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A LC–ESI–MS/MS analysis procedure coupled with solid phase extraction and MeOH extraction method for determination of pyrrolizidine alkaloids in *Tussilago farfara* and *Lithospermi erythrorhzion*

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Abstract

Pyrrolizidine alkaloids (PAs) that are plant toxin naturally produced for protection against herbivores in some plant families. They are associated with the potential hepatotoxic and carcinogenic diseases serious hepatic disease in humans and animals. As the concern of human health risk posed by exposure to PAs has been gradually increased, precise and reliable analysis is required for monitoring PAs. The present study developed a new and simple pretreatment using 50% MeOH (methanol) for quantification analysis of the PAs contained with high content in the herbal medicines. Another pretreatment method using cation-ion exchange solid-phase extraction (MCX-SPE) was employed for determining most of the PAs that are not contained in the herbal medicines. That is, the proposed LC–MS/MS method coupled with MCX-SPE extraction and 50% MeOH extraction method was developed. And to evaluate the reliability of its application for Farfarae Flos and Lithospermi Radix, a validation study was conducted. In addition, monitory study was performed with ten samples in each herbal medicine. As a result, the proposed method had good linearity with $r^2 \geq 0.997$. Also, the recoveries indicated to be in the ranges of 70.4–118.0% for the Farfarae Flos, 70.2–119.7% for the Lithospermi Radix. In two herbal medicines, the intra-day precision was revealed to satisfy the reference criteria in most of the PAs. In monitoring results, most of the PAs were not contained in two herbal medicines, whereas a part of PAs revealed to have high concentration in Farfarae Flos and Lithospermi Radix. The proposed method is considered as a simple and reliable method to quantify 28 PAs present in two herbal medicines. Especially, the simple MeOH extraction method seems to be available for quantification analysis of certain PAs in herbal medicines with high content.

Keywords: Pyrrolizidine alkaloid, LC–MS/MS, Herbal medicine, Farfarae Flos, *Tussilago farfara*, Lithospermi Radix, *Lithospermi erythrorhzion*, Matrix effect

Introduction

As the use of traditional medicines in developed countries is exponentially growing, the concern associated with the human health risk by exposure of Pyrrolizidine alkaloids (PAs) has gradually increased because raw plant materials widely distributed in the world are used for medicinalpurposes such as dietary supplements and

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traditional medicines. PAs are secondary plant metabolites, produced naturally for protection against herbivores in some plant families [1]. Exposure of several PAs over longer periods has been known to cause hepatotoxicity, genotoxicity and carcinogenic potential in humans and animals for decades [2–5]. The International Agency for Research on Cancer (IARC) classifies that Lasiocarpine, Monocrotaline, and Riddelline as "possibly carcinogenetic to humans (class 2B) and Isatidine, Retrorsine, and Senkirkine that have only limited evidence as "not classifiable as to its carcinogenicity to humans (class 3)" [6, 7].

Farfarae Flos (coltsfoot), derived from the dried flower buds of *Tussilago farfara* of the Compositae (Asteraceae) family, is a famous herbal medicine in oriental countries called and registered as "Kuandonghua" [8]. The folk medicines have been used in teas for the relief of coughs, chest complaints, and humid forms of asthma in other countries as well as oriental countries [9, 10]. Their pharmacological activities associated with, anti-inflammatory [11], anti-oxidative [12], anti-microbial [13], anti-diabetic [14], neuroprotection[15], platelet anti-aggregation [16], and anti-cancer [17] etc. have studied. Lithospermi radix, the root of *Lithospermum erythrorhizon* Siebold. et Zuccarinii that belongs to be Borraginaceae, has been used as an oriental herbal medicine to treat wounds, burns, and dermatitis in Korea, China, Japan [18, 19]. Nowadays, it has been reported to have various pharmacological properties as follows; adjuvant for treatment of cancer [20, 21], anti-inflammatory [20, 22], antifungal [22], and antibacterial [23], antiangiogenic [24], and anti-topoisomerase [25] properties.

Many researchers claim that the most worrisome route of human exposure to PAs containing plants is likely to result from the intake of herbal medicines and herbal teas [2, 26, 27]. The German Federal Institute for Risk Assessment (BfR) reports that 221 samples of herbal teas and certain medicinal teas contained Pas [28]. Due to the risk of intake and the high toxicity, the Herbal Medicinal Products Committee (HMPC) of the European Medicines Agency (EMA) have implemented a limit of intake of PAs from herbal medicinal products(i.e. 1 μg PA per day) as a transitional measure for three years, after which threshold will be set to 0.007 μg of 1,2-unsaturated PA/kg body weight (i.e. 0.35 μg PA per day for 50 kg adult and 0.14 μg PA per day for children) [29]. However, in 2019 HMPC has extended the transitional period for the limit of 1.0 μg PA per day until May 2021 [30, 31]. Also, BfArM publishes that the maximum daily dose of toxic PAs for internal use is set at 1 μg for a duration of maximum of 6 weeks per year and 0.1 μg without any limitation in the duration [32]. A specific method for the determination of PA in herbal drugs and herbal extracts does not yet exist. However, the European pharmacopeia adopted a new general chapter for the description of the analytical methods and validation data of Pas [33]. In Korea, the regulatory decision about quality control related to PAs contamination in herbal medicines has not been established yet, Ministry of Food and Drug Safety has developed the analysis methods for the determination of PAs in herbal medicine to monitor the PAs contamination in herbal medicines.

