 µL

Acquity Sample Manager Instrument Setup

Weak Wash Solvent	95:5 Acetonitrile/Milli-Q 18.2 Ω Water
Weak Wash Volume	600 µL
Strong Wash Solvent	95:5 Milli-Q 18.2 Ω Water /Acetonitrile
Strong Wash Volume	200 µL

LC-MS/MS

The secondary confirmation/quantitiation was the LC-MS/MS Triple Quadrapole method using similar methodology employed by the UPLC/PDA quantitative method. The Acuity calculator program was used to calculate the initial LC conditions for the LC-MS/MS so the resolution of the mitragynine and the other similar alkaloids would demonstrate a similar separation pattern to the UPLC/PDA. The mobile phases were the same as for the UPLC/PDA. The flow rate was 0.30 mL/min and the column as kept at 35°C, injection volume was equal to 10 µL. The mobile phase gradient was as follows:

Time (min)	%A	%B
0	90	10
12	50	50
15	90	10
16	10	90
20	90	10

Positive ions were generated using ESI LC-MS/MS to detect mitragynine in Kratom products. Mitragynine tuning solution (1 ug/mL) was teed in to LC mobile phase (0.3 mL/min 50:50 A:B) and was used to optimize tube lens and collision energies (CE) for mitragynine. The source parameters used for the method include: sheath gas (N_2, 50 arbitrary units); auxiliary gas (N_2, 5 arbitrary units); capillary temperature, 300°C; spray voltage 3.5kV. The LC stream was diverted to waste before 3.00 min and after 15 min in the chromatographic run. The following time segments and scan events were used to detect ions for the mitragynine compound:

Table 3: Retention times (RT) and MS parameters: tube lens for precursor ions and collision energy (CE), and the resulting ion ratios for the product ions of each analyte.

Analyte	RT (min)	Ion (m/z)	tube lens	CE	ion ratio
mitragynine	11.51	399.2→174.1*	157	29	100%
		399.2 → 159.1	139	44	68%
		399.2 → 238.2	157	23	61%

*Quantitation ion

Data Analysis and Reporting

GC/MS Data Analysis

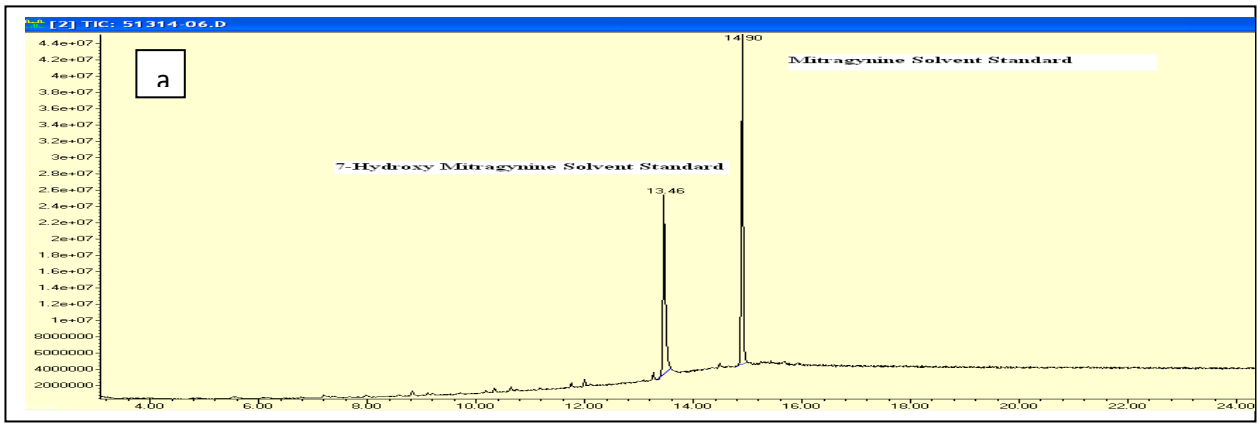
The GC/MS method was adapted from the procedure used by Chan et al [3]. A mitragynine solvent standard was injected at a concentration of 10 µg/mL or 20 µg/mL with a corresponding retention time of 14.90 minutes. A 7-hydroxy mitragynine standard was acquired to determine the retention time of this alkaloid and has a corresponding retention times of 13.46 minutes. Figure 2 is an example of a mitragynine and 7-hydroxy mitragynine solvent standard and the associated full scan mass spectrum. The solvent standards retention time and mass spectrum was used for confirmation of mitragynine in the variety of products received by the Denver Laboratory. Figure 3 is an example of the QEDIT report generated for the reporting of data. Figure 3 thru 5 are examples of a negative control, positive control, and a powder leaf sample. Each figure included the total ion chromatogram (TIC), mass spectrum, and an example of the QEDIT report.

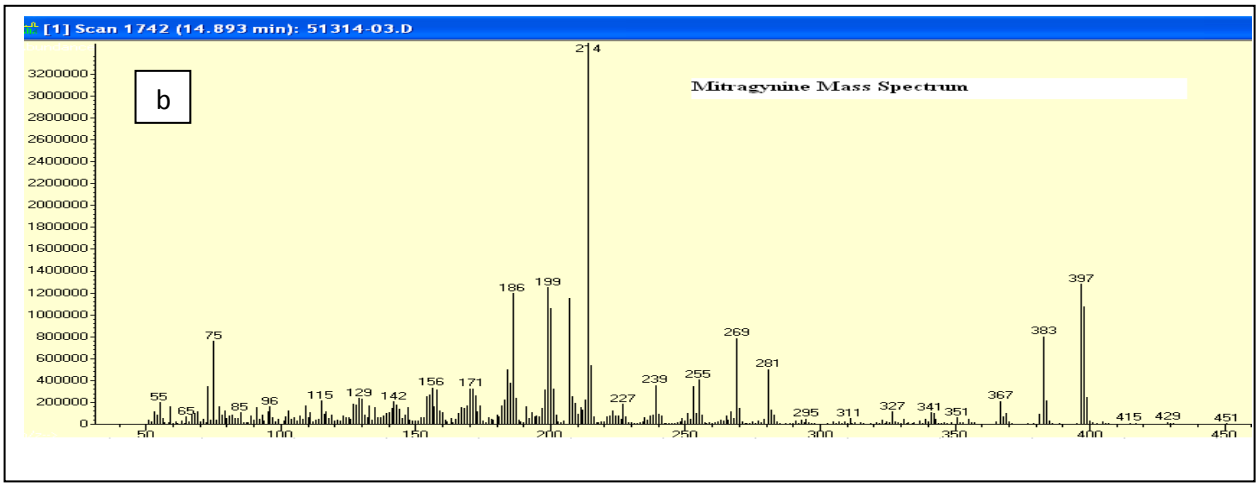
With the GC/MS method, Chromatography/Mass Spectrometry guidance CVM118 [5] for MS^1 full scan data was used to determine confirmation of identity along with retention time matching, 2% GC/MS relative to retention time of the standard and signal–to-noise (s/n) threshold of 3:1. The mass spectrum of the mitragynine standard was compared to the WILEY library and demonstrated a match of greater than 98%. For mass spectral confirmation of mitragynine in the samples analyzed by GC/MS, the mitragynine solvent standard was compared to the matching peak in the chromatogram of the sample. Mitragynine is confirmed as positive if the mass spectrum of the sample matches the spectrum of the mitragynine solvent standard.

Another aspect of ensuring the GC/MS system was suitable for anlysis was performing a visual inspection of the postive control sample. The GC/MS demonstrated a consistent pattern with mitragynine peak eluting first and two other peak eluting at 15.02 minutes and 15.20 minutes, Figure 6. The TIC aquired from the sample was similar to the GC/MS TIC from reference 3. Hence, beside the presence of mitragynine, these two other peaks have been noted with the postive control and all the sample anlyzed at the Denver Laboratory.

Sample that are positive via the GC/MS qualitative screening method are then analyzed by the UPLC/PDA or LC-MS/MS for quantitation.

Figure 2: a) Mitragynine Total Ion Chromatogram (TIC) and 7-Hydroxy Mitragynine b) Mitragynine Mass Spectrum c) 7-Hydroxy Mitragynine Mass Spectrum





