

Appendix No. 1 to the Test certificate ML 286/21

Determination of mitragynine and targeted screening of other alkaloids from *Mitragyna speciosa* plant using U-HPLC-HRMS/MS method

List of samples

ML 286/21 – *Mitragyna Speciosa* leaf powder

Testing strategy

For quantitative determination of the alkaloid mitragynine (CAS Number 4098-40-2) and investigation of the presence of other *Mitragyna speciosa* alkaloids reported in the scientific literature, the strategy of targeted screening followed by quantitation of mitragynine using a commercially available standard was used. The analysis was performed using ultra-high performance liquid chromatography coupled to tandem high-resolution mass spectrometry (KM15, instrumental system (E): U-HPLC-HRMS/MS).

Testing conditions

The compounds of interest were extracted from the sample by a mixture of methanol and water (3x repeated extraction; extraction efficacy verified by 6x repeated extraction procedure). Separation and detection of the compounds present in the extract were performed using a C18 reverse-phase column and a quadrupole/time-of-flight mass analyzer (Agilent 6560 Ion Mobility QTOF LC/MS System, Agilent Technologies). For the quantitation of mitragynine, the external calibration method was used (calibration batch of mitragynine standard dissolved in methanol). The data processing and evaluation were performed using Agilent MassHunter software Qualitative Analysis 10.0 and Agilent MassHunter Workstation software Quantitative Analysis for TOF 10.1. *The standard of mitragynine was purchased from SIGMA-ALDRICH spol. s r.o (Mitragynine solution 100 µg/mL in methanol, certified reference material, Cerilliant®).*

Test results

Table I shows the determined content of mitragynine in the sample ML 286/21, expressed in various units.

Table I: Content of mitragynine in the sample ML 286/21, expressed in various units

Analyte	Result	Expanded uncertainty*	Unit
Mitragynine	7999	800	mg/kg
	8.0	0.8	mg/g
	0.8	0.08	weight %

* Expanded uncertainty was calculated using the coverage factor $k = 2$ corresponding to a coverage probability of approximately 95%. Uncertainty was calculated and stated according to the EA-4/16 and manual Kvalimetrie 11 (issued by EURACHEM CZ). Uncertainty of sampling is not covered. Compliance is evaluated with respect to the uncertainty of the test result according to the Guide ILAC-G8.

The other alkaloids of *Mitragyna speciosa* reported in the scientific literature [1–3] were detected and identified in the sample based on the exact mass m/z of their $[M+H]^+$ ions, isotopic pattern and fragmentation spectra. The list of the screened compounds and their chromatographic records are presented in **Table II** and on **Figures 1 – 7**.

Table II: Alkaloids screened in the sample ML 286/21

Compound name	Molecular formula	Exact mass m/z of the precursor ion $[M+H]^+$	Chromatographic records obtained by ML 286/21 analysis
Mitragynine	$C_{23}H_{30}N_2O_4$	399.2278	Figure 1
Speciogynine			
Speciociliatine			
7-Hydroxymitragynine	$C_{23}H_{30}N_2O_5$	415.2227	Figure 2
7 β -Hydroxy-7H-Mitraciliatine			
Isospeciofoline	$C_{22}H_{28}N_2O_5$	401.2071	Figure 3
Isorotundifoline			
Isospeciofoleine	$C_{22}H_{26}N_2O_5$	399.1944	(not detected)
Corynoxine B	$C_{22}H_{28}N_2O_4$	385.2122	Figure 4
Corynoxine			
Paynantheine	$C_{23}H_{28}N_2O_4$	397.2122	Figure 5
3-Isopaynantheine			
Corynantheidine	$C_{22}H_{28}N_2O_3$	369.2173	Figure 6
Isocorynantheidine			
Mitraphylline	$C_{21}H_{24}N_2O_4$	369.1809	Figure 7

