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Quantitation Method Research for the Pyrrolizidine Alkaloids (PAs) Determination in Botanicals by HPLC-MS/MS

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Abstract: A HPLC-MS/MS method for the determination of Pyrrolizidine alkaloids (PAs) in green tea, orange pekoe tea, hibiscus flower, chamomile flower, and cardamom seed was developed and validated. Its precision, accuracy, linearity, specificity, LOQ, LOD, and ruggedness were validated following AOAC Guidance for Single Laboratory Validation Procedure. The recovery of the analytes is in a range of 70%–130%. LOQ for 21 PAs in the above botanicals is in a range of 0.8 to 6.5 ppb.

Keyword: Pyrrolizidine alkaloids (PAs); HPLC-MS/MS; Botanicals

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1. Introduction

Pyrrolizidine alkaloids (PAs) were among the first naturally occurring plant carcinogens [1–2]. They are known as a class of compounds existing in over 6000 species of botanicals, and the toxicity of PAs on animals and humans is well reported [3–4]. However, methods for accurate measurement of PAs in different matrices of foods and ingredients are challenging in terms of the specificity, recovery, and accuracy due to the sample impurity interference [5–6].

Currently, most methods published for PAs analysis in botanicals and food are focused on tea, traditional Chinese medicine, and honey, represented by the BfR method, which is also mostly adapted for method development and validation on different food matrices and botanical materials [7–10]. The challenge for accurate measurement of PAs in different botanical matrices is how to clean up the samples and reduce the interference of phytochemicals from different botanical materials. The low recovery for the PAs determination was mainly caused by the matrix interference. So a method procedure development focuses on cleaning up the sample by using a combination of cartilages, adjusting the sample concentrations to maximize the ion response of target analytes, and

the purpose is to reduce the matrix interference. In this study, the authors focus on cleaning up the sample by using a combination of cartilages, adjusting the sample concentrations to maximize the ion response of target analytes. In brief, the sample was extracted with 0.05 M sulfuric acid in water by sonication and followed by purification by SPE column, and then the sample solution was subject to HPLC-MS/MS [11–13]. With a C18 column separation by gradient elution of 0.1% formic acid in methanol and 0.1% formic acid in water to analyze twenty-one (21) PAs in the botanicals. In this method, a combination of purification procedure and HPLC-MS/MS is good for better separation and achieves higher detection sensitivity.

2. Experimental

2.1. Reagents and standards

Sulfuric acid, GR grade or equivalent; Ammonia (25%-28%), AR grade or equivalent; Methanol, HPLC grade or equivalent; Water, Ultrapure or equivalent; Formic acid, LC-MS or equivalent; Acetonitrile HPLC grade, or equivalent; Standard list (**Table 1**).

Table 1. Standards and information

Compound	Brand (or equivalent)	Catalog# (or equivalent)	CAS#
Intermedine	BEPURE	RMT10912	10285-06-0
Intermedine N-oxide	BEPURE	RMT10913	95462-14-9
Lycopsamine	BEPURE	RMT17384	10285-07-1
Lycopsamine N-oxide	BEPURE	RMT17385	95462-15-0
Senecionine	BEPURE	RMT24591	130-01-8
Senecionine N-oxide	BEPURE	RMT24593	13268-67-2
Senecivernine	BEPURE	RMT24602	72755-25-0
Senecivernine N-oxide	BEPURE	RMT24604	101687-28-9
Seneciphylline	BEPURE	RMT24595	480-81-9
Seneciphylline N-oxide	BEPURE	RMT24597	38710-26-8
Retrorsine	BEPURE	RMT13555	480-54-6
Retrorsine N-oxide	BEPURE	RMT13557	15503-86-3
Echimidine	BEPURE	RMT17535	520-68-3
Echimidine N-oxide	BEPURE	RMT17536	41093-89-4
Lasiocarpine	BEPURE	RMT24697	303-34-4
Lasiocarpine N-oxide	BEPURE	RMT24698	127-30-0
Europine hydrochloride	BEPURE	RMT13964	570-19-4
Europine N-oxide	BEPURE	RMT13965	65582-53-8
Heliotrine	BEPURE	RMT10774	303-33-3
Heliotrine N-oxide	BEPURE	RMT10775	6209-65-0
Senkirkin	BEPURE	RMT2461	2318-18-5

