

Supporting Information

J. Chem. Metrol. XX:X (20XX) XX-XX

Determination of gymnemic acid level in *Gymnema inodorum* leaves using multiple reaction monitoring mass spectrometry

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S1: Methods

Preparation of G. inodorum and G. sylvestre extracts

The samples of *G. inodorum* leaves (GI1, GI2, GI3 and GI4) were gently cleaned with water and dried in a hot-air oven at 60°C for 24 hours. The dried leaves were ground into a powder and kept at 4 °C. One hundred grams of *G. inodorum* (GI1, GI2, GI3 and GI4) were soaked with 1,000 mL of 50% v/v ethanol solution in water and allowed to stir at 500 rpm, 25°C. A sample was extracted three times every twenty-four hours and the extracted solutions were then pooled. The residual ethanol was removed from the extracted solution by rotary evaporator (RV10 IKA®, USA), followed by the removal of residual water by freeze drying method. The crude extracts of *G. inodorum* were kept at -20°C before further analysis.

Hydrolysis of G. inodorum and G. sylvestre extracts

One hundred milligram extract of *G. inodorum* (GI1, GI2, GI3 and GI4) or *G. sylvestre* (GS1, GS2 and GS3) was refluxed with 30 mL of 2.5 N HCl in 1:1 (v/v) methanol and water at 85°C for 2 hours. The solution was then placed on ice for an hour and centrifuged at 10,000 \times g (AF-5004CS rotor, KUBOTA model 3700, Japan) at 4°C to collect precipitates. The precipitates were further refluxed with 2% w/v KOH in methanol at 85°C for 2 hours. After that, the solution was cooled down to room temperature and 150 mL of ethyl acetate was added to dissolve gymnemagenin. The ethyl acetate fraction was collected and evaporated to dryness. The precipitates were reconstituted in methanol by volumetric flask and filtrated with a nylon filter (0.2 μ m pore diameter) before quantitative analysis.

S2 : Analysis of gymnemagenin/gymnemic acid

Preparation of gymnemagenin standard

0.001, 0.010, 0.024, 0.048, 0.096, 0.241, 0.480 mg/mL of gymnemagenin solution were accurately prepared in methanol using a volumetric flask. The solution was filtered with a nylon filter (0.2 μ m pore diameter) before quantification.

UPLC-ESI-MS/MS method using a multiple reaction monitoring mode for quantification of gymnemagenin

The liquid chromatography (LC) system (1290 Infinity, Agilent Technologies, USA) comprised of a pump, autosampler, and the mass spectrometer comprised of an Agilent Jet Stream Electrospray Ionization and 6495 Triple Quad LC/MS detection systems. The integration of peaks were performed using Quantitative Analysis B.07.00 software. A 0.5 μ L (full loop) sample was injected. A SB-C₁₈ 2.1 mm \times 5 mm, 2.7 μ m column (Agilent Technologies) was used in combination with a Poreshell 120 C₁₈ 2.1 mm \times 100 mm, 2.7 μ m (Agilent Technologies) column at 25 °C as trapping and analytical columns, respectively.

Positive ion mode electrospray ionization and multiple reaction monitoring (MRM) mode were used in this study. The MRM transitions of gymnemagenin were obtained from the manual injection of gymnemagenin standard solution into the mass system. The gymnemagenin molecule will be automatically ionized upon the collision energy in range of 5 V to 80 V. The top four high abundance of ion fragments will be reported as shown in Figure S2. Four MRM transitions of gymnemagenin ([M+H]⁺ = 507.72) were selected: [M+H]⁺ of 489.5, 471.9, 454.0 and 145.4 using collision cell energy of 9 V, 13 V, 17 V and 45 V, respectively. Other mass parameters such as ion source voltage (IS), declustering potential (DP), entrance potential (EP), and collision exit cell (CXP) were 4500 V, 144 V, 9 V, 54 V and 22 V, respectively.