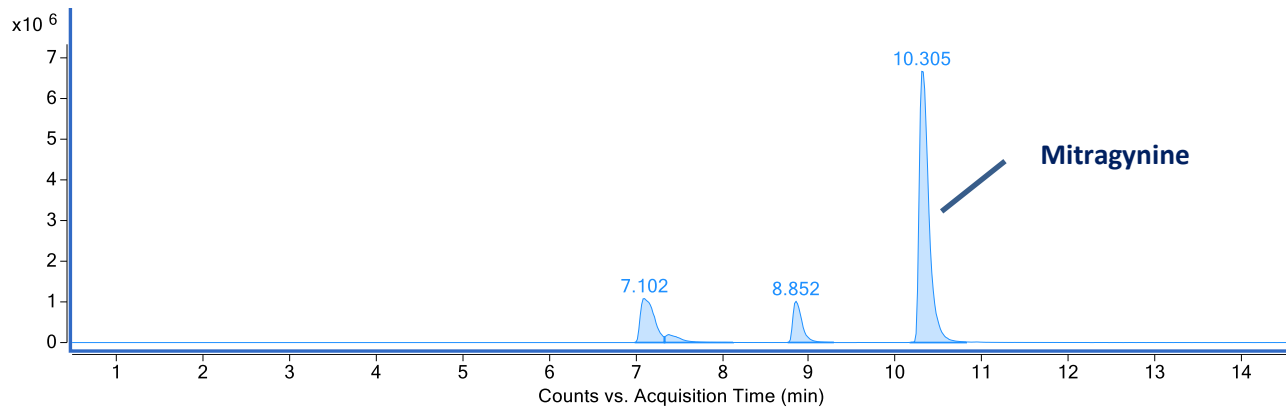


Figure 1: Extracted ion chromatogram for (XIC) m/z 399.2278 ($[M+H]^+$ ions of alkaloids mitragynine, speciogynine and speciociliatine), exact mass window 5 ppm; 100x diluted extract of sample ML 286/21

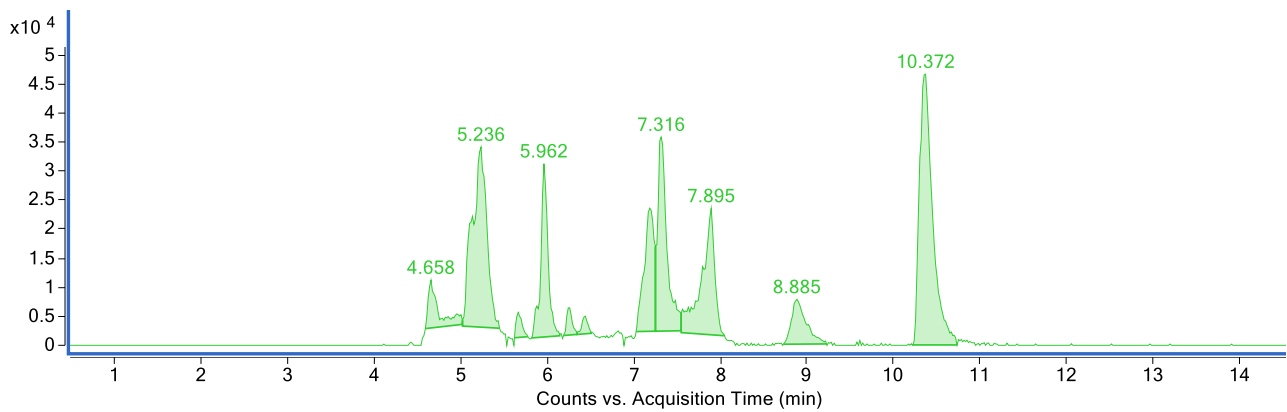


Figure 2: Extracted ion chromatogram (XIC) for m/z 415.2227 ($[M+H]^+$ ion of alkaloids 7-hydroxymitragynine and 7 β -hydroxy-7H-mitraciliatine), exact mass window 5 ppm; 100x diluted extract of sample ML 286/21