2.2. Equipment

Analytical balance; Vortex mixer; Pipette; Sonicator; Centrifuge; Volumetric flask; Coffee grinder; SPE cartridge manifold; Graduate Cylinders; Ultra PW system, Purelab flex, or equivalent; Bond Elut SCX cartridges (500 mg, LRC, p/n 14113039); HPLC System: Agilent Technologies 1260 Series Rapid Resolution HPLC, consisting of Binary Pump, Degasser, and Autosampler or equivalent; Agilent Technologies 6420 Triple Quad MS or equivalent; Agilent MassHunter Workstation Software or equivalent; HPLC Column: Agilent InfinntyLab Poroshell 120 EC-C18, 2.7 µm, 2.1 x 100 mm, Catalog# 695775-902 or equivalent; Guard Column: Phenomenex HPLC Krudkatcher Ultra Column In-line Filter 0.5µ Porosity x 0.004in ID, catalog# AF0-8497 or equivalent

2.3. HPLC chromatographic conditions

Mobile Phase: A= 0.1% of formic acid in water; B= 0.1% of formic acid in methanol; HPLC Gradient conditions (**Table 2**).

Table 2. HPLC Gradient conditions

	Time, min	Flow, mL/min	A, %	B, %
1	0.00	0.2	95	5
2	5.00	0.2	95	5
3	10.00	0.2	93	7
4	15.00	0.2	90.0	10.0
5	25.00	0.2	80.0	20.0
6	30.00	0.2	60.0	40.0
7	32.00	0.2	0.0	100.0
8	38.00	0.2	0.0	100.0
9	38.10	0.4	0.0	100.0
10	42.00	0.4	0.0	100.0
11	42.10	0.4	5.0	5.0
12	54.00	0.4	5.0	5.0
13	54.10	0.2	5.0	5.0
14	62.00	0.2	5.0	5.0

The MS triple quadrupole parameters are shown in **Table 3**.

Table 3. MS triple quadrupole parameters

Parameter	Value
Ionization mode	Positive ESI
Scan type	MRM
Gas temperature	300 °C
Gas Flow	13 L/min
Nebulizer pressure	30 psi

Table 1 (Continued)

Parameter	Value
Capillary voltage	3500 V
Cell acceleration	5 V
Cycle time	500 ms
Dwell time	5 ms
Maximum dwell time	165.8 ms
Resolution	Unit

The MRM transitions and conditions for each PAs are shown in **Table 4** [4].

Table 4. Transitions and conditions for each PAs

Compound	Abbreviation	Precursor ion	Product ion (s)	Fragment	Collision energy
Echimidine	Em	398.2	120.2 220.2 118.1	104	24 16 56
Echimidine-N-oxide	EmN	414.2	254.2 352.1 137.1	104	32 24 40
Europine	Eu	330.2	138.1 156.1 94.1	94	20 32 48
Europine-N-oxide	EuN	346.2	172.1 94.1 111.2	94	36 60 52
Heliotrine	Hn	314.2	138.1 156.1 120.1	98	20 32 40
Heliotrine N-oxide	HnN	330.2	172.1 138.1 111.1	102	32 28 48
Intermedine	Im	300.1	138.2 156.3 120.1	106	20 32 28
Intermedine N-oxide	ImN	316.2	172.1 94.0 111.1	112	32 52 44
Lasiocarpine	Lc	412.2	120.1 220.1 336.1	118	32 20 16
Lasiocarpine N-oxide	LcN	428.2	120.1 254.2 94.0	108	40 32 56
Lycopsamine	La	300.1	138.2 156.3 120.1	104	20 32 24

Table 4 (Continued)

Compound	Abbreviation	Precursor ion	Product ion (s)	Fragment	Collision energy
Lycopsamine N-oxide	LaN	316.2	172.1 94.0 111.1	98	32 48 44
Retrorsine	Re	352.2	120.0 138.1 324.2	112	32 32 28
Retrorsine N-oxide	ReN	368.2	118.0 136.1 119.0	106	36 36 36
Senecionine	Sc	336.2	120.1 138.1 308.2	135	28 32 28
Senecionine N-oxide	ScN	352.3	120.2 136.2 94.2	185	44 38 56
Seneciphylline	Sp	334.2	120.1 138.1 306.1	140	28 32 28
Seneciphylline N-oxide	SpN	350.1	120.1 136.1 118.1	108	36 36 36
Senecivernine	Sv	336.2	120.1 138.1 308.2	130	32 33 28
Senecivernine N-oxide	SvN	352.2	94.0 120.2 136.2	110	56 48 40
Senkirkine	Sk	366.2	168.1 150.1 122.1	128	32 32 36

The MS triple quadrupole time segment is shown in **Table 5**.

