Application Note

Chromatographic Separation of 7-Hydroxy Mitragynine from Mitragynine Pseudoindoxyl

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Key Features

- The co-elution of 7-hydroxy mitragynine and mitragynine pseudoindoxyl (two important mitragynine-related alkaloids) by traditional Gas Chromatography Mass Spectrometry (GC-MS) methods makes testing a challenge for analysts. If testing methods are unable to resolve these two substances in casework, identification and quantification can become compromised.
- The rise of consumer products on the drug market containing potent semi-synthetic opioids derived from kratom, such as 7-hydroxy mitragynine and mitragynine pseudoindoxyl, is of great public health and safety concern.
- Several chromatographic methods to achieve separation are explored in this application note. Method information presented herein may be used to assist labs in developing methods to identify and quantify 7-hydroxy mitragynine and mitragynine pseudoindoxyl in seized drug analysis and/or toxicology cases.

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Introduction

Mitragynine is an indole-based alkaloid and is one of the main psychoactive constituents in the Southeast Asian plant *Mitragyna speciosa*, commonly known as kratom. It is an atypical opioid that is typically consumed *via* kratom for its pain-relieving and euphoric effects. It has also been researched for its use to potentially manage symptoms of opioid withdrawal and as a treatment for alcohol use disorder (AUD).[1,2]

Mitragynine is the most abundant active alkaloid in kratom. In the Thai varieties, mitragynine accounts for up to 66% of total alkaloid content, while 7-hydroxy mitragynine (7-HMG) is a minor constituent (up to 2% of total alkaloid content when dried). Mitragynine is metabolized to 7-HMG and further to mitragynine pseudoindoxyl (MP) (**Figure 1**). 7-HMG and MP are reportedly more potent than mitragynine on the order of 10x and 100x, respectively.[1,3]

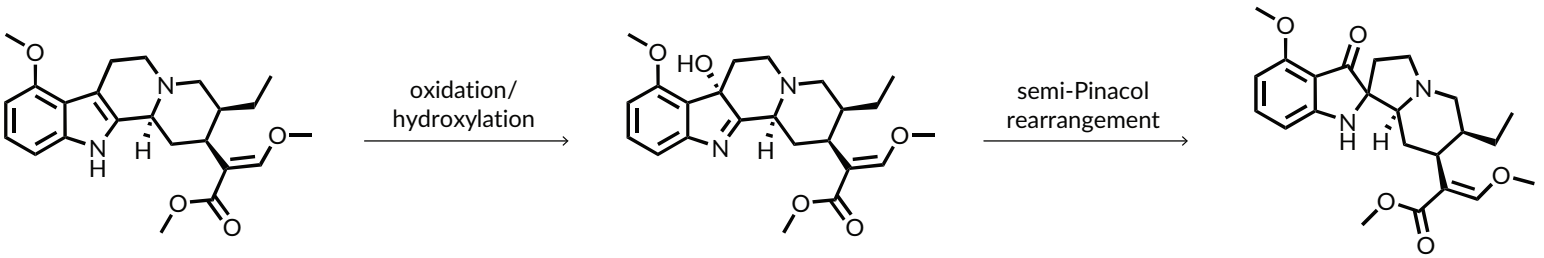

Figure 1. Conversion of mitragynine to 7-hydroxy mitragynine (7-HMG) and mitragynine pseudoindoxyl (MP).

Analysis of commercial products marketed to the consumer as 7-HMG and under other names show that these products contained high amounts of 7-HMG, MP, paynantheine, other isomers of mitragynine, and other kratom alkaloids. The presence of high concentrations of 7-HMG and MP in such products suggests semi-synthetic production. Traditional GC-MS analysis using a generic temperature program found that 7-HMG and MP were indistinguishable.[2,3] The co-elution of these substances poses a challenge for forensic chemists and can distort results when they cannot be resolved. This application note recognizes the co-elution observed between 7-HMG and MP and presents multiple chromatographic techniques and conditions enabling baseline separation of the two species.

Results: GC-MS

Both 7-HMG and MP were individually prepared at 1 mg/mL in acetonitrile. The individuals and a mixture of the two solutions were analyzed on an Agilent 8890 GC equipped with a 5977B MS detector. The injector temperature was set at 300°C, and the oven temperature was programmed to start at 240°C, hold for 1 minute, and then increase at a rate of 30°C per minute up to 300°C. The total run time was 30 minutes. The injection mode was set to a 15:1 split. Helium was used as the carrier gas at a constant flow rate of 2 mL per minute. The single component of the 7-HMG and MP solution was used to confirm the retention time order in the mixture and to ensure no interconversion. A mixture consisting of equal parts 7-HMG and MP was injected on an Rtx-5MS (30.00 m x 0.32 mm x 0.50 µm) column, a non-polar GC column equivalent to 5% phenyl 95% dimethyl arylene siloxane (USP phase G27), which resulted in significant co-elution of 7-HMG and MP (**Figure 2**).