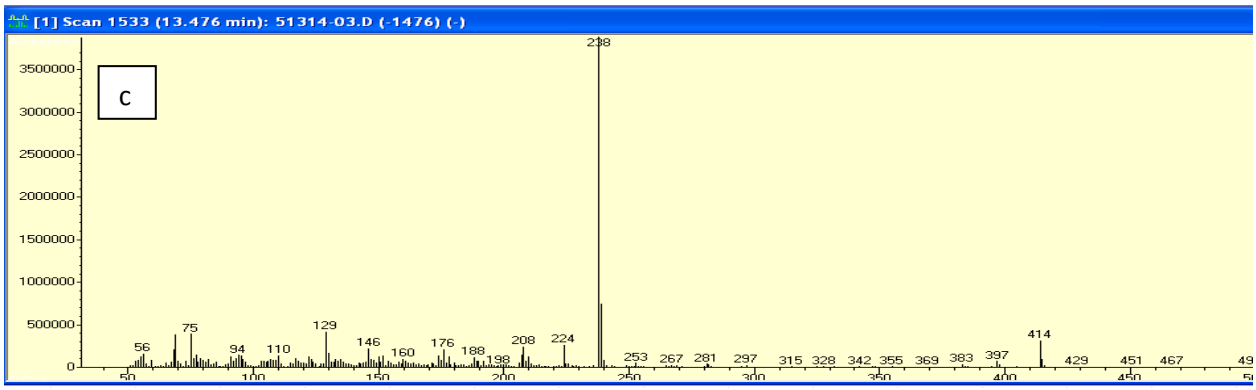
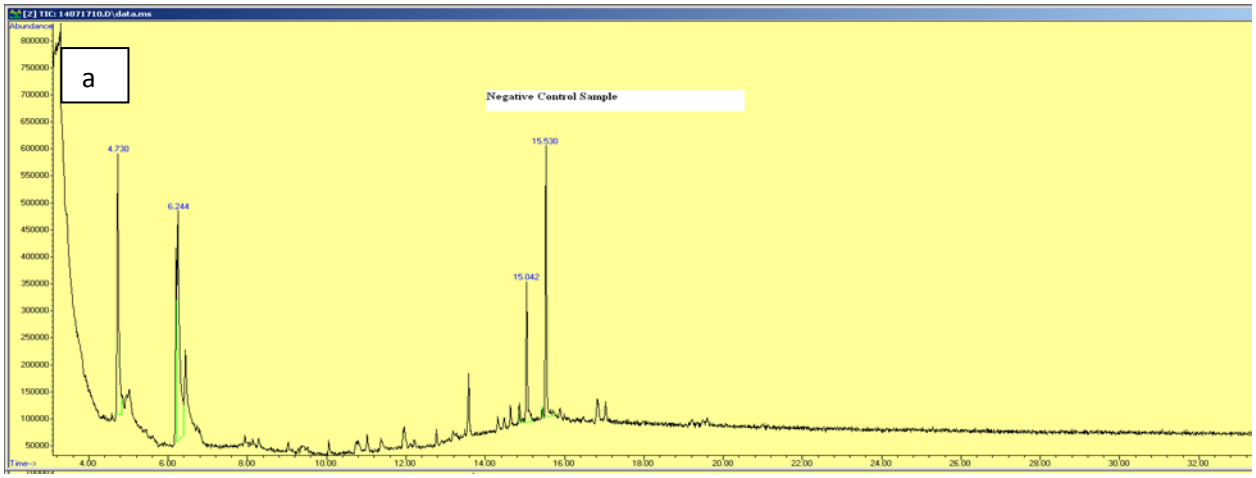


Figure 3: a) Negative Control TIC b) Negative Control QEDIT Report



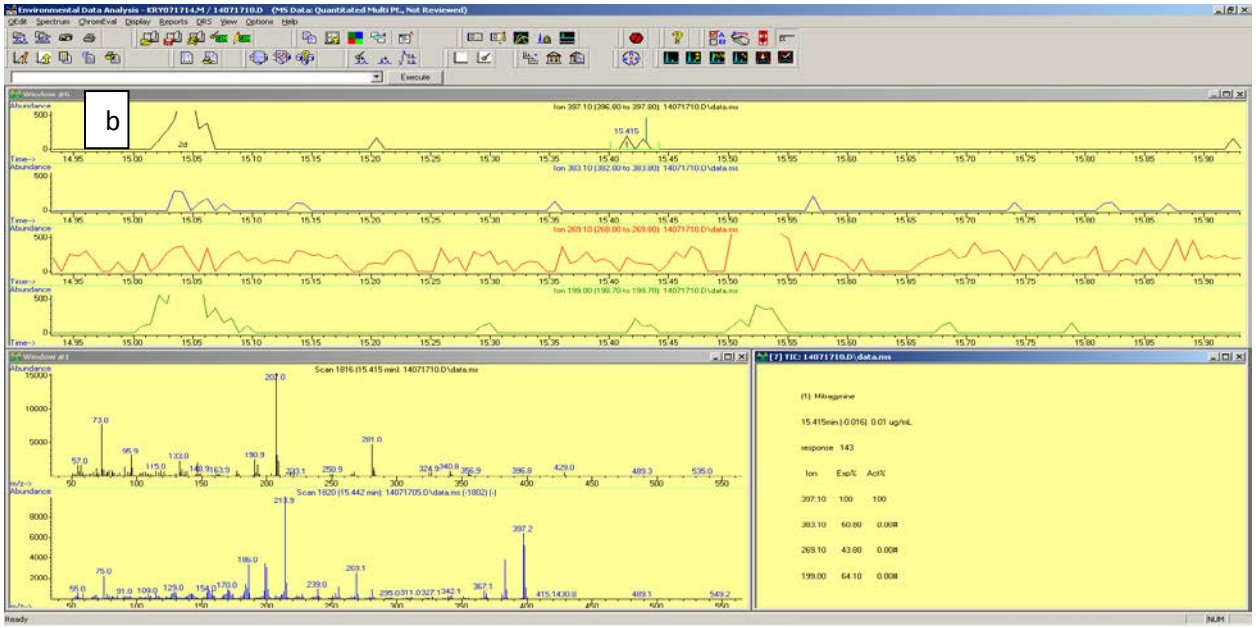
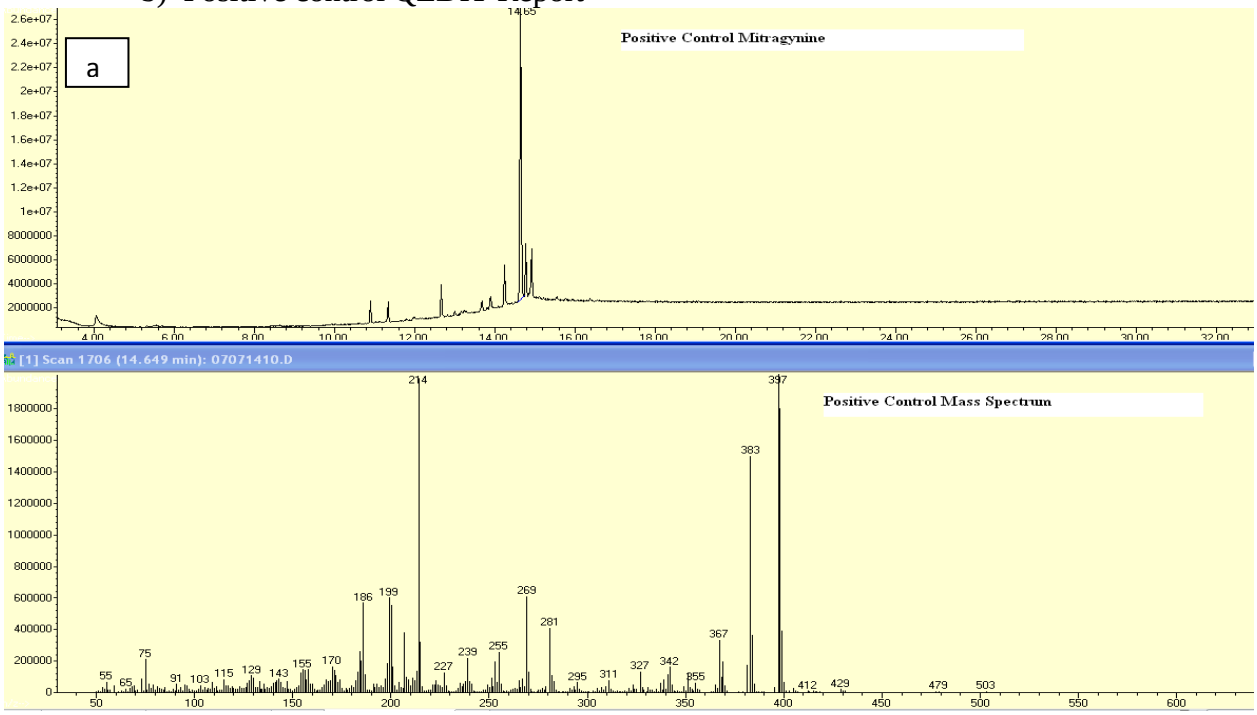


Figure 4: a) Positive control sample TIC and mitragynine mass spectrum postive control
b) Positive control QEDIT Report