Table 5. MS triple quadrupole time segment

	Time	Scan Type	Div Valve	Delta EMV +	Stored
1	0	MRM	To Waste	0	No
2	8	MRM	To MS	200	Yes
3	42	MRM	To Waste	0	No

Column temperature: 40 °C; Sample temperature: ambient; Injection volume: 2 μL; Run time: 62 minutes

2.4. Solution preparation

0.05 M sulfuric acid: Add 2.665 mL of sulfuric acid to 800 mL of water slowly, diluting to 1000mL with water, mix well. Note: If a negative matrix is available, blank plant material extract can instead be 0.05M sulfuric acid.

0.5 % ammonia in methanol: Transfer 10 mL of water and 2 mL of ammonia into a 100 mL volumetric flask, diluting to the mark with methanol, and mix well. This solution must be freshly prepared and used per

working day.

2.5. Standard preparation

PAs Standard Stock Solution: Accurately weigh 1 mg of each pyrrolizidine alkaloid standard to a 10 mL volumetric flask individually, dissolve to mark with acetonitrile, and mix well. The concentration of the stock solution is 0.1 mg/mL. Stock Solution in freezer for 30 days [4, 14–16].

PAs Mixed Mid Stock Standard Solution (PAs mixture, 1 μg/mL): Pipette each of 1 mL of PAs Standard Stock Solution into a 100 mL volumetric flask, diluting to mark with extraction solution to mark, mix well

Working Standard solution:

Working STD 3: Pipette 200 μL of PAs Mixed Mid Stock Standard Solution and add 800 μL of 0.05M sulfuric acid;

Working STD 2: Pipette 100 μL of PAs Mixed Mid Stock Standard Solution and add 900 μL of 0.05M sulfuric acid;

Working STD 1: Pipette 500 μL of Working STD 2 Solution and add 950 μL of 0.05M sulfuric acid.

2.6. Method development

The low recovery for PA determination was mainly caused by the matrix interference. So, the focus of method development is on how to reduce the matrix interference. The method is divided into 3 parts as shown in **Figure 1**.

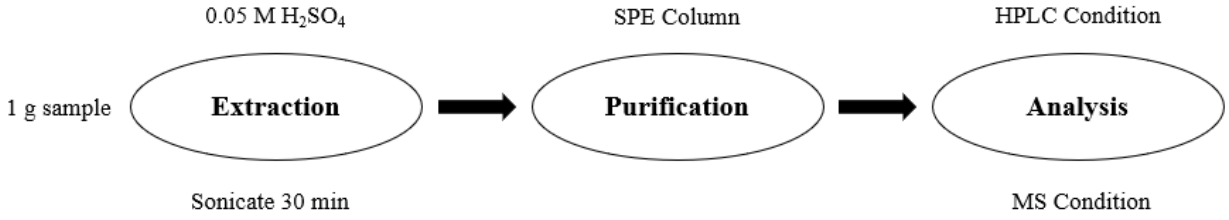

Figure 1. Method development

Extraction: Adjust the sample size to balance the matrix interference and the extraction rate of target analytes (PAs).

Purification: Optimization of the concentration of ammonia in methanol is critical for sample purification. When the matrix contains other alkaline components, such as caffeine. An optimization of the concentration of ammonia in methanol was conducted to maximize the elution of PAs, but to remove most other alkaline components' interference. Four concentrations of ammonia in methanol solution were tested; the results show that 0.5 % ammonia in methanol is the best option (**Table 6**).

Table 6. Recovery result for different concentrations of ammonia in methanol solution

	0.3 % Ammonia	0.5 % Ammonia	1.0 % Ammonia	2.5 % Ammonia
Average Recovery	92%	93%	89%	85%
The Lowest Recovery	75%	80%	76%	65%

Analysis: The flow rate was reduced from 0.3 to 0.2 mL/min. This can improve the ionization efficiency of ESI. The higher the ionization efficiency, the smaller the influence of matrix interference.