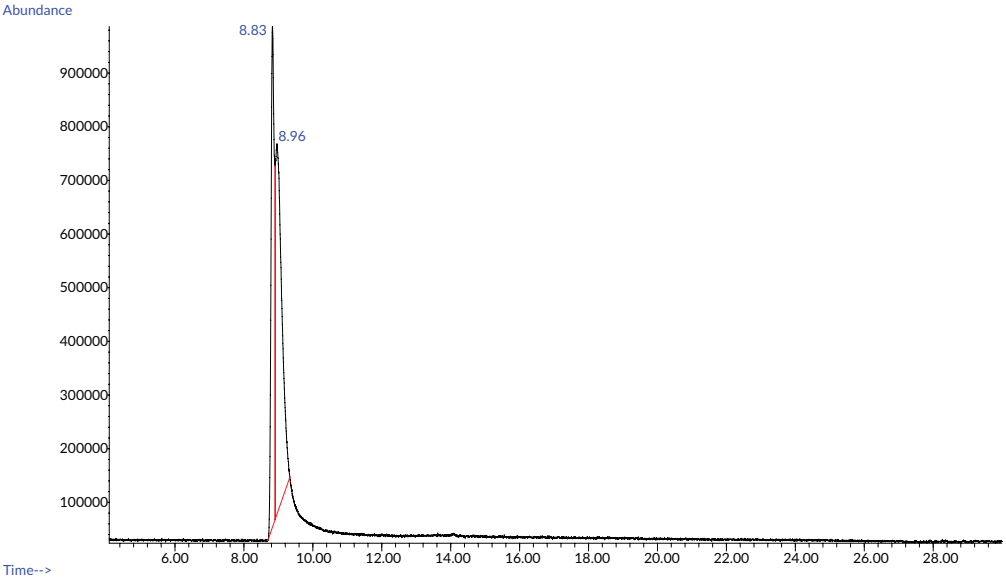

Figure 2. Co-elution of 7-HMG (8.96 min) and MP (8.83 min).

The mass spectrum of the individual components (**Figure 3**) showed the exact same fragmentation patterns which makes differentiating these two compounds by mass spectrometry even more challenging.

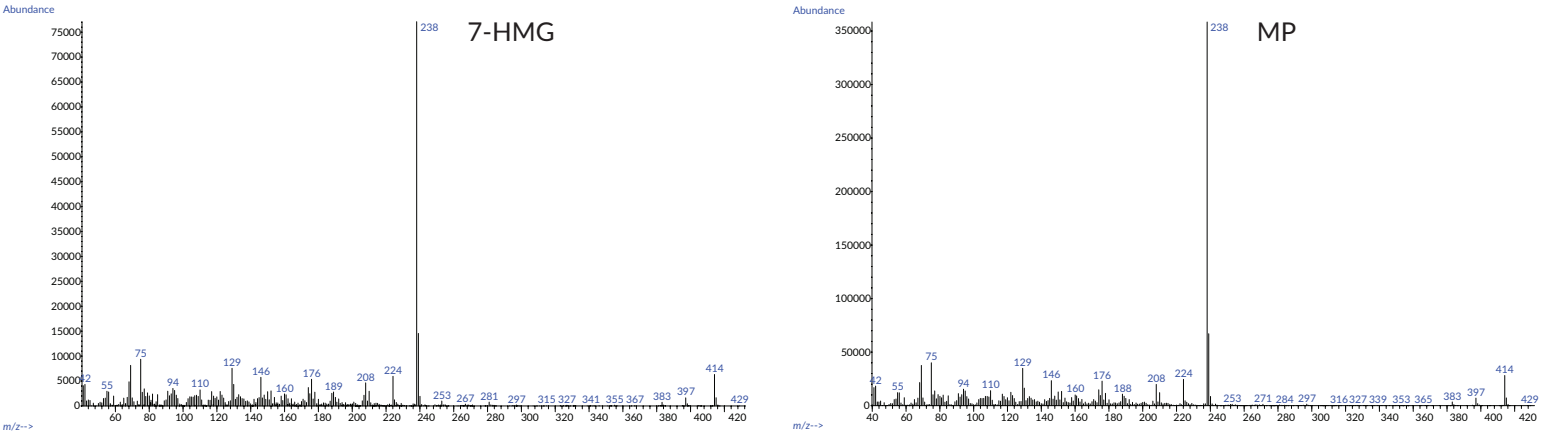

Figure 3. Mass spectrum of 7-HMG (left) and MP (right) reference standards.