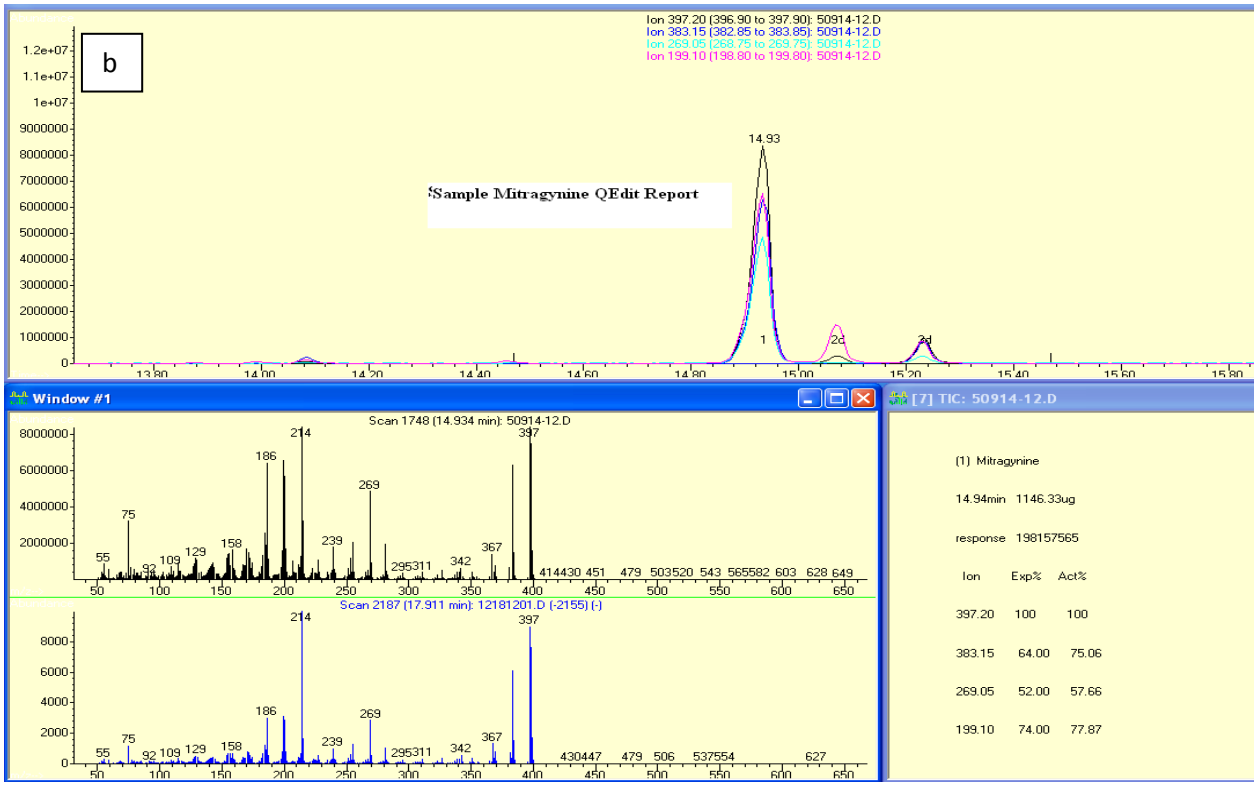
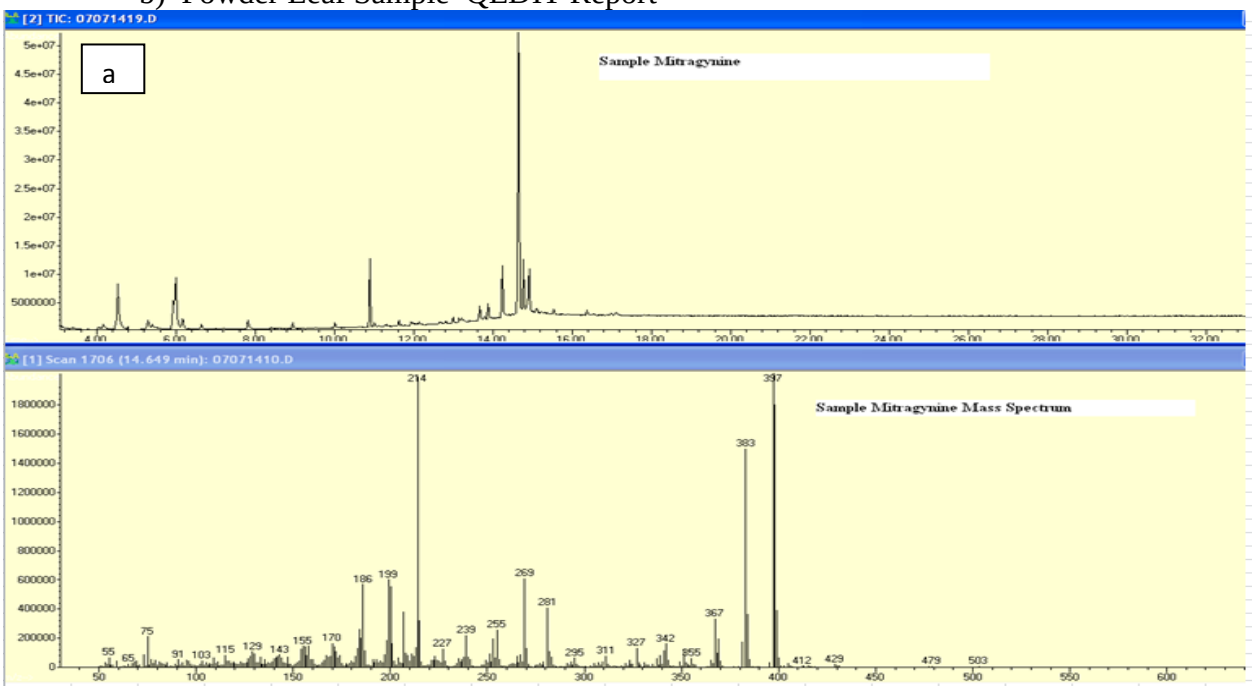


Figure 5: a) Powder Leaf Sample TIC and mitragynine mass spectrum powder leaf sample
b) Powder Leaf Sample QEDIT Report