The injection volume was reduced from 10 to 2 μL. This greatly reduces the burden on ESI, resulting in better ionization efficiency.

The analysis time was extended to reduce matrix interference per unit time. And each PA peak was effectively separated (**Figure 2**).

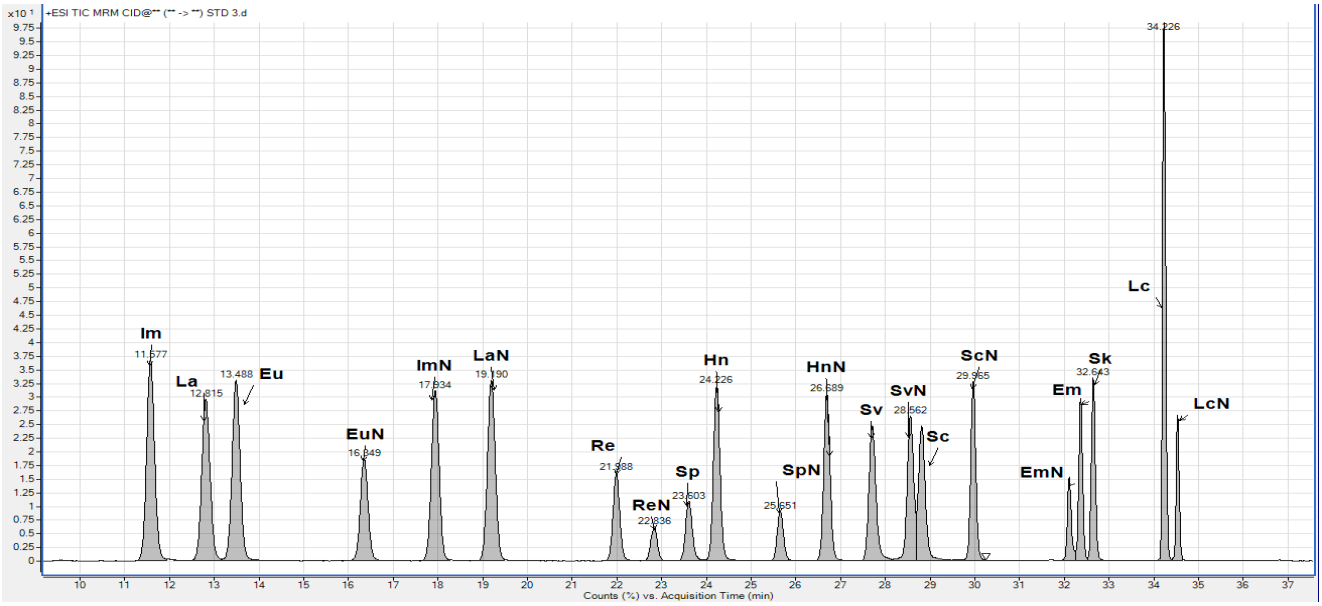

Figure 2. Chromograph of each Pas

2.7. Sample preparation

2.7.1. Extraction

Weigh 1 g ± 0.1 g of the sample, accurate to 0.01 g, the amount of the ground and homogenized samples. Transfer the sample to a 50 mL centrifuge tube. Add 38 mL of 0.05 M sulfuric acid solution, vortex for a few seconds. Sonicate the sample in the tube for 30 minutes in an ultrasonic bath at room temperature. Adjust the pH value to 7.0 using ammonia. Add 0.05 M sulfuric acid solution to the 40 mL mark, mix well. Centrifuged at 7000 rpm for 5 minutes. Filter the supernatant through 0.45 μm membrane filters [14–17].

2.7.2. SPE-Procedure

Install the SPE cartridge on top of the manifold. SPE column condition: Add 5 mL of methanol on top of the SPE cartridge and let the methanol go through by gravity. Add 5 mL of water on top of the SPE cartridge and let the solution go through by gravity. Collect the solution to waste. Load 20 mL sample solution (filtrate of extract), let the solution go through by gravity, and collect the solution in the waste. Rinse the cartridge with a sample loaded by 4 mL of water, let the water go through by gravity, and collect the solution into waste. Rinse once more. Rinse the cartridge with the sample loaded by 4 mL of methanol, let the methanol go through by gravity, and collect the solution in the waste. Rinse once more. Elute the SPE column with 4 mL of 0.5 % ammonia in methanol, let the 0.5 % ammonia in methanol go through by gravity, and collect the solution in the sample tube. Elute once more. Dry the elute solution under a nitrogen stream at 50 °C ± 5 °C.