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When several attempts were made to resolve the peaks by first adjusting the rate of temperature increase, then adjusting the initial temperature, and lastly adjusting the flow rate, all of which were not successful, the solution mixture was then introduced onto a DB-35MS column (30.00 m x 0.320 mm x 0.25 µm), a mid-polar GC column equivalent to 35% phenyl 65% dimethyl arylene siloxane (USP phase G42). The best results were obtained using our initial parameters on this column. This gave a peak resolution of 0.78 (**Figure 4**). Further optimization attempts were made using this column but did not yield better results. The conditions resulting in the separation shown in **Figure 4** may be useful for qualitative analysis.

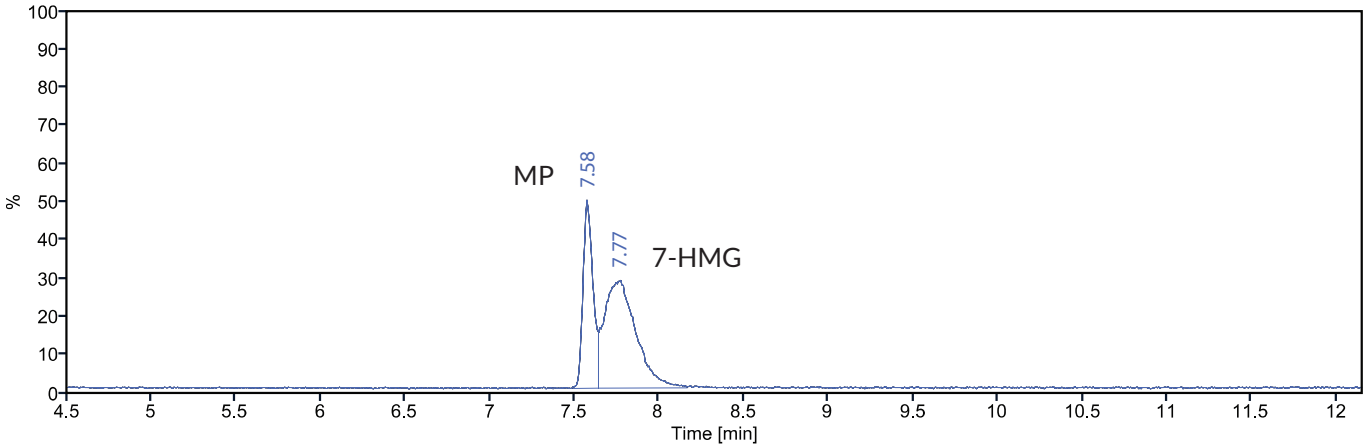


Figure 4. Co-injection of MP (7.58 min) and 7-HMG (7.77 min) on a DB-35MS column with a peak resolution of 0.78.

Results: GC-FID

Due to the non-ideal results from the GC-MS, we wanted to see if the two mixture components would perform better on Gas Chromatography-Flame Ionization Detection (GC-FID) and explore other chromatographic separation methods.

A 1:1 mixture of both components (1 mg/mL in acetonitrile) was analyzed by GC-FID using an Agilent 8890 gas chromatograph equipped with a cool-on-column inlet. The sample solution was introduced directly onto the column. The oven temperature was programmed from 50°C, hold for 1 minute, to 300°C at a rate of 30°C per minute, with a total run time of 30 minutes. Helium was used as the carrier gas at a constant flow rate of 2 mL/min.

The best results using this method gave a peak resolution of 1.07, with MP eluting first at 17.35 min and 7-HMG eluting after at 18.58 min (**Figure 5**).

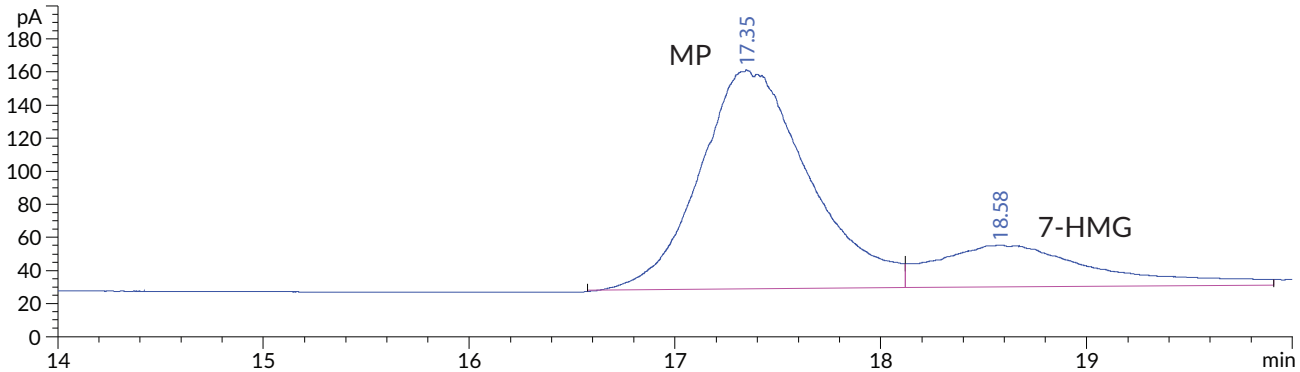


Figure 5. Co-elution of MP (17.35 min) and 7-HMG (18.58 min) on the GC-FID with a peak resolution of 1.07.

