Phase I Metabolism of Mitragynine using In Vitro Methods

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Disclaimer

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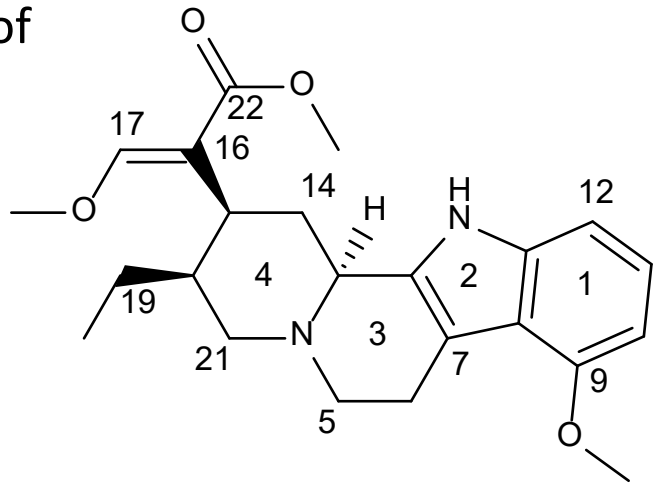
Mitragynine

- The main psychoactive compound in kratom
  - A powder, resin, or leaf cuttings from the leaves of *Mitragyna speciosa*
- Mitragynine is a potent μ-agonist
  - Potency is one-fourth that of morphine
- Originally a cultural practice in Southeast Asia, its use is increasing in the western world
  - Used recreationally as an opiate replacement and for the non-medically supervised treatment of opiate addiction
- Currently, kratom is unscheduled in the United States at the federal level
  - State legislation varies



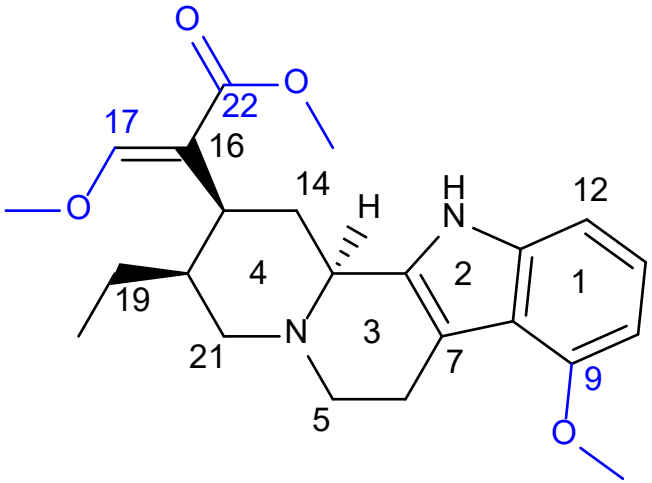
Pharmacology

- Kratom's effects are dose-dependent
  - Low doses produce a stimulant effect
  - High doses produce opiate-like effects
- In rats, doses of over 1000 mg/kg, increased blood pressure and caused severe hepatotoxicity and nephrotoxicity
  - Decreased body weights, behavioral changes and changes in blood chemistry
- In mice, mitragynine's opiate like effect for reducing stress has shown potential for addiction and abuse
  - ED_{50} 22 mg/kg
  - LD_{50} 477 mg/kg

Metabolism

- **Zarembo et al.1974:**
  - *Helmin-thosporum sp*
  - Oxidation and hydroxylation were the primary metabolic routes

- **Philipp et al. 2009:**
  - In vivo study in human and rats
  - Hydrolysis of the methylester groups (16 position)
  - Demethylation of the methoxy groups (9 and 17 positions)
  - Identified a number of hydroxylated metabolites



Metabolism

- Role of CYP450 isoenzymes has not been fully investigated

- **Manda et al. (2014, 2017):**
  - Mitragynine could have drug-drug interactions with compounds that are P-glycoprotein substrates
  - Activate the pregnane X receptor which could increase the activity of 3A4 and 1A2

- **The purpose of this study was to identify the specific CYP450 isoforms involved in the metabolism of mitragynine**

Experimental Design

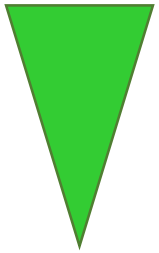
- Microsomal Incubation
  - 50 µM Mitragynine (500 µL reaction)
  - Bactosomes – eight P450s
    - rCYP1A2, rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C18, rCYP2C19, rCYP2D6, and rCYP3A4
  - Control bactosomes (no P450 gene)
  - Blanks (enzyme added but no drug)
  - 500 µL reaction volume
    - 100 µM pH 7.4 potassium phosphate buffer
    - 50 pM enzyme
    - 1.3 mM NADP+
    - 3.3 mM glucose-6-phosphate and magnesium citrate
    - 0.4 U/mL glucose-6-phosphate dehydrogenase

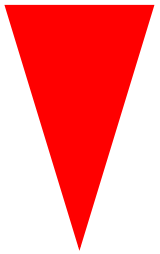
- Inhibition Assay
  - 20 µM Ketoconazole
    - rCYP3A4, rCYP2C18, rCYP2C19
  - 20 µM Fluvoxamine
    - rCYP2D6

Incubation Setup

Phosphate buffer (pH 7.4)
50 µM Mitragynine
NADP+
Glucose-6-phosphate
Magnesium citrate
Glucose-6-phosphate dehydrogenase

Phosphate buffer (pH 7.4)
50 µM Mitragynine
NADP+
Glucose-6-phosphate
Magnesium citrate
Glucose-6-phosphate dehydrogenase
Inhibitor





Pre-incubate 5 min at 37°C
Add enzyme

Uninhibited

Inhibited

Time Stops: 0, 60, 120, 180, and 240 minutes
Remove 25 µL and add to 25 µL of cold acetonitrile with 5 µM mitragynine-D_3 (Internal Standard)

Centrifuge for 3 minutes (4◦C and 10,000G)
Reconstitute supernatant (1:1) in 50:50 mobile phase

LC/Q-TOF-MS Method

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Identification of five Mitragyna alkaloids in urine using liquid chromatography-quadrupole/time of flight mass spectrometry