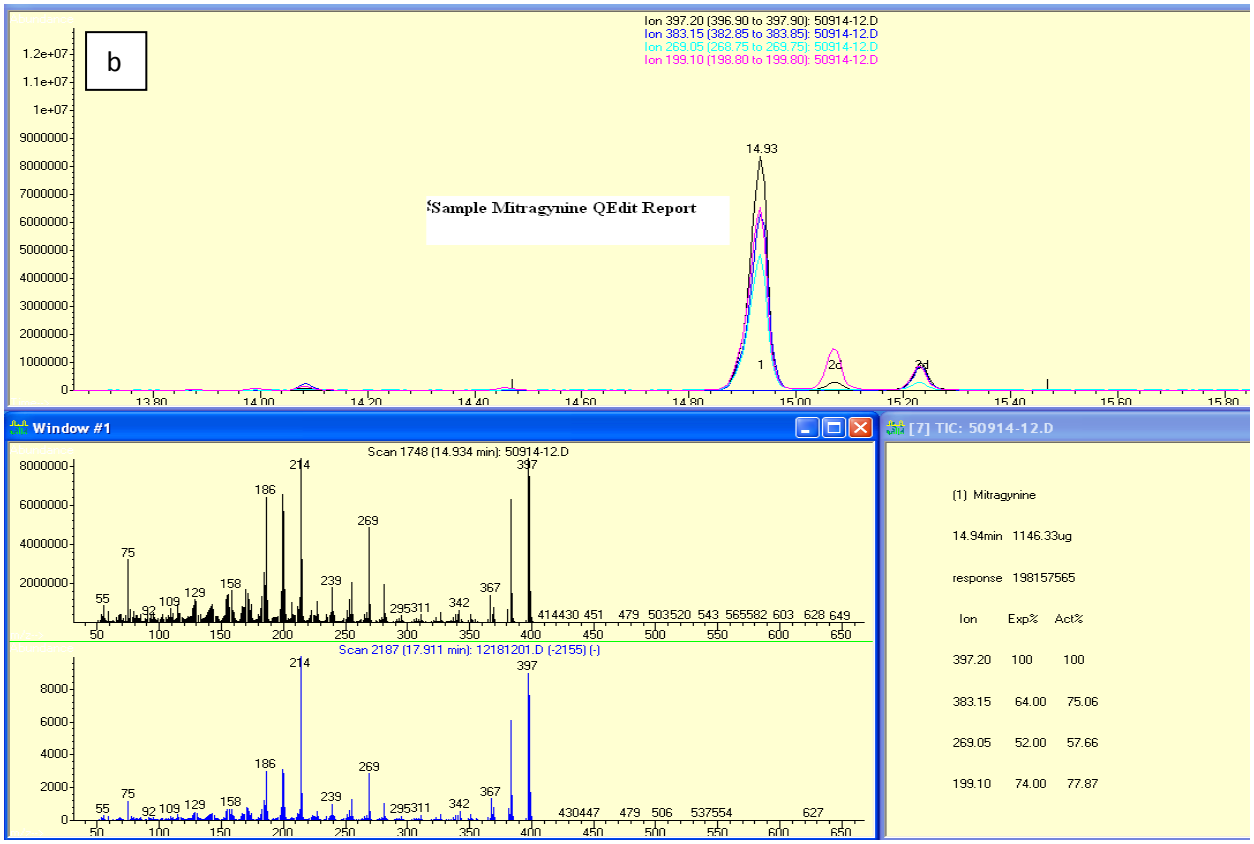


Figure 6: Positive Control Sample TIC and mass spectrum of peak at Rt=15.04

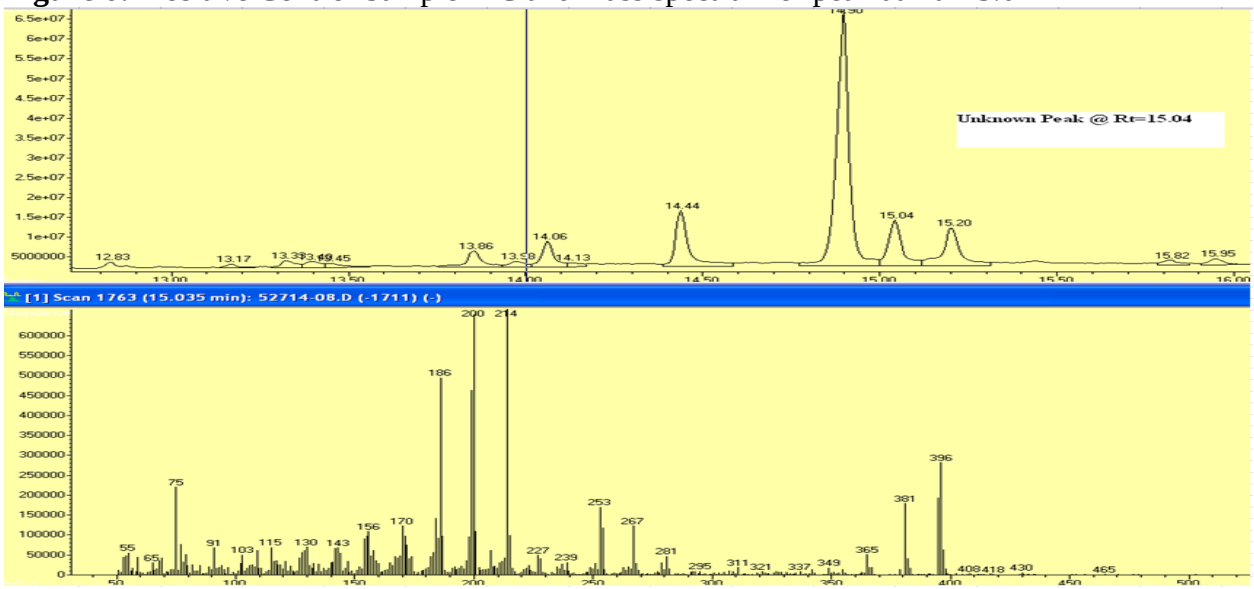
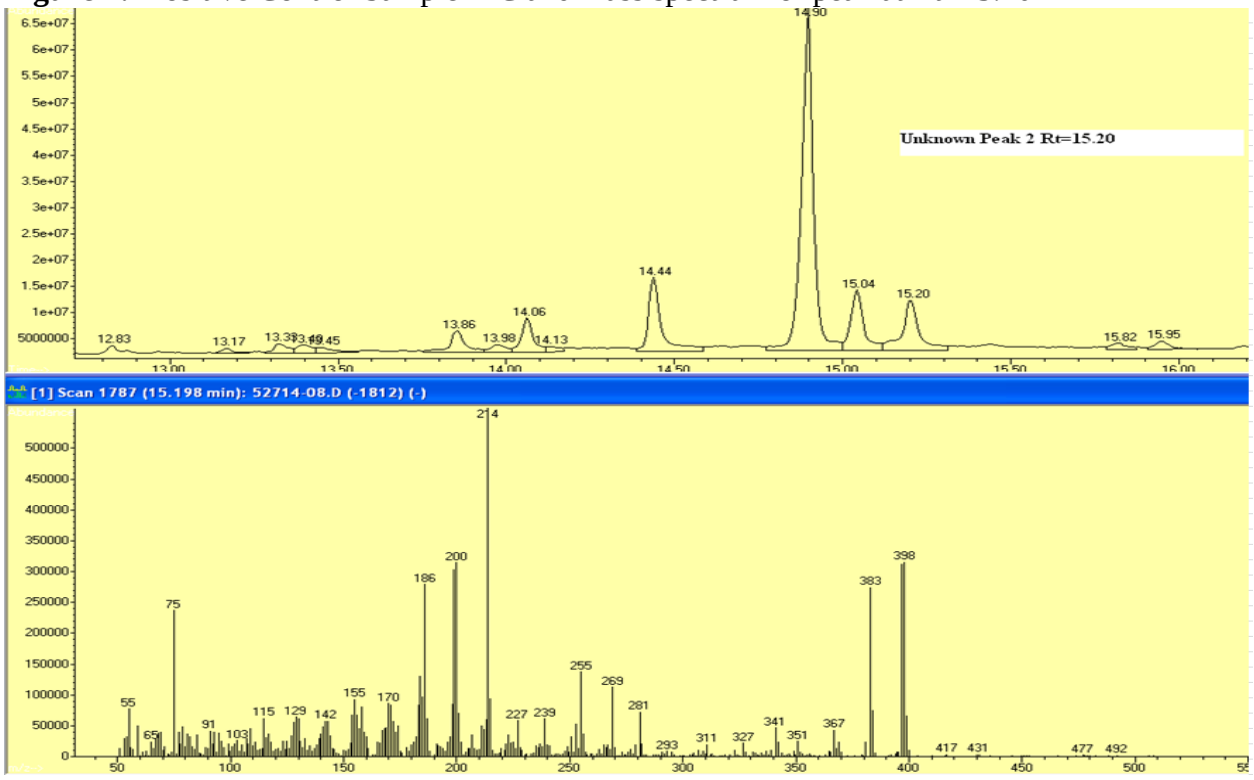


Figure 7: Positive Control Sample TIC and mass spectrum of peak at Rt=15.20



UPLC/PDA Data Analysis

The retention time and UV spectrum was determined by injecting a solvent standard of mitragynine and 7-hydroxy mitragynine, Figure 8 and 9. A negative control was analyzed to ensure mitragynine was not present in the negative control (n=7), Figure 10. A positive control (n=21) was analyzed and mitraginine was present and quantitated in the product obtained via the internet. The retention time of mitragynine was 3.444 minutes and UV spectra purity was used for the quantitation and confirmation of mitragynine in the positive control and samples analyzed for the presence of mitragynine in Kratom. The UV spectrum was similar to spectra published in reference [2] and the UV spectrum obtained for the solvent standard. For quantitation, a five point calibration curve ranging from 1 µg/mL – 10 µg/mL was performed with every batch of samples and must have a correlation coefficient greater than or equal to than 0.995. Sample concentrations demonstrating responses outside the calibration range were diluted for the response to fall within the calibration curve range.

Quality Control was assessd by anlayzing reagent blanks (Figure 11), method blanks, secondary standard preparation, and postive control (Figure 12) analyzed in duplicate. A visual inspection of the positive control sample was perfomred as part of the QA/QC for the UPLC/PDA ssystem to determine suitability of the system for this analysis. The postive sample control contained the mitragynine peak at a Rt=3.44 minutes and three other peaks with the approximate Rt=3.533 minutes, 3.634 minutes, and 3.783 minutes. The peak at 3.533 must have a resolution factor between 1 and 1.5 for the chromatogram to be satifactory for the quantitiation of mitragynine in Kratom. The other two peaks, 3.643 minutes and 3.783 minutes, should demostrate baseline resolution. The purpose of inspecting the chromatogram is due to the complex sample matrix commonly observed in herbal /biotanical products. Figure 14 is an overlay of the mitragynine standard, negative control, postive control, and a sample.

Calculating Concentration of mitragynine

The concentration calculated from the calibration curve reflects the amount of mitragynine in the extract and the sample preparation and dilution needs to be taken into account to calculate the amount of mitragynine in the sample. For example, if the in vial concentration is 8.847 µg/mL, dilution was 50 µL into 1.00 mL (20x), 102.23 mg of sample, 10 mL of extraction solvent, the calculations are as follows:

ug/mL mitragynine in Initial Dilution

$$\frac{8.847\ \mu g}{mL}\ \text{mitragynine} \times \frac{1000\ \mu L}{50\ \mu L} = \frac{176.9\ \mu g}{mL}\ \text{mitragynine}$$

ug/g Mitragynine in Product

$$\frac{176.9\ \mu g}{mL}\ \text{mitragynine} \times \frac{10.00\ mL}{102.23\ mg} \times \frac{1000\ mg}{1\ g} = \frac{17{,}310\ \mu g}{g}\ \text{mitragynine}$$

% mitragynine (w/w)

$$\frac{17{,}310\ \mu g}{g}\ \text{mitragynine} \times \frac{1\ mg}{1000\ ug} \times \frac{1\ g}{1000\ mg} \times 100 = 1.73\ \%\ \text{mitragynine (wt/wt)}$$