Results: HPLC

High-Performance Liquid Chromatography (HPLC) was explored using the Agilent 1260 HPLC-DAD (HPLC with Diode Array Detection). 7-HMG and MP (each at 1 mg/mL in acetonitrile) were analyzed using an Ascentis Express 90 Å C18 column (150 × 3.0 mm, 2.7 µm) maintained at 30°C.

The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (**A**) and acetonitrile (**B**), delivered at a flow rate of 1.0 mL per minute. The gradient program started at 5% B, increased to 50% B over 10 min, then to 95% B at 12 min, and was held for 3 min before returning to 5% B at 15.1 min, with a total run time of 20 min. The UV wavelength was set at 232 nm to analyze both components. The result, as shown in **Figure 6**, indicates that MP and 7-HMG are very well resolved under these chromatographic conditions, making this a very useful technique. **Figure 6** includes the corresponding UV absorptions, indicating another useful method for differentiation of the two species.

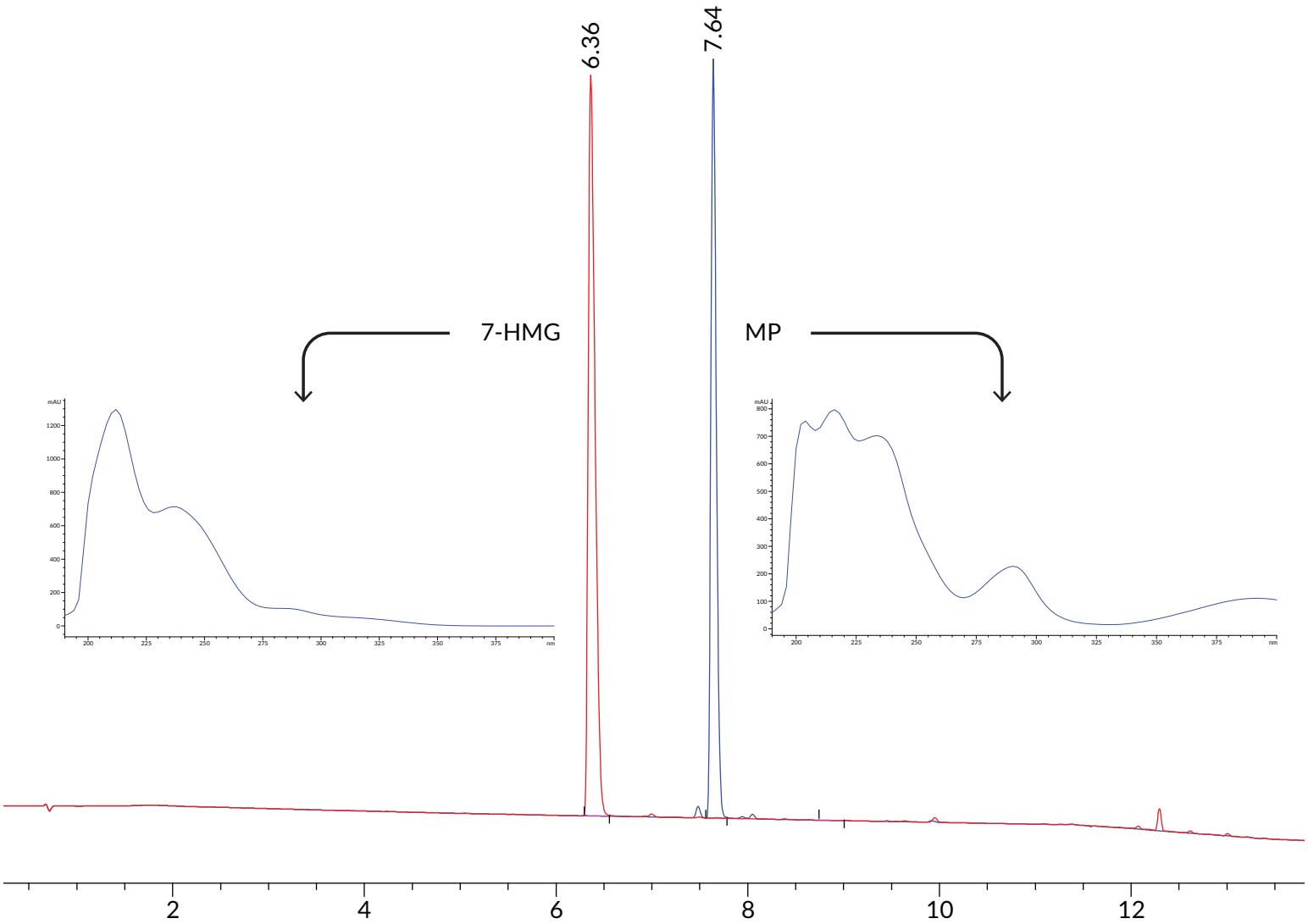


Figure 6. HPLC separation of 7-HMG (6.36 min) and MP (7.64 min) including their corresponding distinct UV absorptions.

Results: LC-MS/MS

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) was explored. The components were analyzed on a Sciex Exion LC-AC HPLC system, with a Sciex 4500 QTrap MS. Conditions for the LC and MS components are provided in the table below.

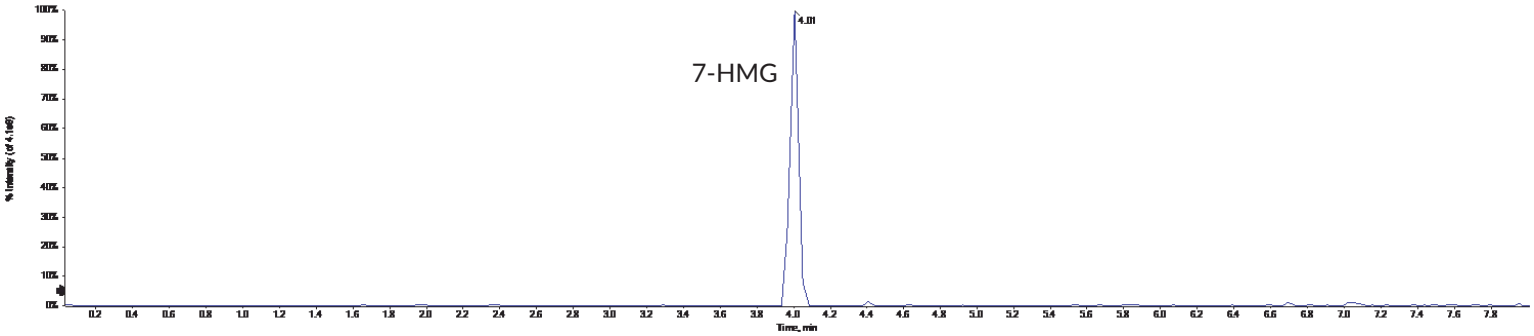