Stephanie Basiliere, Kelsie Bryand, Sarah Kerrigan

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LC/Q-TOF-MS Conditions

Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS

LC Separation

- Poroshell 120 EC-C18 Column (2.1x100 mm, 2.7 µm)
- Mobile Phase A: 5 mM Ammonium Acetate in DiH_20
- Mobile Phase B: Acetonitrile
- Flow Rate: 0.4 mL/min
- LC Gradient: 20% B (0.5 min); 20-90% B (0.5-10 min)

Q/TOF Parameters

- Gas Temperature: 350˚C
- Gas Flow Rate: 13 L/min
- Sheath Gas Temperature: 400˚C
- Sheath Gas Flow Rate: 12 L/min
- Nebulizer Pressure 45 psig

Acquisition

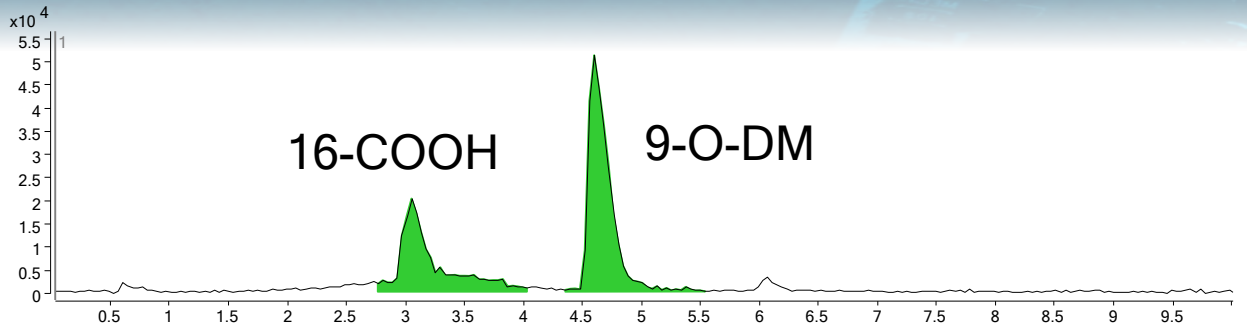
- Full Scan Auto
- Targeted Mode (7-MG-OH)
- Run Time: 10 minutes
- MS Scan Rate: 8 spectra/sec
- MS/MS Scan Rate: 2 spectra/sec
- MS Scan Range: 100-1000 m/z
- ESI Mode: Positive

MS

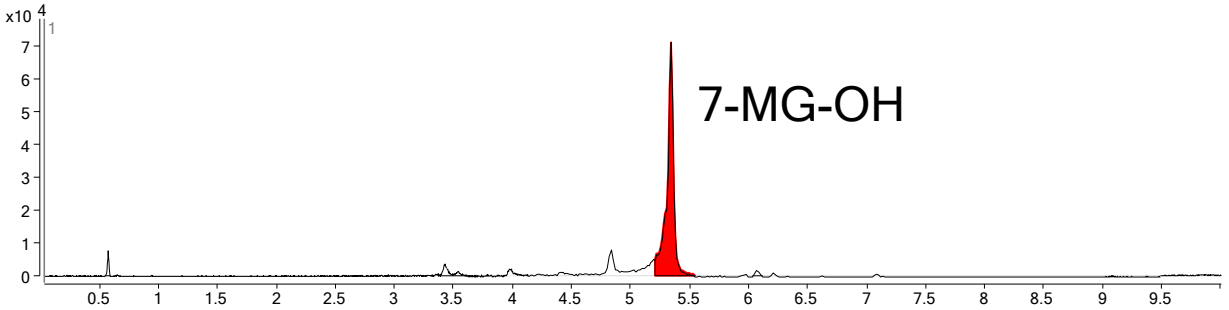
- Capillary Voltage: 4000 V
- Fragmentor Voltage: 150 V
- Nozzle Voltage: 0 V
- Collision Energy: 25 eV, 27, eV 28 eV