Figure 8: Mitragynine Solvent Standard and UV Spectrum

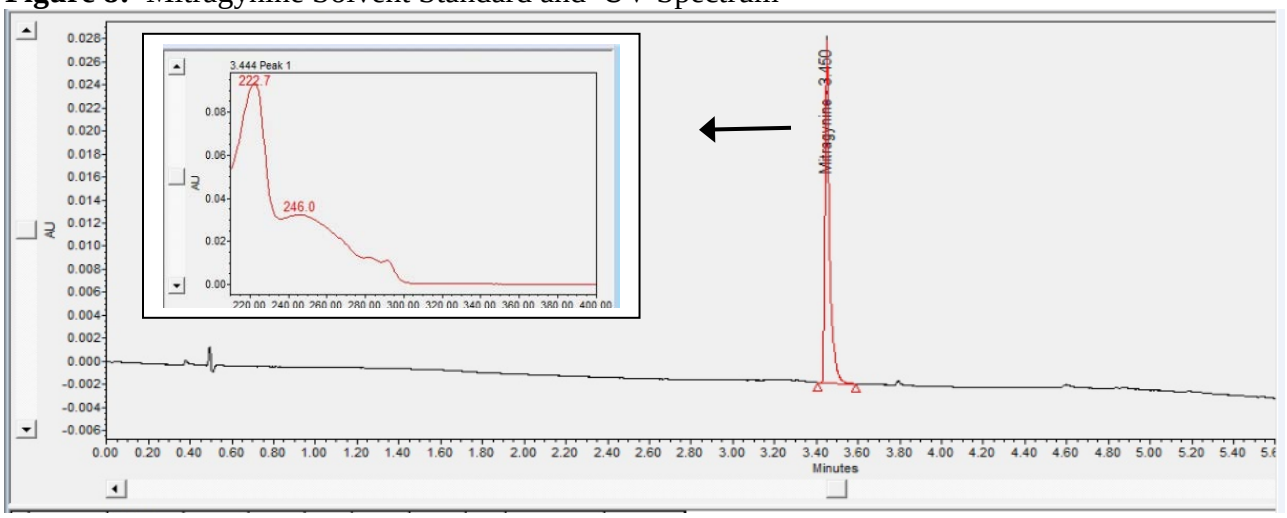


Figure 9: 7-Hydroxy mitragynine Solvent Standard and UV Spectrum

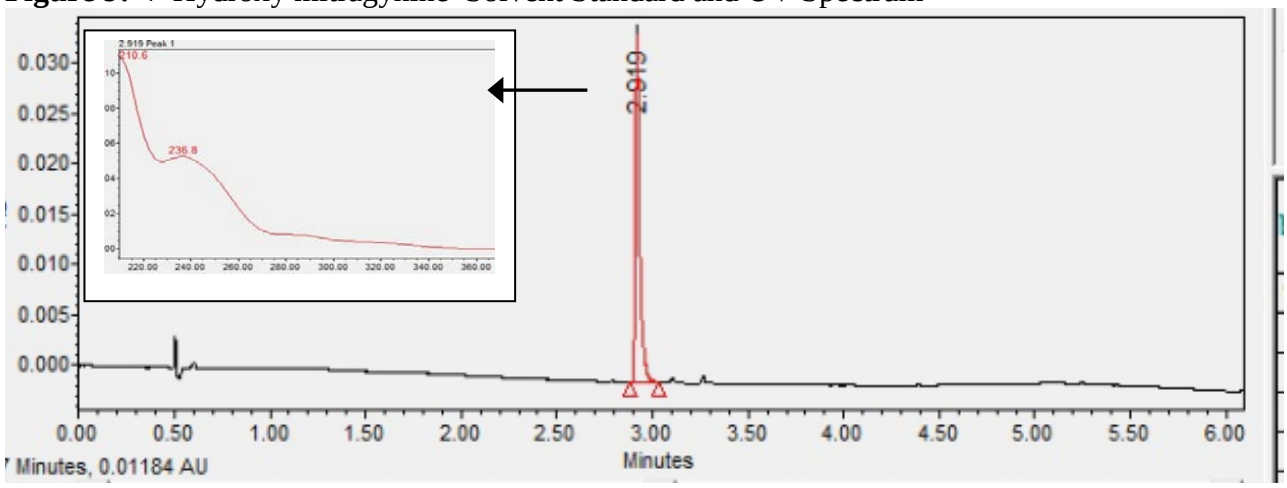


Figure 10: Reagent Blank

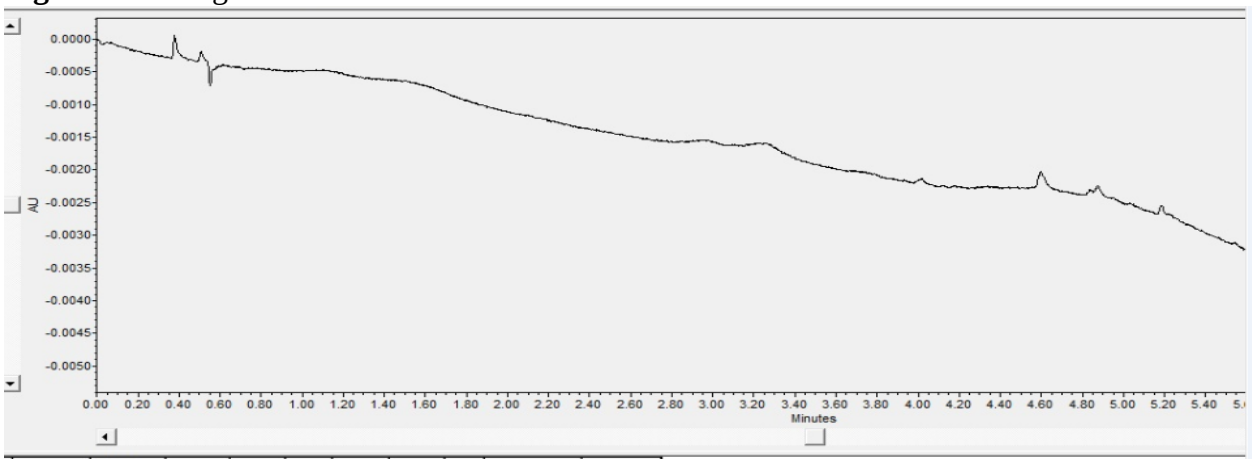


Figure 11: Negative Control

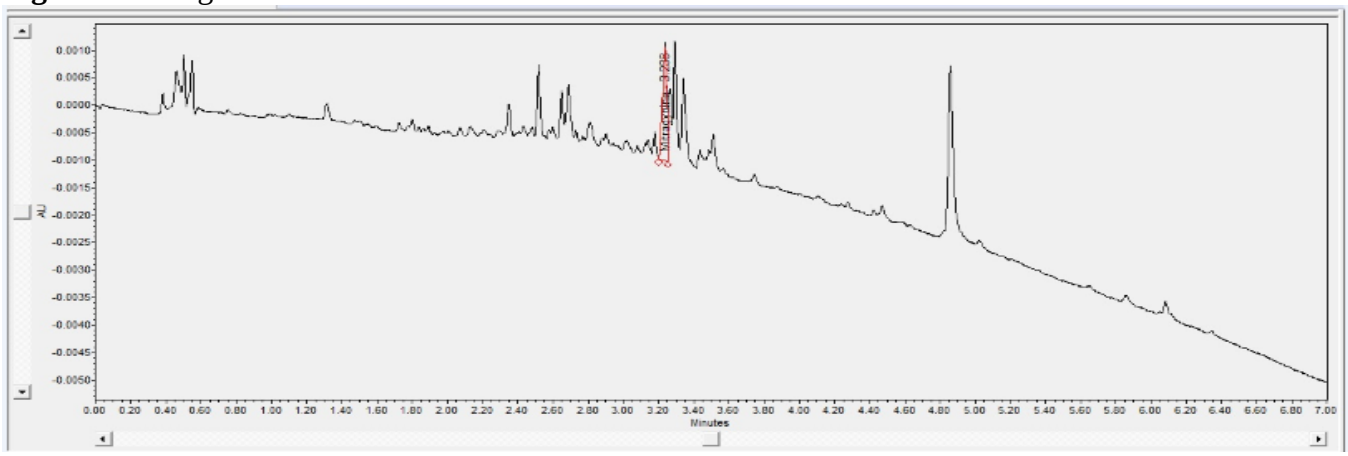


Figure 12: Positive Control and Mitragynine UV Spectrum

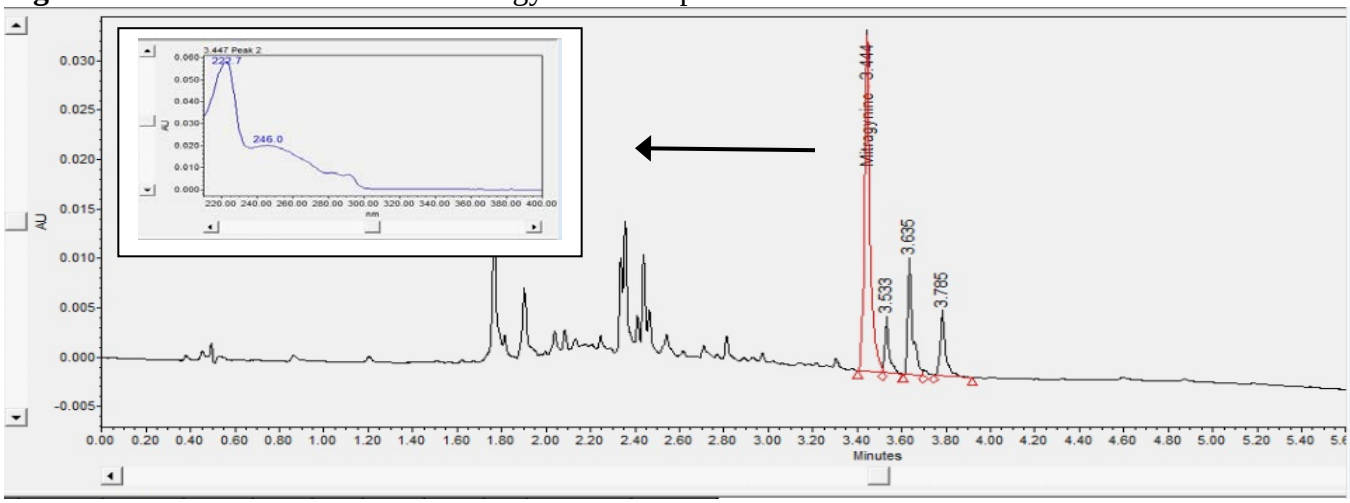


Figure 13: Powder Leaf Sample and Mitragynine UV Spectrum

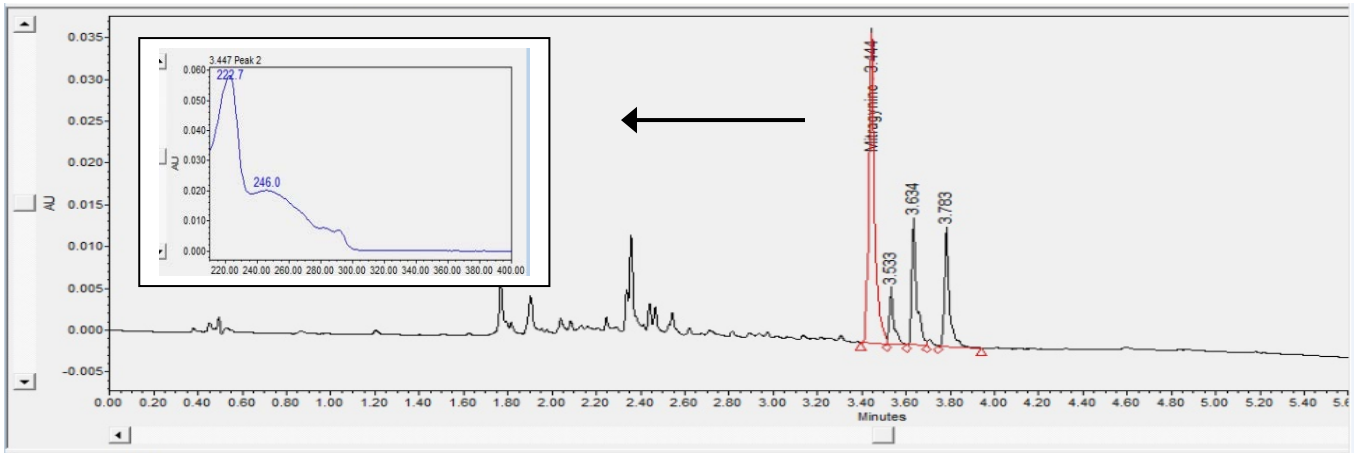
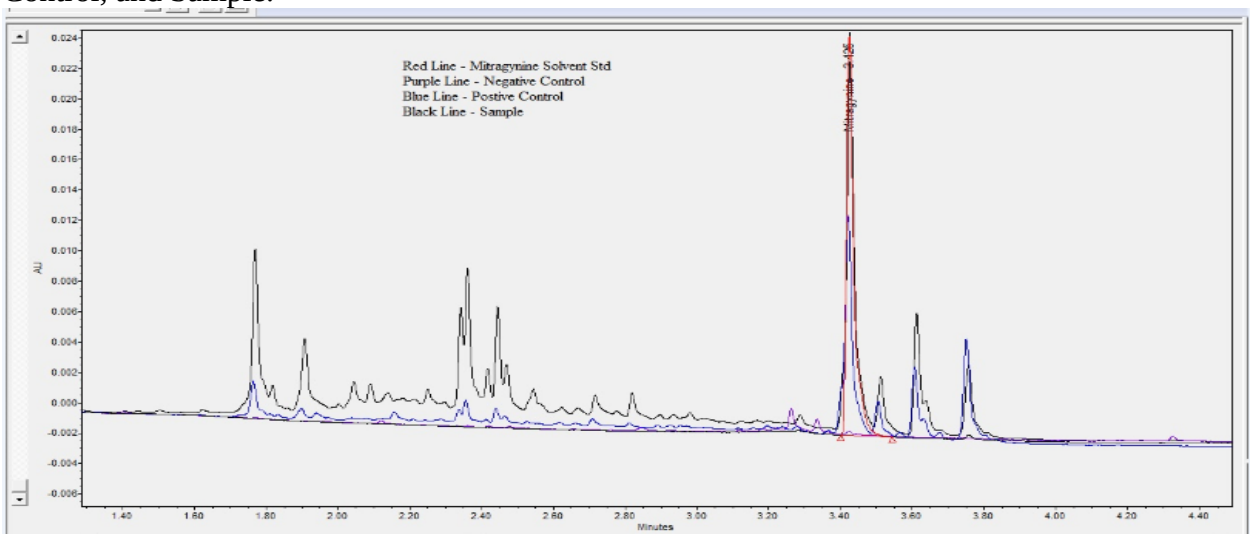