Table 1. Conditions and gradient for the LC and parameters for the MS.

HPLC		MS/MS	
Conditions		**Parameter**	**Value**
Column	Ascentis Express C18 100 x 2.1 mm, 2.7 µm	CUR	50
Column Temp	30°C	CAD	High
Sample Diluent	Methanol	IS Voltage	5500V
Inj. Vol.	1 µL	TEM	650
Flow Rate	0.5 mL/min	GS1	70
Mobile Phase	A: 0.1% Formic acid in water B: 0.1% Formic acid in acetonitrile	GS2	70
Gradient	0.0-6.5 min: 5-50% B 6.5-8.0 min: 50% B hold 8.0-8.1 min: 50-5% B 8.1-10.0 min: 5% B hold	Declustering Potential	60V
		Entrance Potential	10V
		Collision Energy	40V

Regarding the MS/MS experiment, we followed the Enhanced MS > Enhanced Resolution > IDA Criteria > Enhanced Product Ion. This experimental set up is designed to generate a chromatogram that also contains fragmentation data. The Enhanced MS and Enhanced Resolution experiments set the parameters for ion collection in QTrap. The IDA Criteria refines the method so that the 1 to 2 most abundant ions are utilized for the Enhanced Product Ion experiment. This produces a mass spectrum for each peak that can be used for identification purposes.

The IDA chromatogram below is an overlay of the individual samples at a concentration of 5 µg/mL in methanol. This chromatogram provides confirmation of retention time and shows there is minimal interconversion between the two compounds in this work (**Figure 7**).

5 µg/mL 7-hydroxy mitragynine for retention time purposes only.