Extracted Ion Chromatograms

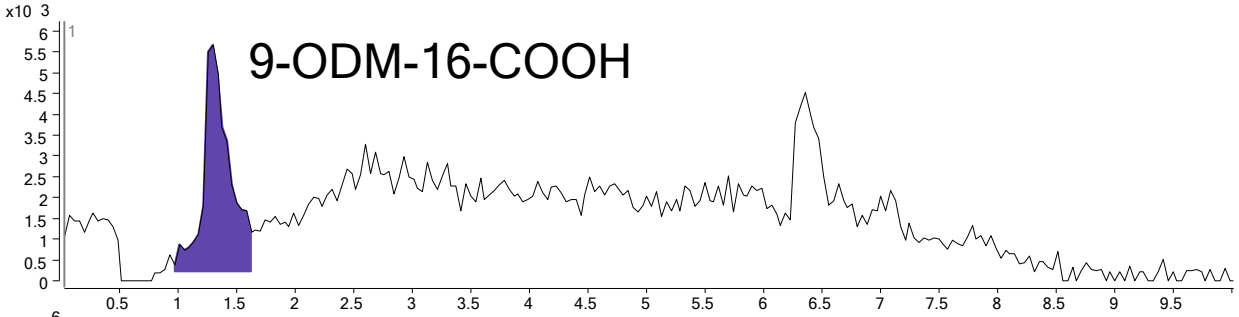
$[M+H]^+$ 385.2122



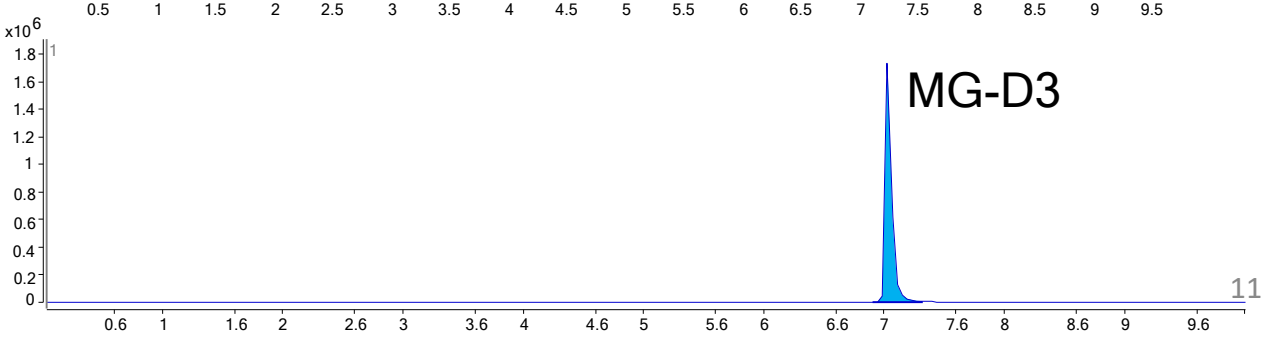
$[M+H]^+$ 415.2227