Figure 14: Overlayed Chromatograms of Mitragynine Standard, Negative Control, Positive Control, and Sample.



LC-MS/MS Data Analysis

The LC-MS/MS method was adapted from the procedure published Kikura-Hanajiri et al. **[2].** Sample extraction followed the same process as the UPLC/PDA, but included an extra dilution step. For quantitation, a five point calibration curve ranging from 10 ng/mL –100 ng/mL was performed with every batch of samples and must have a correlation coefficient greater or equal to than 0.995. Sample concentrations demonstrating responses outside the calibration range will be diluted so the response will fall within the calibration curve range. Below is an example of the calculations:

µg/mL mitragynine in initial dilution

$$\frac{49.208\ \text{ng mitragynine}}{\text{mL}} \times \frac{1.00\ \text{mL (100X)}}{0.010\ \text{mL}} \times \frac{1.00\ \text{mL (50X)}}{0.020\text{mL}} = 246{,}000\ \text{ng/g mitragynine}$$

µg/g mitragynine in product

$$\frac{246{,}000\ \text{ng mitragynine}}{\text{mL}} \times \frac{10.00\ \text{mL}}{102.23\ \text{mg}} \times \frac{1000\ \text{mg}}{1\ \text{g}} \times \frac{1\ \text{ug}}{1000\ \text{ng}} = 24{,}063\ \text{µg/g mitragynine}$$

% mitragynine (w/w)

$$\frac{24{,}063\ \text{µg mitragynine}}{\text{g}} \times \frac{1\ \text{mg}}{1000\ \text{µg}} \times \frac{1\ \text{g}}{1000\ \text{mg}} \times 100 = 2.41\ \%\ \text{mitragynine (wt/wt)}$$

For confirmation via the LC-MS/MS method, CVM 118 for scan data was used to determine confirmation of identity along with retention time matching. The ratio of m/z 159, 174, and 238 for mitragynine in the positive control and samples were compared to the solvent standard. The relative abundances from the spectral tabulations in the sample were compared to the standard

and needed to be within 10%; the ions found were in comparison m/z 174 hence the ion ratios are as follows: m/z 159 (68%) and 238 (61%). Mitragynine was confirmed in the positive control and the samples analyzed by the Denver Laboratory. The use of the TSQ provided the selectivity and sensitivity and mitragynine was not detected in the solvent blank and negative control.

Figure 15: a) Mitragynine Solvent Standard - Total Ion Chromatogram (TIC)
b) Mitragynine Reconstructed Ion Chromatogram (RIC)

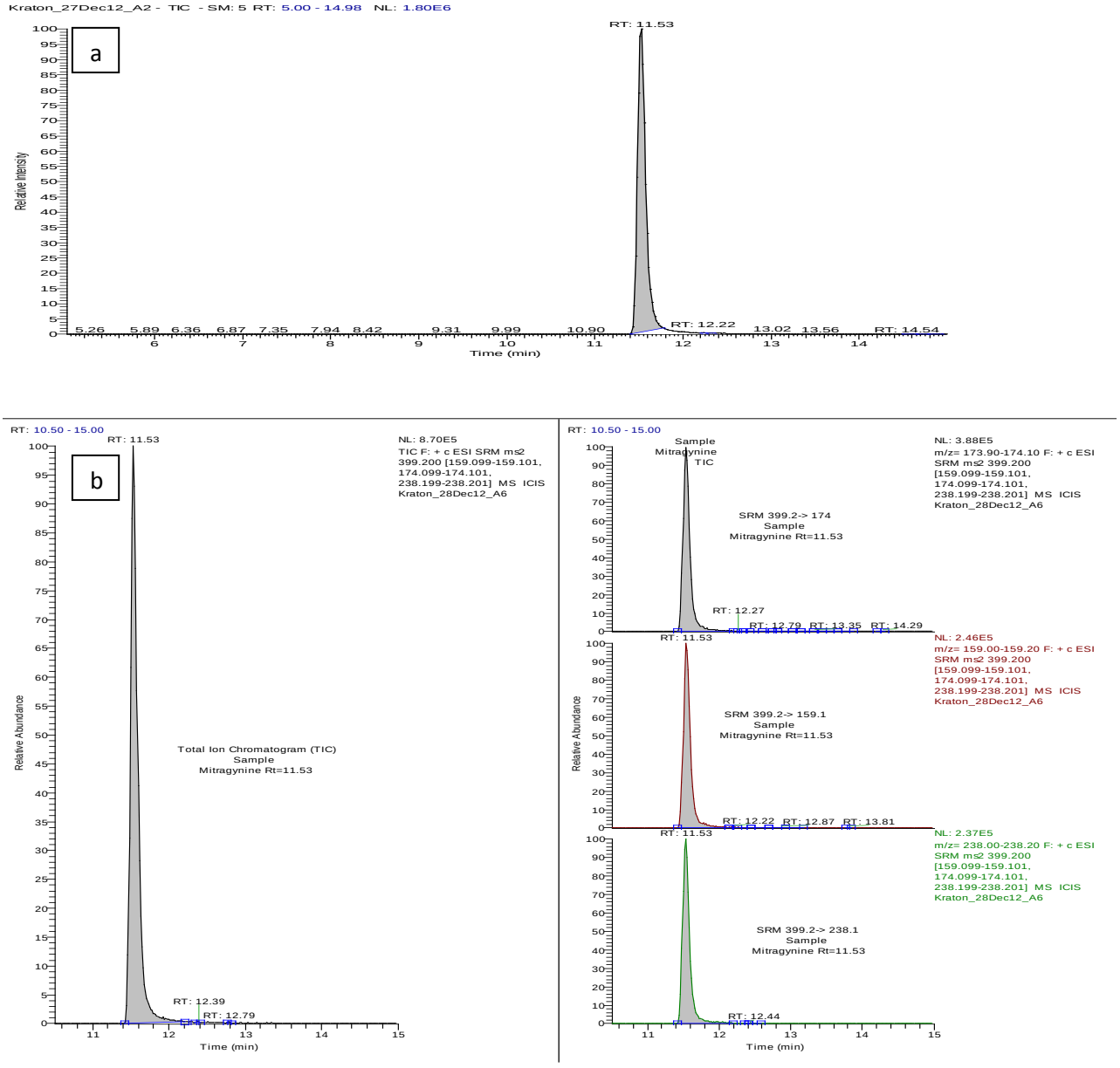
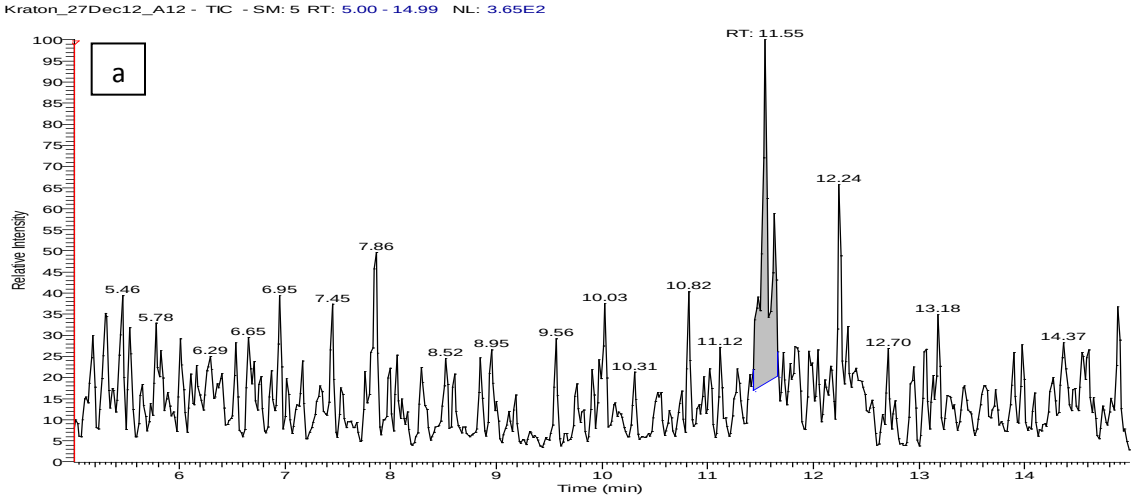


Figure 16: a) Negative Control - Total Ion Chromatogram (TIC)
b) Negative Control Reconstructed Ion Chromatogram (RIC)