5 µg/mL mitragynine pseudoindoxyl

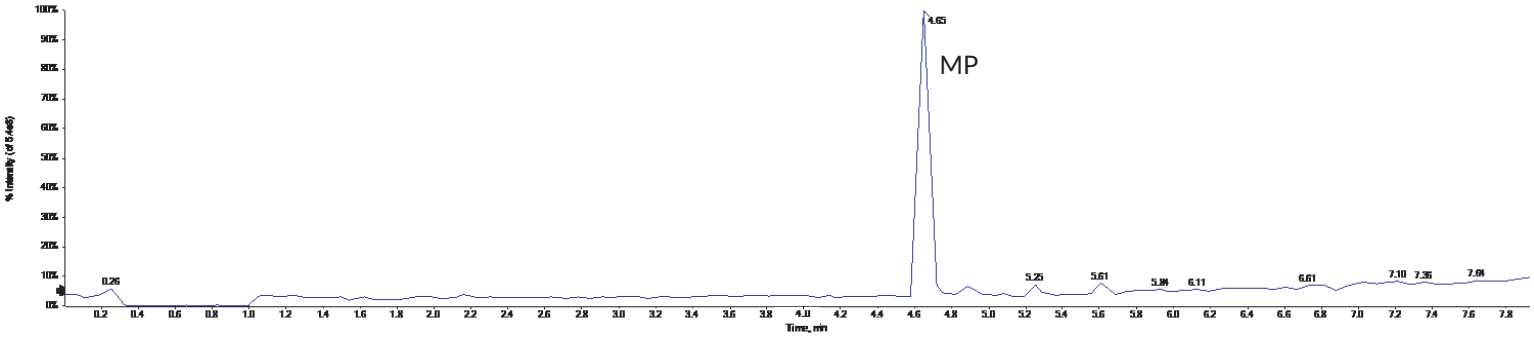


Figure 7. Separate injections of 7-HMG (4.01 min) and MP (4.65 min).

The chromatogram below (**Figure 8**) is an overlay of the IDA chromatogram and the Enhanced Product Ion mass spectra from the 5 µg/mL mixture of the two compounds. The retention times match the individual standard above.