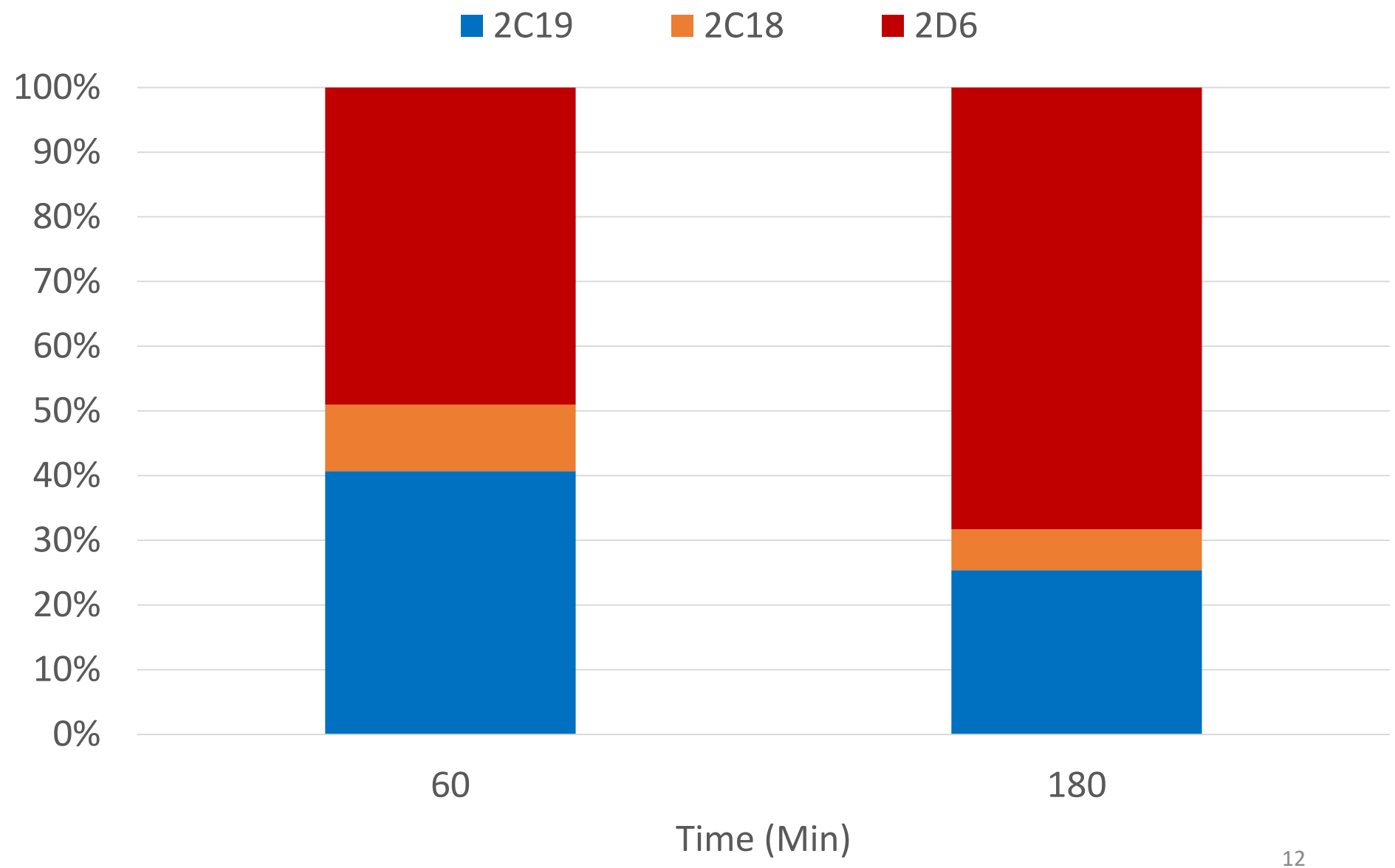
$[M+H]^+$: 371.1965



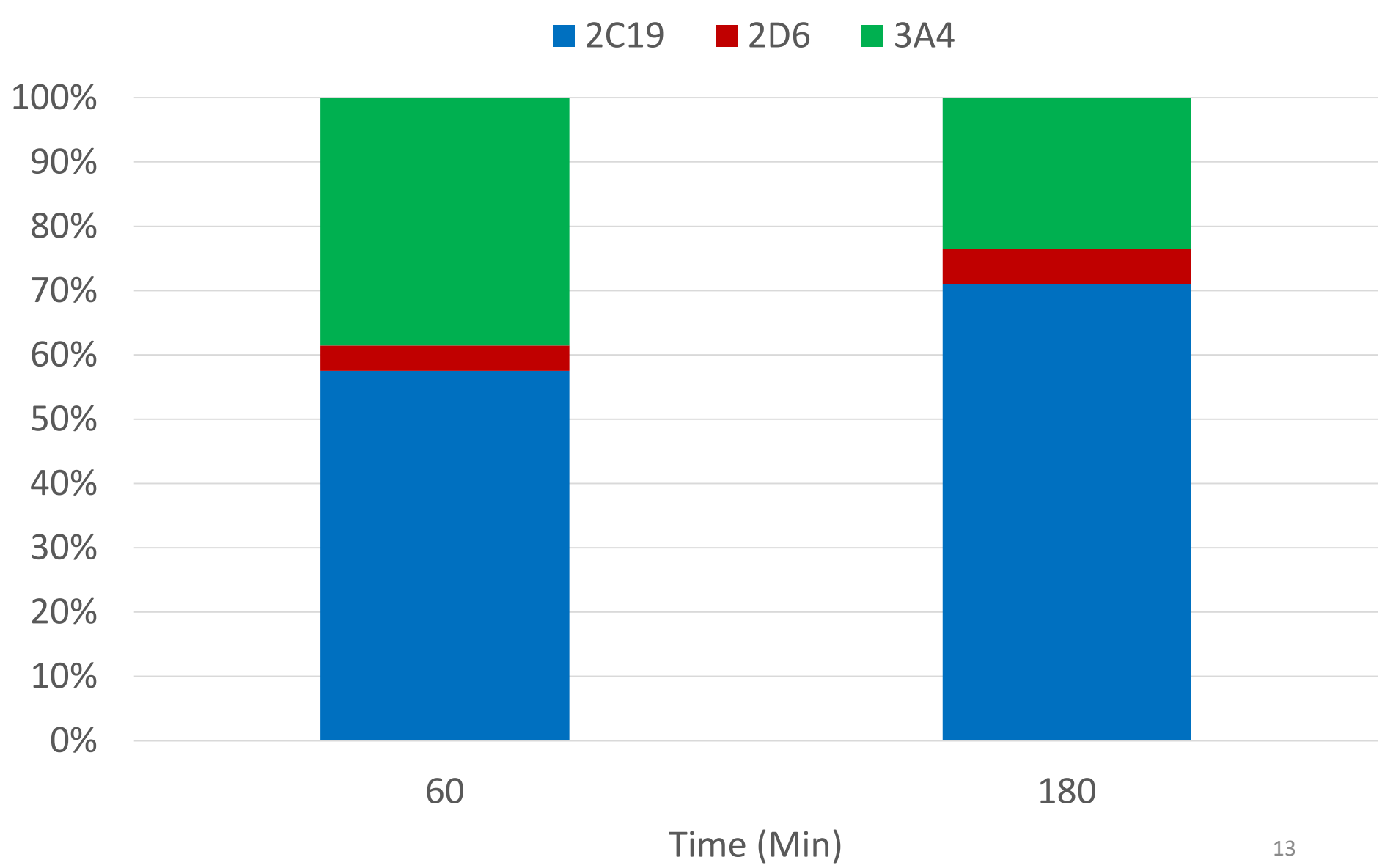
$[M+H]^+$: 402.2467



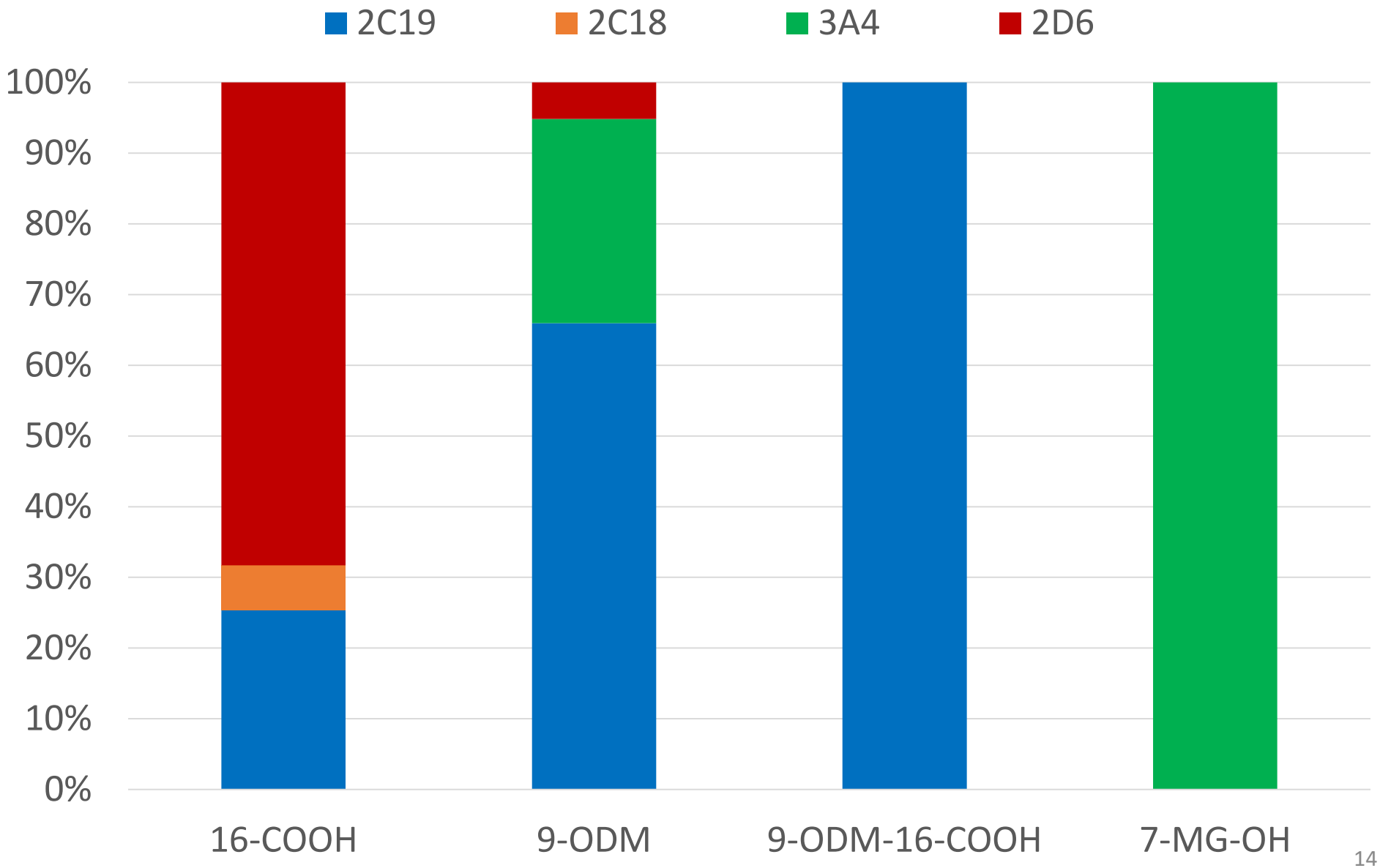