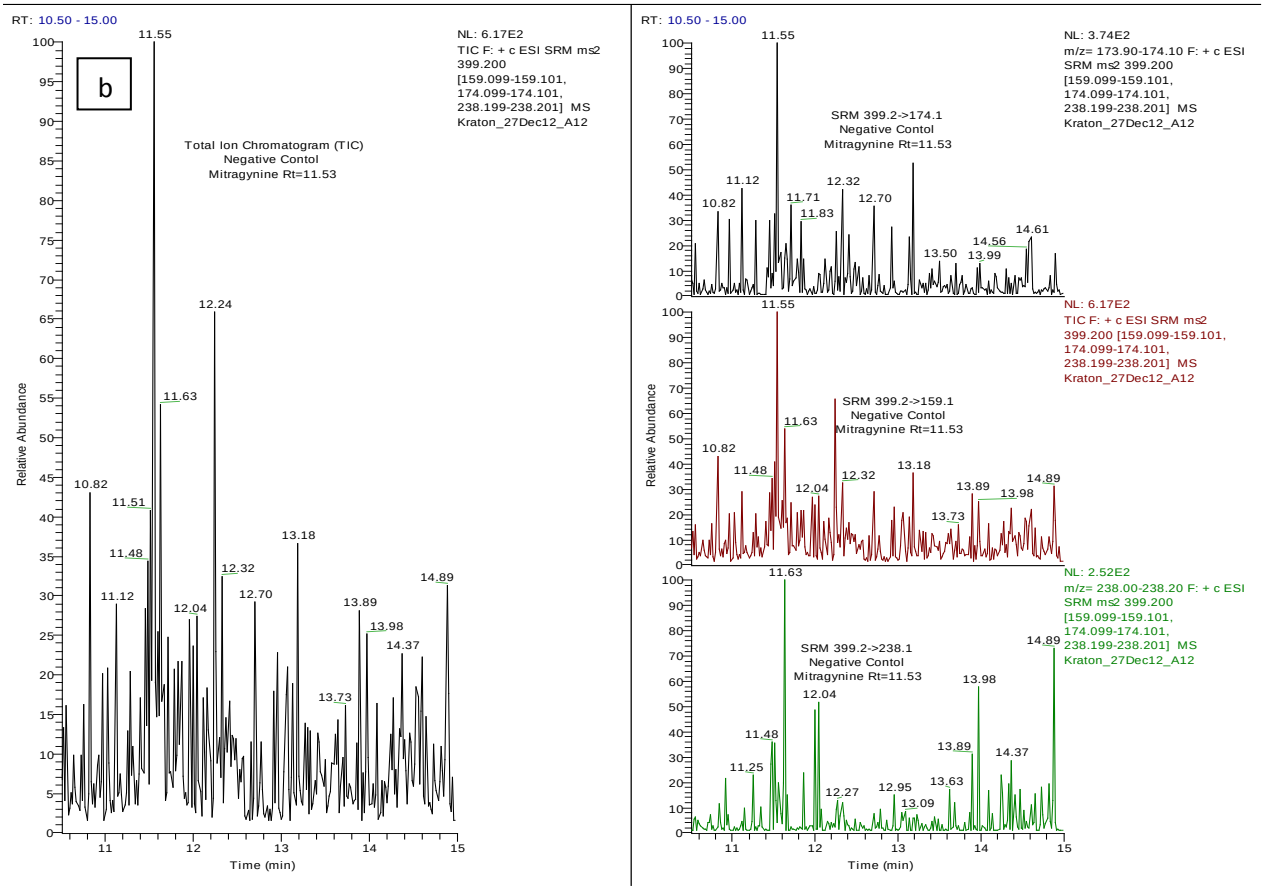


Figure 17: a) Positive Control - Total Ion Chromatogram (TIC)
b) Positive Control - Reconstructed Ion Chromatogram (RIC)

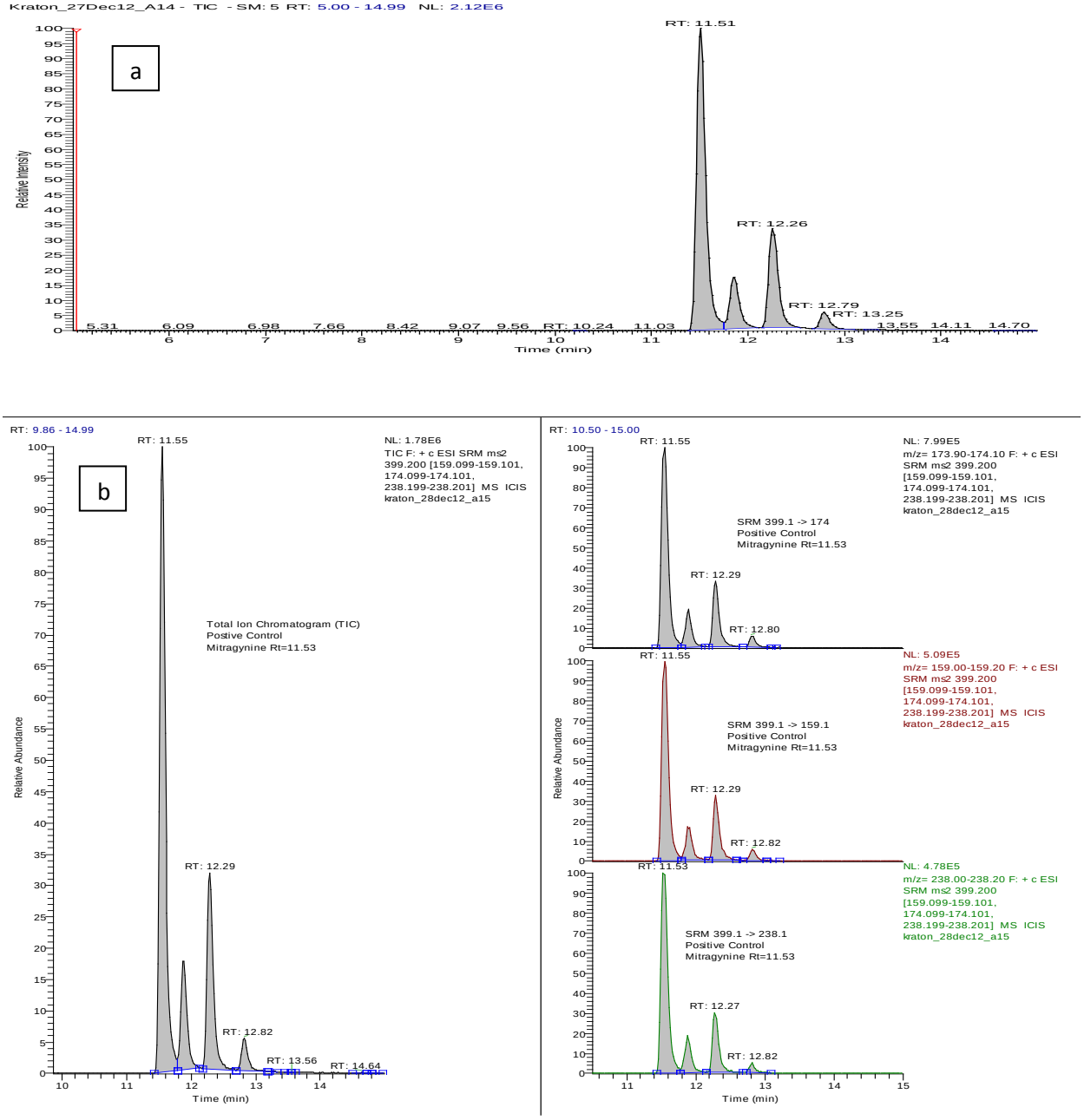
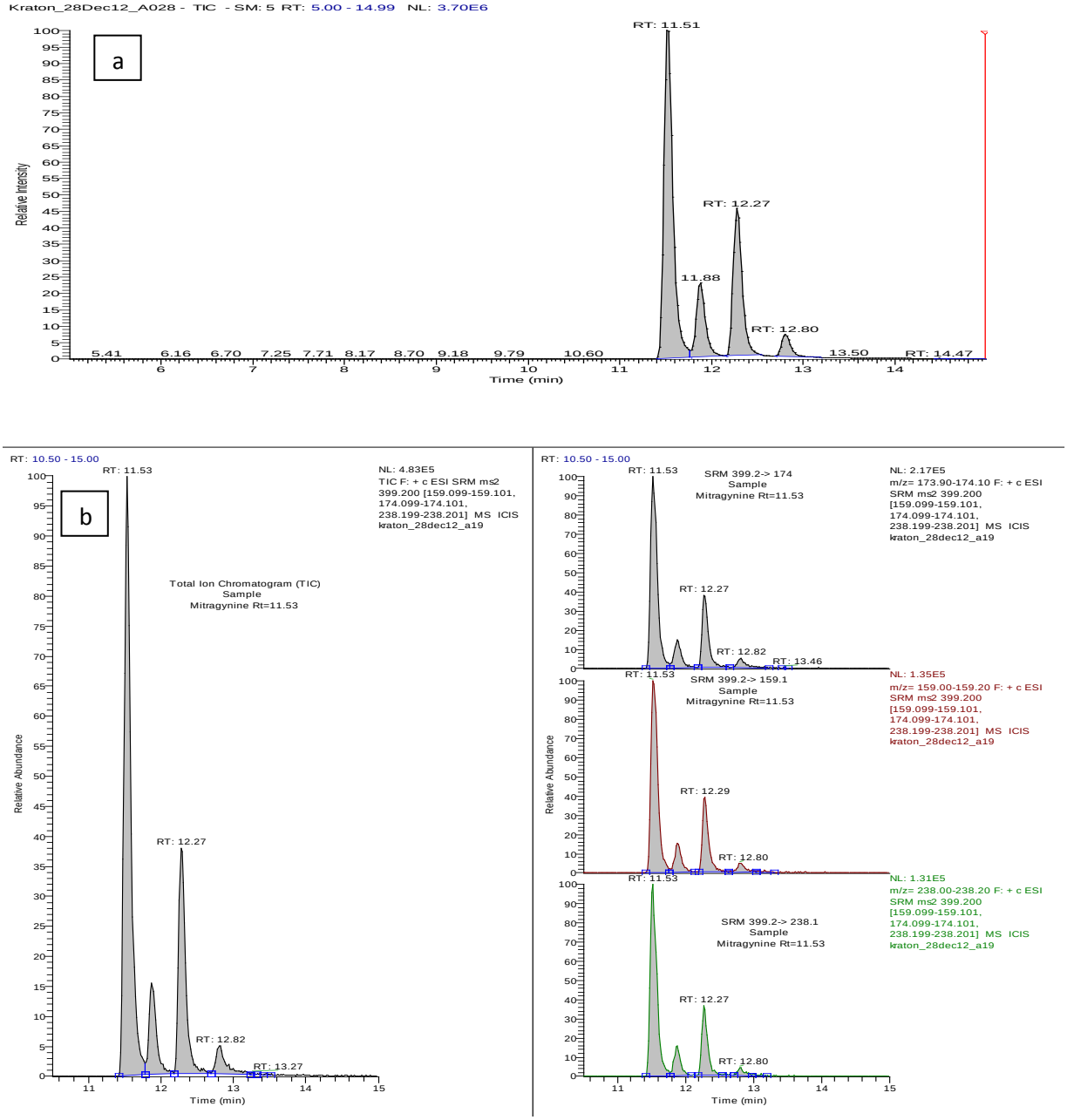