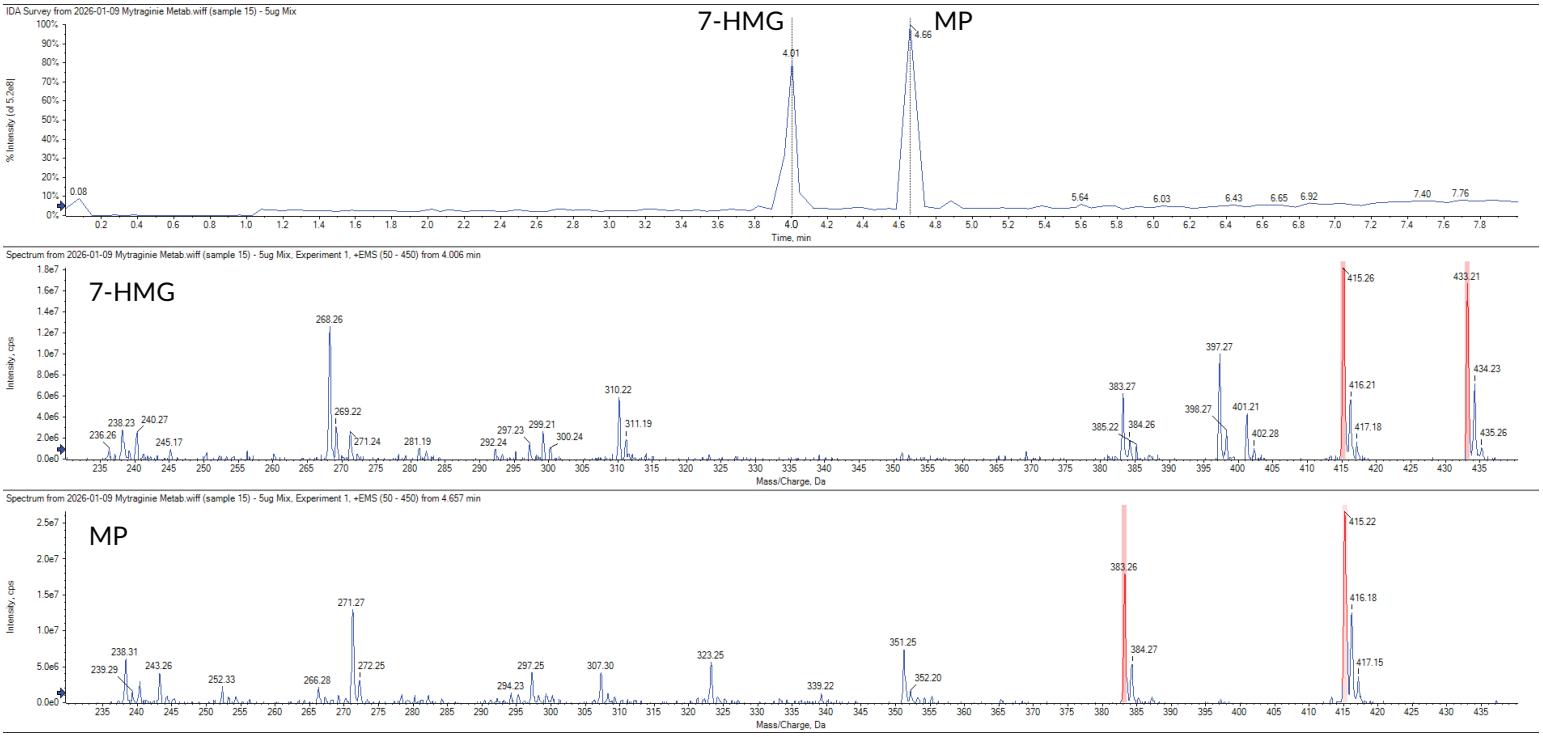


Figure 8. Overlay of the IDA chromatogram and the Enhanced Product Ion Mass Spectra of 7-HMG and MP.

It is important to note that 7-HMG readily forms a water adduct (*m/z* = 433.2 Da) using these conditions, but MP does not.

A comparison of the mass spectra shows that 7-HMG has two fragments (397.2, 401.2 Da) that are not present in the MP mass spectra. A comparison also shows that there are two MP fragments (323.2, 351.2 Da) that are not present in 7-HMG. These differences in fragmentation patterns allow for the identification of each compound in a mixture of these compounds.

Results: HRMS

Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS) was explored to determine if chromatographic separation was achievable and if fragmentation patterns differed on this instrumental platform. 7-HMG and MP were analyzed on a Sciex Exion LC HPLC system with a Sciex X500R QTOF-MS. Conditions for the LC and MS components are described in the literature.[4]

The chromatogram below (**Figure 9**) is an XIC chromatogram for the exact mass of these analytes. Two peaks are observed illustrating the resolution between the two analytes. 7-HMG first and MP second, which is expected based on their functional group chemistry.

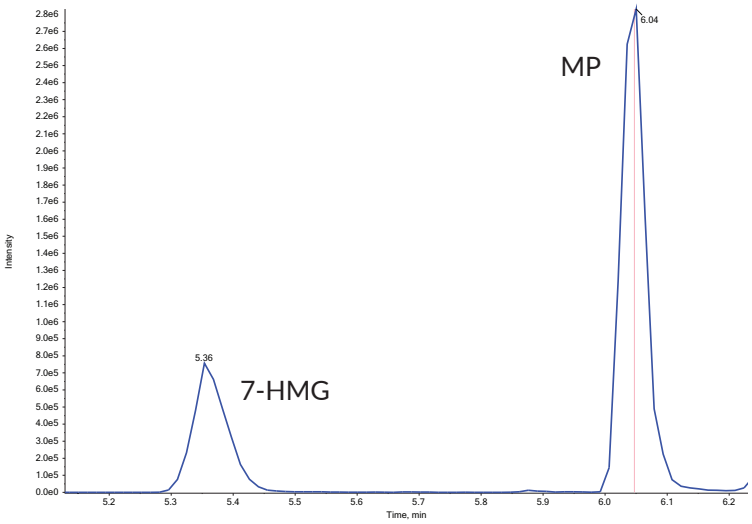


Figure 9. Extracted ion chromatogram showing 7-HMG (5.36 min) and MP (6.04 min) chromatographic separation.

The mass spectra below (**Figure 10**) represent the fragment ions acquired for 7-HMG and MP by LC-QTOF-MS. The mass spectrometer utilized a non-targeted acquisition mode. Fragment ions were acquired from *m/z* 50-510 using a collision energy spread to promote the development of an array of fragment ions. The two isomers showed similar fragment spectra; however, 7-HMG exhibited an *m/z* 397 consistent with the loss of water, while MP exhibited a more distinctive *m/z* 383.