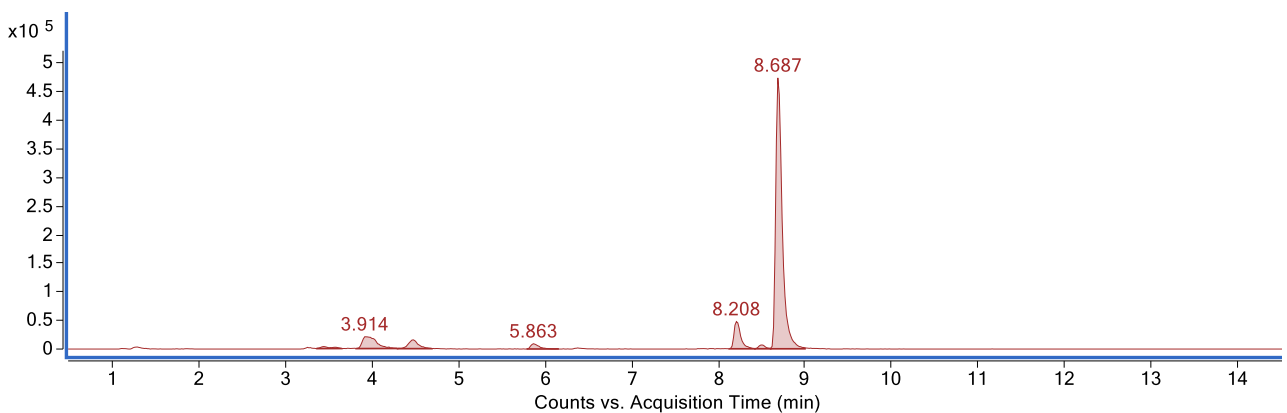


Figure 3: Extracted ion chromatogram (XIC) for m/z 401.2071 ($[M+H]^+$ ions of alkaloids isospeciofoline and isorotundifoline), exact mass window 5 ppm; 100x diluted extract of sample ML 286/21

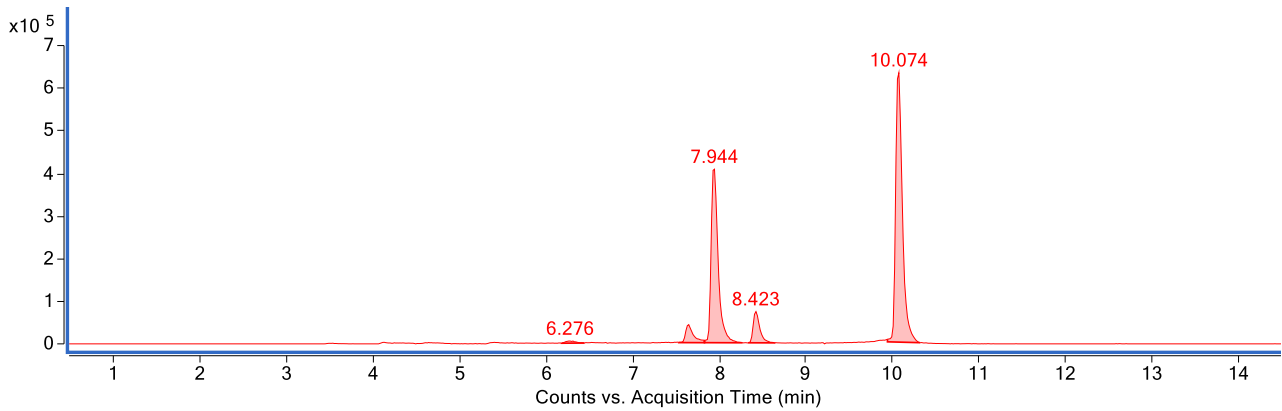


Figure 4: Extracted ion chromatogram (XIC) for m/z 385.2122 ($[M+H]^+$ ions of alkaloids corynoxine B and corynoxine), exact mass window 5 ppm; 100x diluted extract of sample ML 286/21