16-Carboxymitragynine



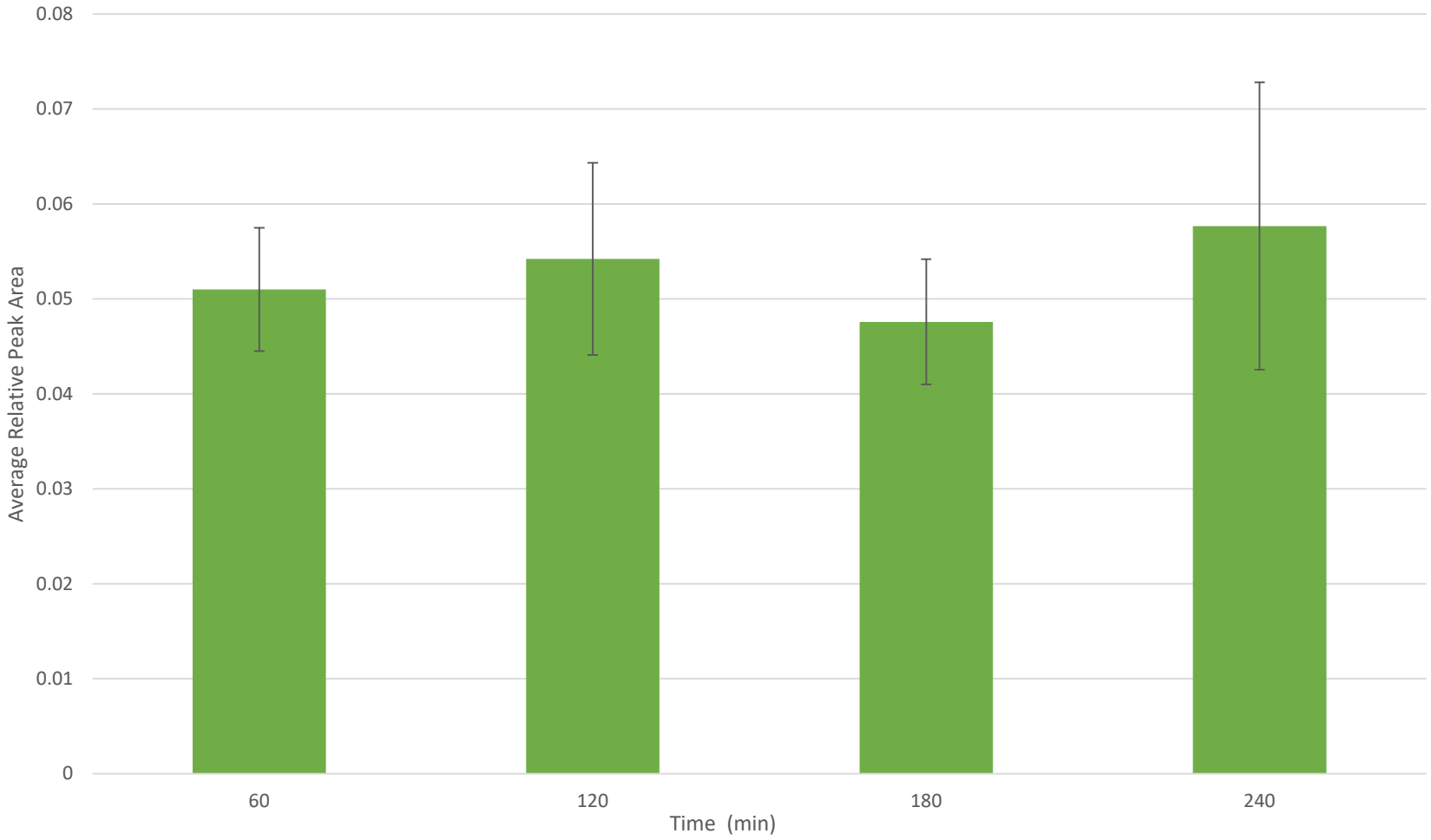
9-O-Demethylmitragynine



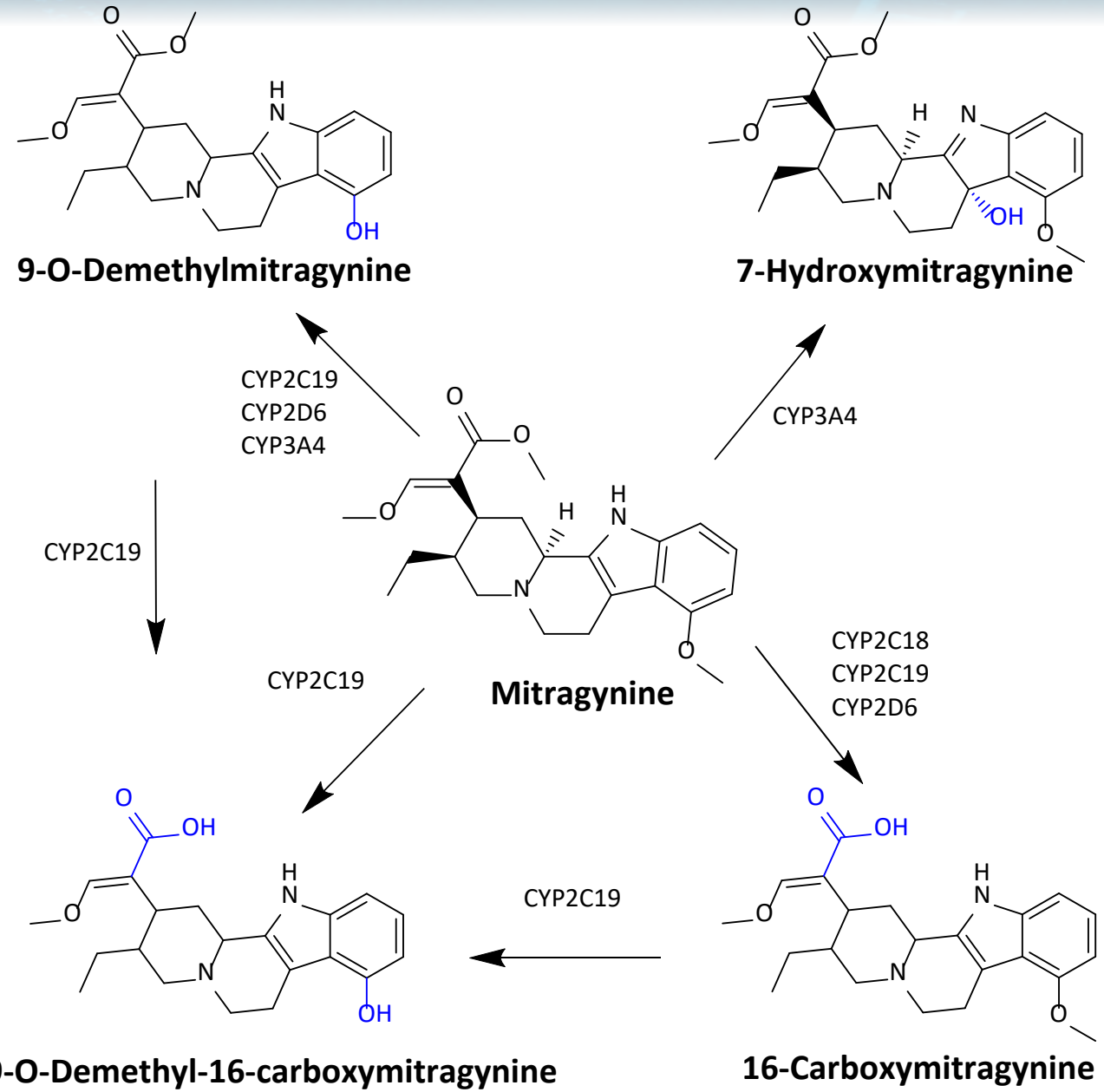
CYP Activity (180 min)