2.7.3. Reconstitution of the sample

Dissolve the residue in 2 mL of 0.05 M sulfuric acid solution by sonication and shaking. Filter a portion of the

sample through a 0.2µm syringe filter, discard the first few drops of the filtrate, and then fill an HPLC vial.

2.8. System suitability

The R^2 for each PAs component peak should not be less than 0.995. Run a standard check (working standard solution 2) after every six sample injections, and at the end of the run. The peak area of each compound from each check standard should be within 90%–110% of the peak area of Working Standard 2 from the calibration curve.

2.9. Calculations

Obtain each PA Standard Curve(s) by plotting corresponding PA Standard Concentrations vs. their Peak Area. Interpolate the sample concentration (ng/mL) from the standard curve(s). Calculate the amounts of each PA per the formulas below. Calculate the amount of Total PA by summing all 21 PAs.

$$\text{PA (ng/serving)} = \frac{\text{Spl. Conc.}_{(\text{ng/mL})} \times \text{D.F.}_{(\text{mL})} \times \text{SS}_{(\text{g/serving})}}{\text{Spl. Wt.}_{(\text{g})}}$$

Spl. Conc.= Sample concentration from the standard curve
D.F. = Dilution factor for sample
SS = Serving size
Spl. Wt.(g) = Sample weight

3. Results and discussion

3.1. Specificity

Blank, standard, and sample (spiked standards) solutions were prepared and analyzed following the test method. Run HPLC-MS/MS analysis of blank, standard, and sample solutions. Each transition of blank was overlaid with those of the standard and sample solutions. Two Qualifier Response Ratios of each PA from the sample solutions to those of the standard solution were compared. No significant interfering peak was found in each PA transition at the retention time of each PA peak from the standard solution. All Qualifier Response Ratios of each PA from the sample solutions were 84-117% of those from the standard solution.

3.2. Precision

Six (6) replicate samples of each sample (spiked standards) were prepared and analyzed according to the test method. The amount of each PA from the samples was calculated. The RSD of six replicates of green tea was not more than 14%, Green tea extract was not more than 8%, and Herbal concentrate tea was not more than 10%.

3.3. Accuracy

The spiked samples were prepared by adding known quantities of Spike Standard Solution to the samples. The spiked samples (three concentrations and three replicates of each concentration) were analyzed according to the test method. The amount of each compound of PA in the spiked samples measured versus the spiked amounts of each compound added was calculated as % Recovery. The recoveries for all spiked levels of green tea were within 71%–104%. The recoveries for all spiked levels of green tea extract were within 73%–105%. The recoveries for all spiked levels of cardamom seed extract were within 71%–105%. The recoveries for all spiked levels of herbal concentrate tea were within 72%–130%. The recoveries for all spiked levels of orange pekoe extract were within 72%–128%. The recoveries for all spiked levels of chamomile powder were within 79%–130%. The recoveries for

all spiked levels of hibiscus flower powder were within 93%–129%.

3.4. Linearity/Range

Standard solutions were prepared and diluted to the 5 levels of PAs. Three (3) replicate injections were made for each of the five (5) standard solutions. The peak areas obtained for each solution were plotted against their corresponding theoretical concentrations. Linear regression analyses on the five coordinates were performed. The R^2 of the linear curve was between 0.998 to 1.000

3.5. Ruggedness

The same lots of each sample were analyzed in duplicate by a second analyst on a different day.

The RSD of the eight test results (6 Precision + 2 Ruggedness) was calculated. The RSD of eight replicates of green tea was no more than 13%, green tea extract was no more than 8%, and herbal concentrate tea was no more than 10%.

3.6. LOQ & LOD

Standard solutions at the estimated LOQ level were prepared and analyzed three (6) times. Signal-to-noise ratios were calculated by MassHunter. The lowest concentration of each compound, which Signal-to-noise ratio NLT 10:1 was selected as the limit of quantitation. All LOQs in the solution were within 0.18–1.61 ng/mL. All LOQs in the sample were within 0.8–6.5 ppb.

Disclosure statement

The authors declare no conflict of interest.

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