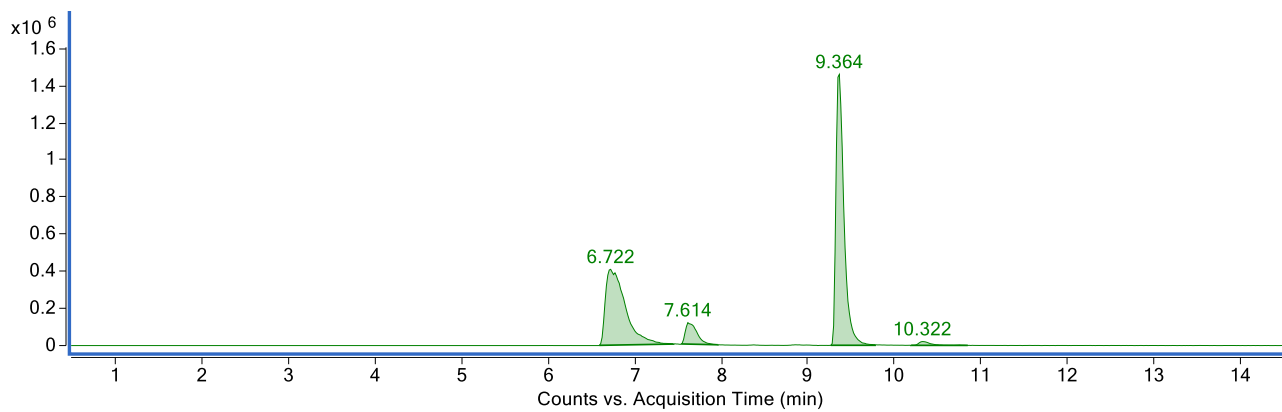


Figure 5: Extracted ion chromatogram (XIC) for m/z 397.2122 ($[M+H]^+$ ions of alkaloids paynantheine and 3-isopaynantheine), exact mass window 5 ppm; 100x diluted extract of sample ML 286/21

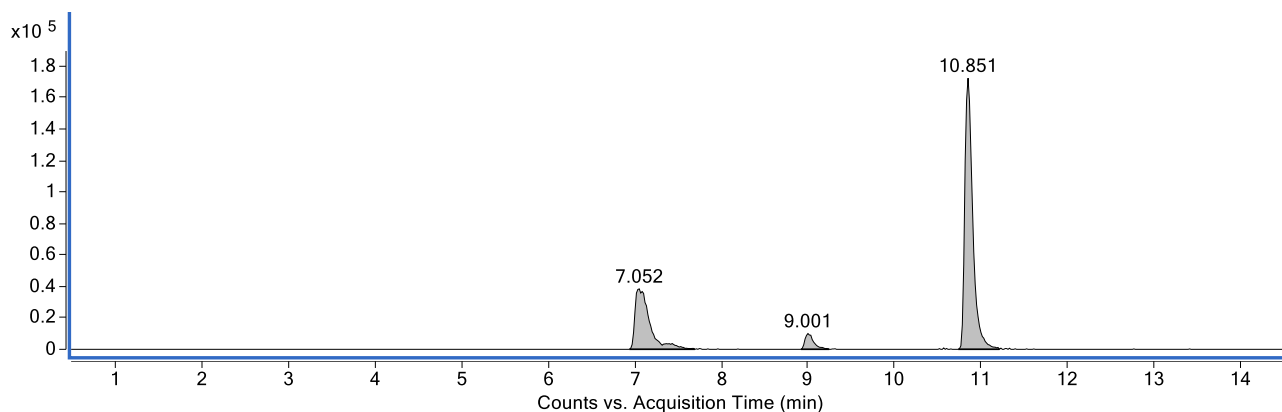


Figure 6: Extracted ion chromatogram (XIC) for m/z 369.2173 ($[M+H]^+$ ions of alkaloids corynantheidine and isocorynantheidine), exact mass window 5 ppm; 100x diluted extract of sample ML 286/21