The mobile phases consisted of solution A (0.1% v/v formic acid in water) and solution B (0.1% v/v formic acid in acetonitrile). At the first minute, isocratic conditions at 100% A were used at a 0.2 mL/min flow rate. A linear gradient elution was performed from 0% to 50% of solution B in 10 min and from 50% to

90% of solution B in 8 min. Finally, the column was washed and re-equilibrated for 7 min at 100% of solution A. Injection time was 25 min.

The concentration of detected gymnemagenin was directly converted to gymnemic acid quantity. The gymnemic acid concentration in the extract and dry leaf materials is calculated regarding to the equation below.

$$\text{Gymnemic acid (mg/kg)} = (C \times V)/W$$

Where C is the concentration of a sample solution as compared to the standard curve (mg/L), V is the final volume of a sample solution (ml), W is the weight of the extract or dry leaves (g).

S3. Method validation

Calibration curve

A stock solution of the gymnemagenin standard was prepared by dissolving an accurate weight in methanol using a volumetric flask and diluting to an appropriated concentration range. The standard curve was in range of 0.001 - 0.480 mg/L. The solution was filtrated with a nylon filter (0.2 μm pore diameter) before quantification. A calibration curve was determined by seven concentrations in triplicate and plotted between peak area *versus* concentration. Linear regression analysis of the standard was calculated with $y = ax + b$, where x was standard concentration and y was peak area. The linearity of the calibration curve was performed by the correlation coefficient (R^2).

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined in accordance with International Conference of Harmonization. LOD and LOQ values were calculated from the standard curve which was analyzed three times. Both LOD and LOQ were determined based on the following formula,

$$\text{LOD} = (3.3\sigma)/S$$

$$\text{LOQ} = (10\sigma)/S$$

Where σ is the standard deviation of the Y-intercept and S is the average slope of the regression line determined from the calibration curve in triplicate.

Precision

Intra-day and Inter-day precision values were determined by seven replicate injections of two concentration levels in the same day (Intra-day) and different day (Inter-day). The variations of retention time was measured and presented as the percentage of relative standard deviation (%RSD).

Accuracy

The accuracy of this method was evaluated by %recovery. A known amount of the gymnemagenin standard was added to samples at three concentrations of 0.048, 0.096 and 0.145 mg/mL to represent the low, medium and high levels. Each solution was injected into the LC-MS system in triplication. After extraction, the spiked samples were analyzed and the recovery percentage of gymnemagenin in each sample was evaluated by the equation as shown below,

$$\% \text{recovery} = [(\text{observed amount} - \text{initial amount}) \times 100] / \text{spiked amount}$$

Table S1 : Method validation of gymnemagenin detection using liquid chromatography with electrospray ionization mass spectroscopy in multiple reaction monitoring procedure

Linear range (mg/L)	Linear regression equation	Correlation coefficient (R ²)	LOD (mg/L)	LOQ (mg/L)	%RSD*				%Recovery		
					Intraday		Interday		Low level	Medium level	High level
					0.048 mg/L	0.241 mg/L	0.048 mg/L	0.241 mg/L			
0.001 - 0.480	y = 366799x - 815.51	0.9999	0.0017	0.0052	0.10	0.098	0.96	0.95	100.54	90.87	94.64

*Based on seven replicate injections at a certain concentration.