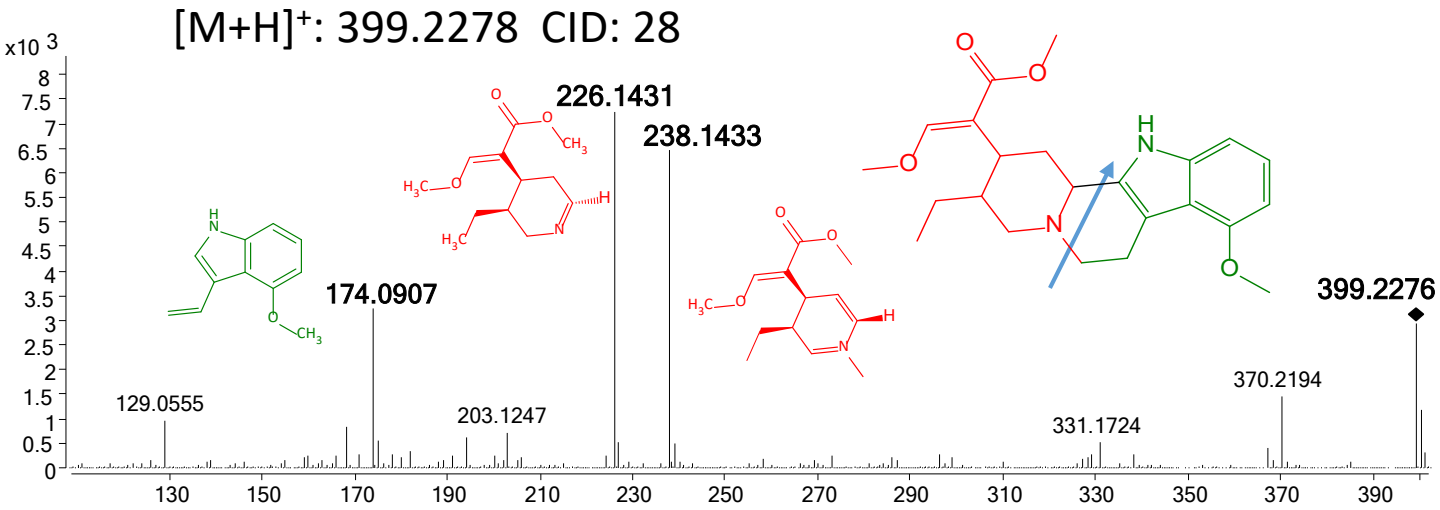
7-Hydroxymitragynine (N=6)