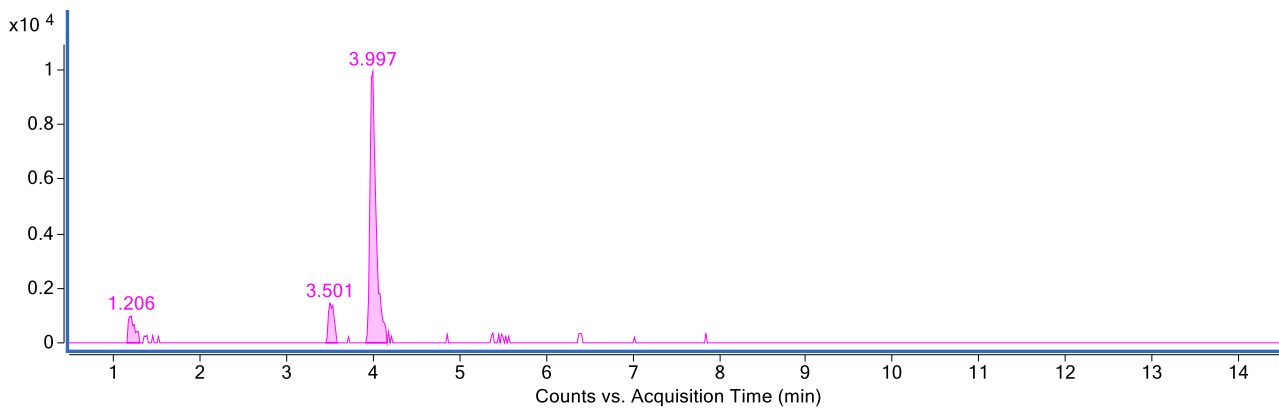


Figure 7: Extracted ion chromatogram (XIC) for m/z 369.1809 ($[M+H]^+$ ion of alkaloid mitraphylline), exact mass window 5 ppm; 100x diluted extract of sample ML 286/21

Interpretation of test results

The determined content of the alkaloid mitragynine in the *Mitragyna speciosa* leaf powder (ML 286/21) is 0.8 weight %. A number of other *Mitragyna speciosa* alkaloids were also detected in the sample. Due to the observed presence of possible isomeric compounds (multiple chromatographic peaks detected for the same exact mass m/z of $[M+H]^+$ ions of the monitored alkaloids), the similarity of the obtained fragmentation spectra and the absence of corresponding standards, it was not possible to confirm the identity of these compounds or determine their content in the sample.

References

- [1] B. Avula, S. Sagi, Y.-H. Wang, M. Wang, Z. Ali, T.J. Smillie, J. Zweigenbaum, I.A. Khan, Identification and Characterization of Indole and Oxindole Alkaloids from Leaves of *Mitragyna speciosa* Korth Using Liquid Chromatography–Accurate QToF Mass Spectrometry, *J. AOAC Int.* 98 (2015) 13–21. <https://doi.org/10.5740/jaoacint.14-110>.
- [2] A. Sharma, S.H. Kamble, F. León, N.J.-Y. Chear, T.I. King, E.C. Berthold, S. Ramanathan, C.R. McCurdy, B.A. Avery, Simultaneous quantification of ten key Kratom alkaloids in *Mitragyna speciosa* leaf extracts and commercial products by ultra-performance liquid chromatography–tandem mass spectrometry, *Drug Test. Anal.* 11 (2019) 1162–1171. <https://doi.org/10.1002/dta.2604>.
- [3] R. Kikura-Hanajiri, M. Kawamura, T. Maruyama, M. Kitajima, H. Takayama, Y. Goda, Simultaneous analysis of mitragynine, 7-hydroxymitragynine, and other alkaloids in the psychotropic plant “kratom” (*Mitragyna speciosa*) by LC-ESI-MS, *Forensic Toxicol.* 27 (2009) 67–74. <https://doi.org/10.1007/s11419-009-0070-5>.

End of the appendix