Appendix No. 1 to the Test certificate ML 2321/21

Determination of alkaloids from *Mitragyna speciosa* plant using U-HPLC-HRMS/MS method

List of samples

ML 2321/21 – Kratom Emerald

Testing strategy

The analysis of kratom sample ML 2321/21 aimed at detection and quantitation of mitragynine (CAS Number 4098-40-2) and 7-hydroxymitragynine (CAS Number 174418-82-7), i.e. major bioactive alkaloids occurring in *Mitragyna speciosa* plant [1–3]. Target screening strategy employing advanced technique, ultra-high performance liquid chromatography coupled to tandem high-resolution mass spectrometry (U-HPLC-HRMS/MS) was chosen for sample extract investigation in the first phase. In the next step signals (peaks) of targeted analytes were SW extracted and quantified using commercially available analytical standards. Accredited method KM15, instrumental system E, was used for this purpose.

Testing conditions

The compounds of interest were extracted from the sample by aqueous methanol (three times repeated extraction; extraction efficacy verified by six times repeated extraction procedure). Separation and detection of the compounds present in the combined extracts were performed using a C18 reverse-phase chromatographic column and a quadrupole/time-of-flight mass analyzer (Agilent 6560 Ion Mobility QTOF LC/MS System, Agilent Technologies). For the quantitation of mitragynine and 7-hydroxymitragynine, external calibration method was used (calibration set of corresponding standards dissolved in methanol). Primary data processing and then evaluation were performed using Agilent MassHunter software Qualitative Analysis 10.0 and Agilent MassHunter Workstation software Quantitative Analysis for TOF 10.1.

Standards of mitragynine (Mitragynine solution 100 µg/mL in methanol, certified reference material, Cerilliant®) and 7-hydroxymitragynine (7-Hydroxymitragynine solution 100 µg/mL in methanol with 0.1N NH3, certified reference material, Cerilliant®) were purchased from SIGMA-ALDRICH spol. s r.o.

Test results

Table I shows the determined content of mitragynine and 7-hydroxymitragynine in the sample ML 2321/21, expressed in various units.

Table I: Content of mitragynine and 7-hydroxymitragynine in the sample ML 2321/21, expressed in various units

Analyte	Result	Expanded uncertainty*	Unit
Mitragynine	11766	1177	mg/kg
	12	1.2	mg/g
	1.2	0.12	weight %
7-Hydroxymitragynine	220	35	mg/kg
	0.22	0.035	mg/g
	0.022	0.0035	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.*

Interpretation of test results

The presence of *Mitragyna speciosa* alkaloids, specifically mitragynine and 7-hydroxymitragynine, was confirmed in the examined kratom sample (ML 2321/21) and their content was quantified. The concentration ratio of these alkaloids in the sample was approximately 55 : 1 (mitragynine : 7-hydroxymitragynine).

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.

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