Proposed CYP Activity Pathway

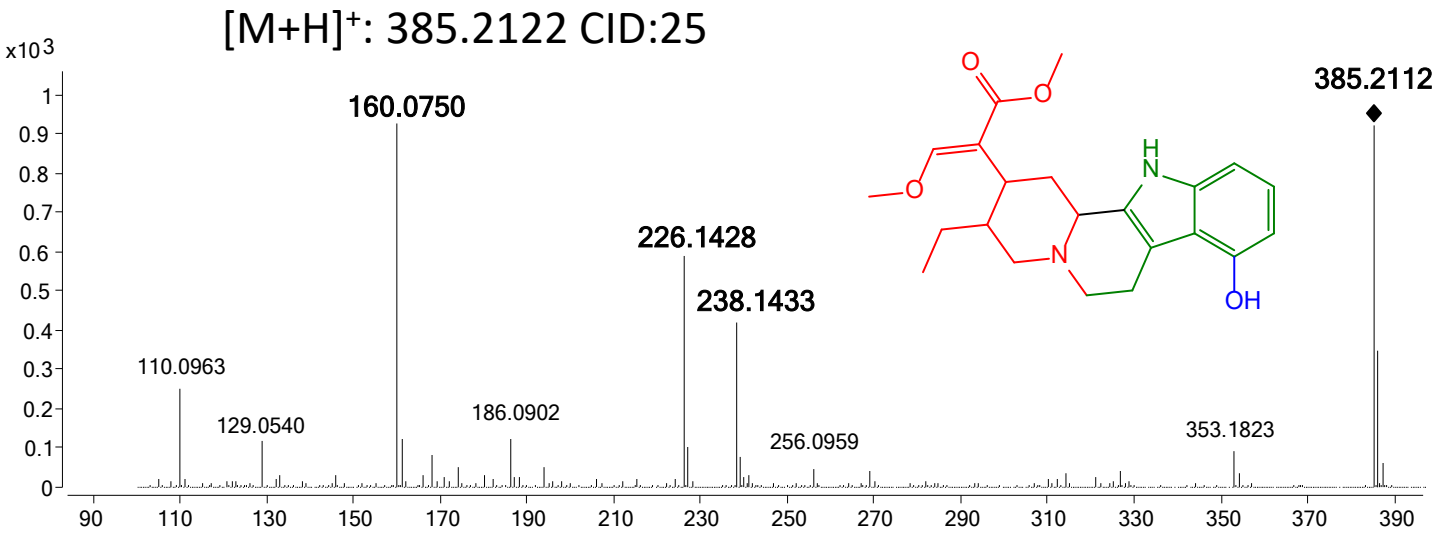


Mitragynine



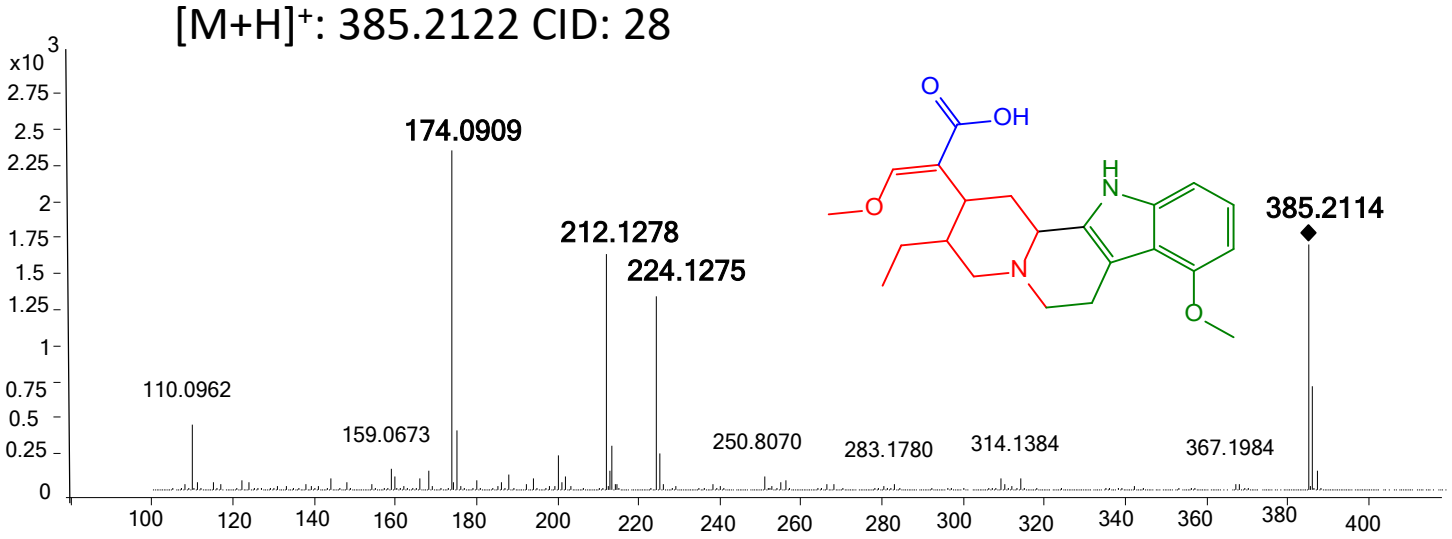
M/Z	Chemical Formula	Accurate Mass	Exact Mass	PPM
399	$[C_{23}N_2O_4H_{30}]^+$	399.2276	399.2278	**-0.1**
238	$[C_{13}NO_3H_{19}]^+$	238.1433	238.1438	**-0.2**
226	$[C_{12}NO_3H_{18}]^+$	226.1432	226.1436	**-0.2**
174	$[C_{11}NOH_{11}]^+$	174.0907	174.0913	**-0.3**

9-O-Demethylmitragynine



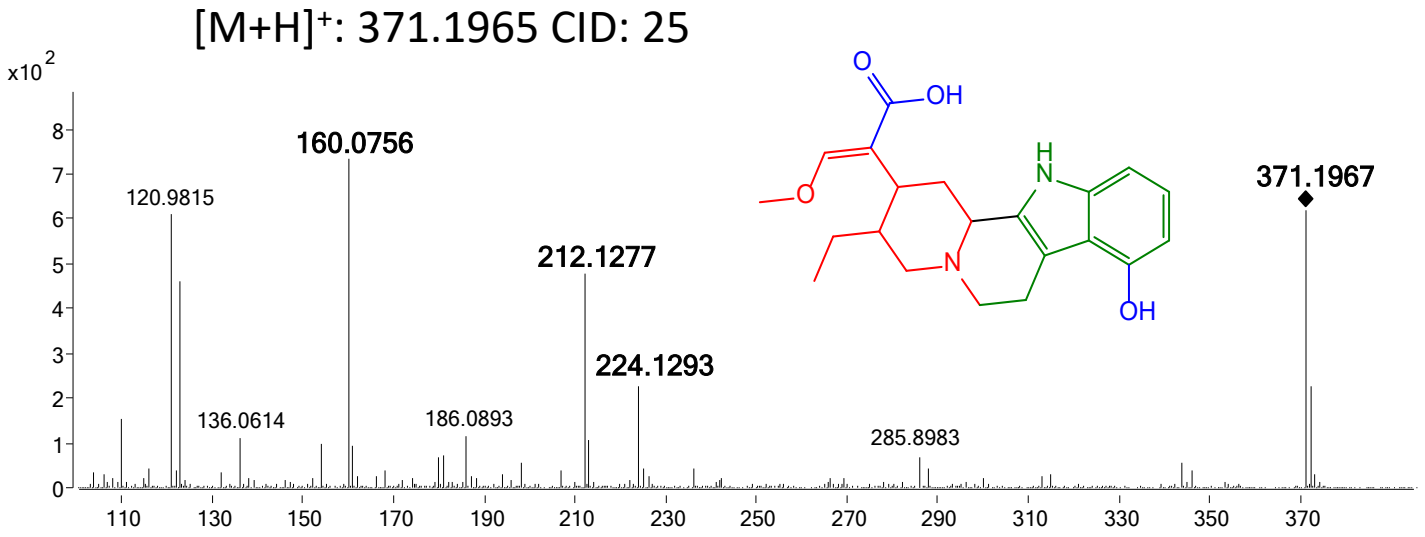
M/Z	Chemical Formula	Accurate Mass	Exact Mass	PPM
385	$[C_{22}N_2O_4H_{28}]^+$	385.2112	385.2122	**-0.3**
238	$[C_{13}NO_3H_{19}]^+$	238.1433	238.1443	**-0.4**
226	$[C_{12}NO_3H_{18}]^+$	226.1438	226.1438	**0.0**
160	$[C_{10}NOH_8]^+$	160.0750	160.0757	**-0.4**