Figure 18: a) Sample Dried Powder Leafs - Total Ion Chromatogram (TIC)
b) Sample Dried Powder Leafs - Reconstructed Ion Chromatogram (RIC)



Method Validation

UPLC/PDA and LC-MS/MS

Mitragynine standard was used for the identification, confirmation and quantitation for the determination of Kratom. Due to the low concentration of the mitragynine stock standard (100 µg/mL) and the high level of mitragynine in the samples, traditional spiking of the standard via a wet/dry spike into a negative control was not feasible. Different types of commercial products that claimed to contain Kratom were purchased via the Internet in 2012 and analyzed for

mitragynine. Validation was performed by characterizing a dry leaf Kratom product and extracting this material seven times a day over a three day period for a total number of extraction n=21, refer to Table 4. Once the positive control was characterized, the same positive control was extracted in duplicate (n=2) for every analytical batch. The UPLC/PDA data demonstrated a mean value of 1.041% (n=21, 4.2%) and the LC-MS/MS 1.140% (n=14, 6.81%) for mitragynine in the positive control.

Table 4 : Validation Data of Mitragynine in Positive Control

Day	Mean % (RSD) UPLC	Mean % (RSD) LC-MS/MS
1 (n=7)	1.061 (3.6%)	1.156 (5.95%)
2 (n=7)	1.056 (3.1%)	1.125 (7.82%)
3 (n=7)	1.004 (4.4%)	n/a
Overall (n=21, 14)	1.041 (4.2%)	1.1403 (6.81%)

The same positive control was used for all the analysis of products received by the Denver Laboratory. Table 5 is a summary of three different chemists extracting the positive control over a 1 year time frame and analyzed on the UPLC/PDA. The overall results demonstrate the extraction procedure generates reproducible results and the positive control is homogenous.

Table 5: Positive Control over 2 years and different analysis

Batches	Analyst	Control	% Mitragynine
1	A	Positive Control	1.177
		Positive Control Duplicate	1.110
2	A	Positive Control	1.182
		Positive Control Duplicate	1.110
3	B	Positive Control	1.092
		Positive Control Duplicate	1.321
4	B	Positive Control	1.241
		Positive Control Duplicate	1.207
5	C	Positive Control	1.101
		Positive Control Duplicate	1.131
		Mean (n=10)	1.1672
		sd	0.074
		%RSD	6.31

The same set of samples were extracted and analyzed via both the UPLC/PDA and the LC-MS/MS. The results for the amount of mitragynine are similar; hence the LC-MS/MS method can be used for the confirmation and quantitation of mitragynine in Kratom.

Table 6: Comparison of UPLC/PDA and LC-MS/MS

Sample	Product Type	UPLC/PDA % (wt/wt)	LC-MS/MS % (wt/wt)	% Difference
Positive Control		1.025	0.973	5.07
Positive Control Duplicate		0.985	0.919	6.67
1	Powder	0.540	0.536	0.74
2	Leaf Powder	0.708	0.699	1.27
4	Dry Incense	3.540	3.100	12.4
5	Powder of Natural Dye	1.015	0.859	15.4
6	Mitragyba Javanica	1.440	1.215	15.6
7	Kulit Kaya	0.681	0.620	8.96
8	Armatopic Herb	1.412	1.064	24.6

Results of Samples Tested

From 2012 to 2014, samples were submitted to the Denver Laboratory ranged from small packets of drinks, capsules, tea leaves, powdered leaves and spent leaves from a manufacturing processing facility. Denver analyzed over 30 samples and Table 7 is a summary of the results.

Table 7 : Results of Samples Tested

Sample Number	Sample	Confirmation GC/MS	Quantitation UPLC
1	Powder	Y	0.54%
2	Leaf Powder	Y	0.71%
3	Powder	Y	0.52%
4	Dry Incense	Y	3.54%
5	Powder of Natural Dye	Y	1.02%
6	Mitragyba Javanica	Y	1.44%
7	Kulit Kaya	Y	0.68%
8	Armatopic Herb	Y	1.41%
9	Liquid Shot Material	Y	241 µg/mL
10	Liquid Shot Material	Y	241 µg/mL
11	Liquid Shot Material	Y	237 µg/mL
12	Liquid Shot Material	Y	223 µg/mL
13	Kratomite	Y	287 µg/mL
14	Kratomite	Y	213 µg/mL
15	Kratomite	Y	201 µg/mL
16	Kratomite	Y	200 µg/mL
17	Kratomite	Y	200 µg/mL
18	Kratomite	Y	189 µg/mL
19	Kratomite	Y	190 µg/mL
20	light green powder	Y	1.54%
21	Kratom	Y	308 ug/mL
22	Dietary Supplement	Y	1.64%
23	Dietary Supplement , capsule	Y	1.68%
24	Dietary Supplement , capsule	Y	1.60%
25	Dietary Supplement , capsule	Y	1.67%

Sample Number	Sample	Confirmation GC/MS	Quantitation UPLC
26	Liquid Extract	Y	1.56%
27	Biotanical Extract powder	Y	1.86%
28	gelatin capsule, powder	Y	1.74%
29	Suspension Oil	Y	0.495%
30	Spent Herbs - Solid Material		
a	Sub 1 # 009	Y	0.049%
b	Sub 2 #003	Y	0.076%
c	Sub2 # 004	Y	0.060%
d	Sub 2 #010	Y	0.101%
e	Sub 2 #011	Y	0.166%
f	Sub 3 #005	Y	0.173%
g	Sub 3 #006	Y	0.174%
h	Sub 3 #012	Y	0.056%
i	Sub 3 # 013	Y	0.080%
j	Sub 4 #007	Y	0.038%
k	Sub 4 # 014	Y	0.037%
31	Brown Liquid	Y	84.95 ug/mL
32	Mitragynine Extract	Y	34.65%
33	Brown Liquid	Y	198 ug/mL
34	Brown Liquid	Y	1.32%
35	Liquid Supplemental Pack	Y	398.1 ug/mL

CONCLUSION

This LIB describes the method development and validation for the detection, quantification, and confirmation of identity for mitragynine in products containing Kratom (*Mitragyna speciosa).* The GC/MS is the primary technique for the initial qualitative determination (screening) of mitragynine in Kratom. Products resulting in a positive response to mitragynine are quantified via the UPLC/PDA. A secondary analytical technique, LC-MS/MS, was validated and used for qualitative identification and quantitation of mitragynine in Kratom products.

REFERENCES

1. Asian leaf 'kratom' making presence felt in US emergency rooms, *NBC News U.S. News Monday* Mar 19, 2012 1:48 PM

2. Kikura-Hanajiri R., Kawamura M., et al *Forensic Toxicoogy*, 27: 2009, 67-74

3. Chan K.B., Pakiam C., Rahim R.A. *Bulletin on Narcotics, vol. LVII, Nos. 1 and 2, 2005, 249-256*

4. US FDA/ Center for Veterinary Medicine *Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues,* Guidance for Industry #118; 2003

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AOAC SMPR 2015.008 – Method Requirements for Alkaloids of Mitragyna Speciosa

The FDA publishes a Laboratory Information Bulletin (LIB) for the quantitative and qualitative analysis of Mitragynine in Kratom. The method was original validated in 2012 and does not have all the requirements requested AOAC SMPR 2015.008. The table below addresses the requirements specified in Table 1 of AOAC SMPR 2015.008 and gives explanation as to why some of these requirements were not addressed in the FDA LIB 4578.

AOAC SMPR requirement	Explanation
Quantitative for 7-hydroxymitragynine	1 - No, at the time of the method validation (2012), 7-hydroxymitragynine was available but expensive. 2- The assay was developed to determine the amount of mitragynine in various forms of dietary supplements. The quantitation range was developed due to the fact that mitragynine was available at 100 ug/mL. To run the assay for 7-hydroxymitragynine could possible contaminates the UPLC/PDA system.
Single Laboratory Validation: Calibration Range: LOQ: LOD:	 1.00 ug/mL – 10.0 ug/mL corresponding to a sample concentration range of 0.01% - 1.00%. 0.01% 10 ppm
Single Laboratory Validation: Recovery:	Due to the low level of the mitragynine standard at the time of validation, spikes were not performed. The Denver Laboratory acquired a Kratom Thai Leaf product and characterized the product. This was used as a positive control and extracted with ever analytical run. The recovery for the mitragynine compared to the concentration determined in the positive control was 94% - 107%.
Single Laboratory Validation: Repeatability:	4.4% for all concentrations of product