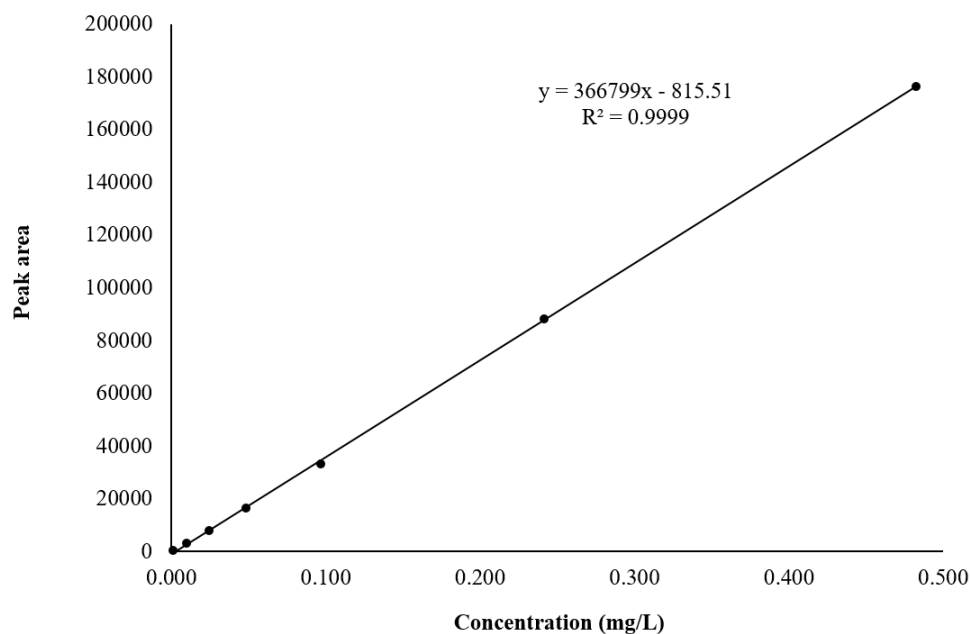


Figure S1: The linear regression between gymnemagenin concentrations and their peak areas

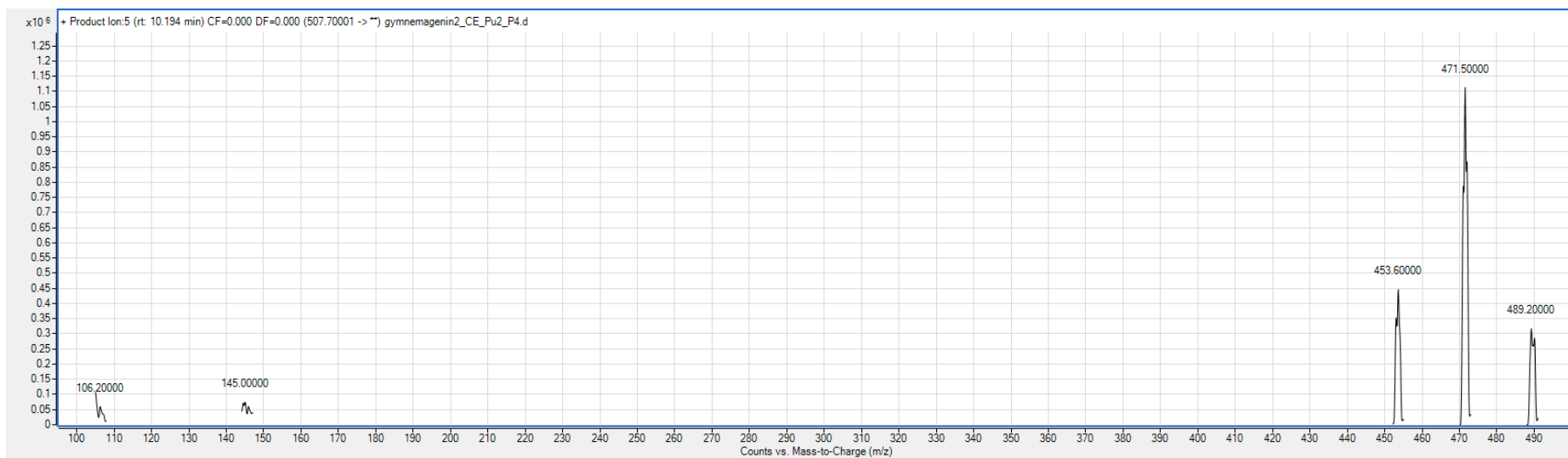


Figure S2: The MRM transitions of gymnemagenin when applying the collision energy in range of 5 V to 80 V