16-Carboxymitragynine



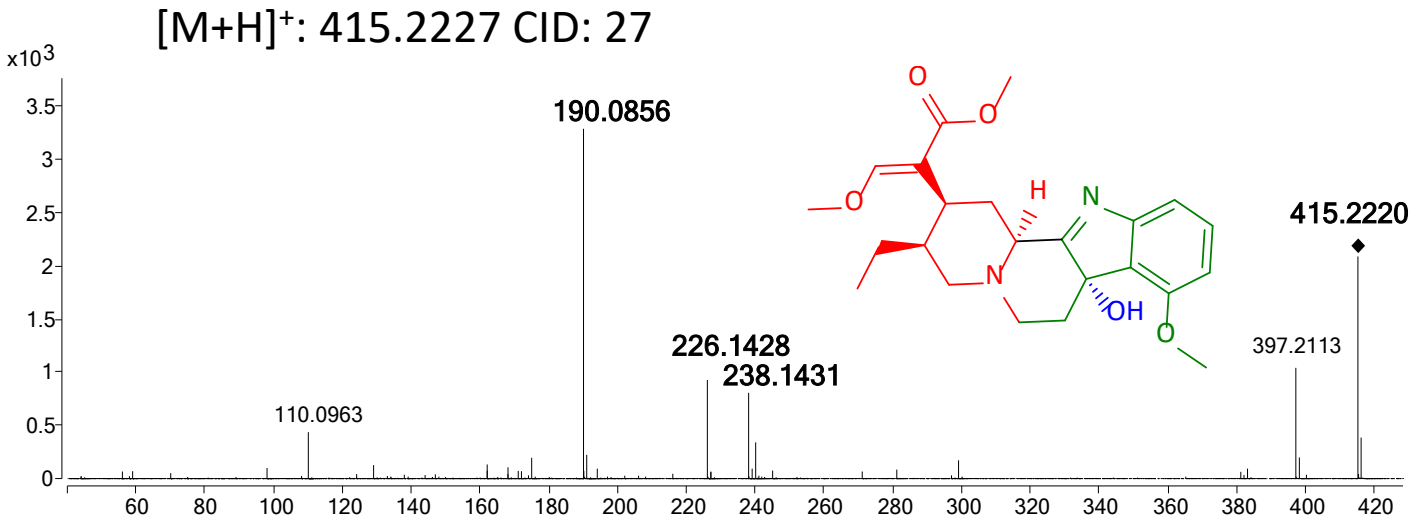
M/Z	Chemical Formula	Accurate Mass	Exact Mass	PPM
385	$[C_{22}N_2O_4H_{27}]^+$	385.2114	385.2122	**-0.2**
224	$[C_{12}NO_3H_{17}]^+$	224.1293	224.1287	**0.3**
212	$[C_{11}NO_3H_{16}]^+$	212.1278	212.1281	**-0.1**
174	$[C_{11}NOH_{11}]^+$	174.0910	174.0913	**-0.2**

9-O-Demethyl-16-carboxymitragynine



M/Z	Chemical Formula	Accurate Mass	Exact Mass	PPM
371	$[C_{21}N_2O_4H_{26}]^+$	371.1967	371.1965	**0.1**
224	$[C_{12}NO_3H_{17}]^+$	224.1293	224.1287	**0.3**
212	$[C_{11}NO_3H_{16}]^+$	212.1277	212.1281	**-0.2**
160	$[C_{10}NOH_8]^+$	160.0757	160.0757	**0.0**

7-Hydroxymitragynine



M/Z	Chemical Formula	Accurate Mass	Exact Mass	PPM
415	$[C_{23}N_2O_5H_{29}]^+$	415.2220	415.2227	**-0.2**
238	$[C_{12}NO_3H_{18}]^+$	238.1431	238.1434	**-0.1**
226	$[C_{13}NO_3H_{19}]^+$	226.1428	226.1434	**-0.3**
190	$[C_{11}NO_2H_{11}]^+$	190.0856	190.0862	**-0.3**

Inhibition Studies

rCYP	Compound	Time (min)	Inhibition (%) Mean ± SD
2C18	16-Carboxymitragynine	120	100% ± 0% (n=4)
2C19	16-Carboxymitragynine	120	77% ± 14% (n=4)
	9-O-Demethylmitragynine	120	78% ± 10% (n=4)
	9-O-Demethyl-16-carboxymitragynine	120	100% ± 0% (n=4)
2D6	16-Carboxymitragynine	120	63% ± 3% (n=4)
	9-O-Demethylmitragynine	120	100% ± 0% (n=4)
3A4	9-O-Demethylmitragynine	120	100% ± 0% (n=4)
	7-Hydroxymitragynine	120	93% ± 1% (n=6)

Conclusions

- Identified 4 CYP450s involved in mitragynine metabolism
  - 9-O-DM (2C19, 2D6, 3A4)
  - 16-COOH (2C18, 2C19, 2D6)
  - 7-MG-OH (3A4)
  - 9-O-DM-16-COOH (2C19)

- 7-Hydroxymitragynine is a notable metabolite of mitragynine
  - Known psychoactive compound in kratom
  - 17-fold more potent then morphine
  - Also verified as a metabolite through inhibition studies

- CYPs 2C18, 2C19, 2D6 and 3A4 involved in metabolism
  - Provides insight into potential toxicity and drug-drug interactions in light of polydrug use among opioid users

Acknowledgements

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Questions

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