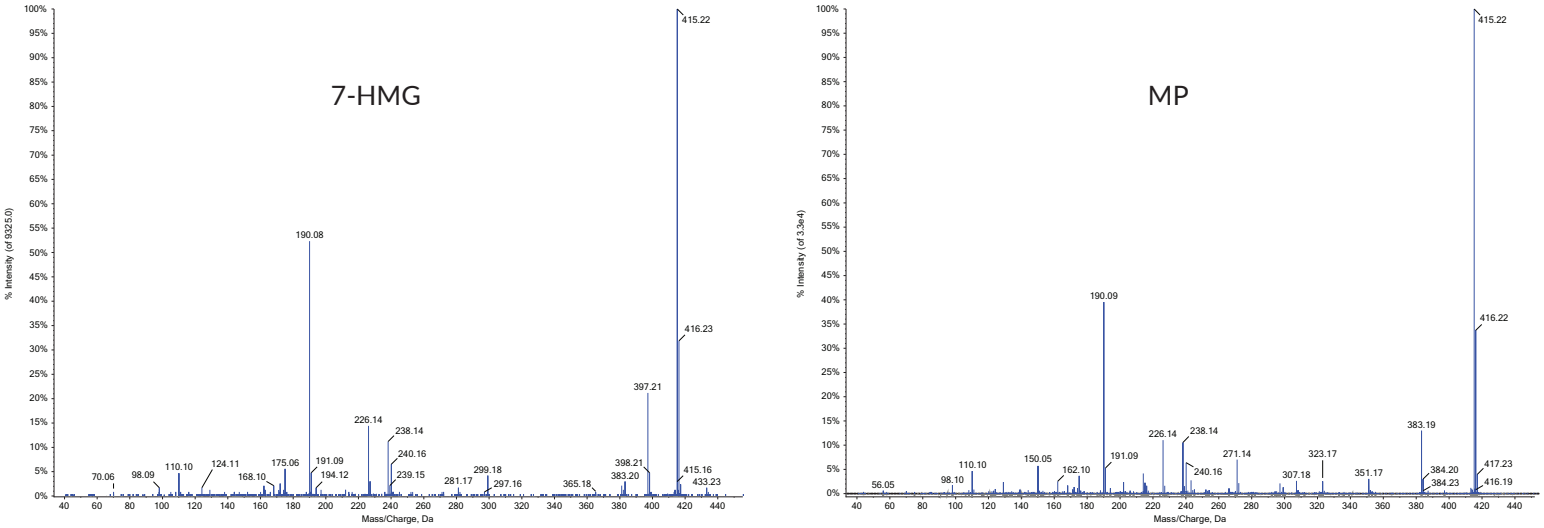


Figure 10. MS/MS spectra for 7-HMG (left) and MP (right) after analysis using an LC-QTOF-MS operating in ESI+ and MS/MS^{ALL} acquisition.

Results: TLC

The simple technique of Thin-Layer Chromatography (TLC) can be a very useful, inexpensive, and rapid tool. Individual solutions of 7-HMG and MP reference standards plus a co-spot were loaded onto a TLC plate and eluted with 3:2 heptane/ethyl acetate with 2% triethylamine. As shown in **Figure 11**, 7-HMG had an R_f of 0.27 and MP had an R_f of 0.21. As visualized using a UV wavelength of 254 nm, note the distinguishable colors, which, in addition to the R_f differences, can serve as a simple qualitative analysis technique.

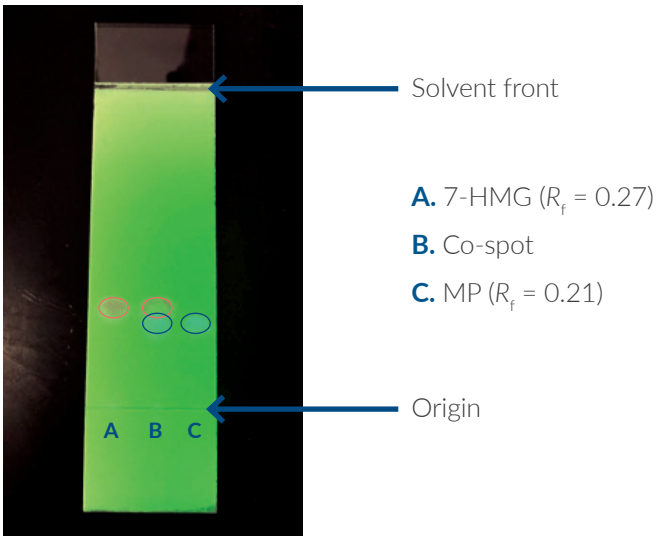


Figure 11. TLC of 7-HMG and MP plus co-spot in center lane.

Cayman products used in this application

Item No.	Product Name
13114	7-hydroxy Mitragynine
20033	Mitragynine Pseudoindoxyl

Conclusion

Kratom alkaloids continue to appear in the recreational drug market across the United States, fueled by the rise of smoke shops, gas stations, and convenience stores selling a variety of mitragynine-related products. 7-HMG and MP are now commonly found in pills, gummies, edibles, and other sample types purporting these substances. Due to their increased potency over mitragynine, these novel kratom alkaloids remain of high public health and safety interest. As states move to schedule these substances and they appear in forensic investigations, accurate identification remains vital. We discovered that 7-HMG and MP were virtually indistinguishable by routine GC-MS methods due to similar retention time and identical mass spectra, leaving the opportunity for misreporting. Our approach to resolve these isomers and developing methods for differentiation were successful in resolution and individual reporting.

References

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