Various analytical methods for determination toxic PAs present in *Tussilago farfara* have been reported as follows; High-performance thin-layer chromatography (HP-TLC) [34], High performance liquid chromatography (HPLC–UV) [35, 36], Gas chromatography–mass spectrometry (GC–MS) [37, 38], High-performance liquid chromatography–mass spectrometry (HPLC–MS) [18], HILIC/ESI-Q-TOF–MS (Hydrophilic interaction liquid chromatography/electrospray ionization-quadrupole time-of flight mass spectrometry) [34]. Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF–MS) [18, 39] claim that Liquid chromatography–mass spectrometry (LC–MS) methods are the most suitable procedures for achieving precise quantification of PAs in herbal medicines because Liquid chromatography with tandem mass spectrometry (LC–MS/MS) technologies allow the limit of quantitative determination of PA to become in a range substantially below 1 mg/kg. A specific European Pharmacopoeia (Ph.Eur.) method for determination of PA in herbal drugs and herbal extracts does not yet exist [31], however, the HMPC at EMA recommends using the LC–MS/MS procedure (BfR-PA-Tea) developed by the BfR for determining pyrrolizidine alkaloids in herbal medicinal products [29]. As raw herbal medicines, which are used for herbal preparations, herbal medicinal products are comprised of multi-component mixtures. For precise quantification of PAs, verification of the analysis method is required for every herbal preparation or herbal drug even if a validated method for determination of pyrrolizidine alkaloids exists [40]. The majority of PAs present in *Tussilago farfaraare* are known to be Senkirkine and traces of Senecionine are also present. *Lithospermum erythrorhizon* is well known to contain several PAs such as intermedine, myoscorpine, and hydroymyoscorpine with a total concentration of ~ 0.02% relative to the dried drug [41].

Recently, our laboratory developed the new LC–MS/MS method coupled with cation-ion exchange solid phase extraction (MCX-SPE) method to determine 28 PAs in herbal medicines. For evaluation of its application, validation studies of the new LC–MS/MS method have been conducted against various herbal medicines having potential PAs contamination. In general, for validation of the analysis method, the blank samples, which

are not present in the analyte, are required to obtain precise recoveries of the analyte compounds. Through the preliminary test, we found that Farfarae Flos contained 3 PAs with high content such as Senkirkine, Senecionine, Senecionine *N*-oxide, whereas Lithospermi Radix possesses 4 PAs including Echimidine, Echimidine *N*-oxide, Intermedine, Intermedine *N*-oxide with high content. Due to these PAs present in the herbal medicines, it was difficult to precisely quantify these PAs due to the saturation occurred by their high content. Therefore, to resolve such a problem, the present study developed new simple pretreatment for quantification analysis of 7 PAs contained the herbal medicines. The developed extraction method used a large volume of 50% MeOH and a small amount of sample so that the unnecessary or interfering materials are sufficiently diluted. The present study was to validate the new LC–ESI–MS/MS method combined with MCX-SPE extraction and MeOH extraction method for confirmation of its application to determine 28 PAs in the Farfarae Flos and Lithospermi Radix.

Materials and methods

Chemicals and reagents

The 28 pyrrolizidine alkaloid (PA) standards used in the present study were purchased from Phytolab (Vestenbergsgreuth, Germany), their abbreviations are shown in Table 1. Methanol (HPLC grade), ammonia solution (25%), and water (LC–MS grade) manufactured from Merck KGaA (Darmstadt, Germany) and ammonium formate, formic acid, and sulfuric acid (H_2SO_4, 98%)

Table 1 The conditions of multiple reaction monitoring (MRM) for determination of 28 PAs in herbal medicines using LC–MS/MS

No.	Compound name	PAs Abbr	RT[a] (min)	Formula	Cas No	Precursor ion $[M+H]^+$	MRM ion transitions (*m/z*)		CE[b] (eV)
							Quantitative ion	Confirmation ion	
1	Echimidine	Em	20.33	$C_{20}H_{31}NO_7$	520-68-3	398.25	120.1	220.1	− 25
2	Echimidine *N*-oxide	EmN	20.33	$C_{20}H_{31}NO_8$	41093-89-4	414.15	254.1	352.2	− 17
3	Erucifoline	Er	7.25	$C_{18}H_{23}NO_6$	40158-95-0	350.20	120.2	138.25	− 31
4	Erucifoline *N*-oxide	ErN	8.49	$C_{18}H_{23}NO_7$	123864-94-8	366.20	94.2	119.15	− 25
5	Europine	Eu	8.05	$C_{16}H_{27}NO_6$	570-19-4	329.70	138.2	156.1	− 29
6	Europine *N*-oxide	EuN	9.10	$C_{16}H_{27}NO_7$	65582-53-8	346.30	172.05	111.1	− 28
7	Heliotrine	Hn	12.29	$C_{16}H_{27}NOs$	303-33-3	314.20	138.15	156.2	− 44
8	Heliotrine *N*-oxide	HnN	14.19	$C_{16}H_{27}NO_6$	6209-65-0	330.10	172.15	111.1	− 31
9	Intermedine	Im	8.01	$C_{15}H_{25}NO_5$	10286-06-0	300.30	94.15	138.05	− 34
10	Intermedine *N*-oxide	ImN	9.80	$C_{15}H_{25}NO_6$	95462-14-9	316.20	172.05	94.1	− 25
11	Jacobine	Jb	8.18	$C_{18}H_{25}NO_6$	6870-67-3	352.30	120.15	155.15	− 31
12	Jacobine *N*-oxide	JbN	9.20	$C_{18}H_{25}NO_7$	38710-25-7	368.10	296.15	120.15	− 44
13	Lasiocarpine	Lc	20.59	$C_{21}H_{33}NO_7$	303-34-4	412.20	120.1	336.15	− 20
14	Lasiocarpine *N*-oxide	LcN	20.68	$C_{21}H_{33}NO_8$	127-30-0	428.20	254.3	93.85	− 28
15	Lycopsamine	La	8.43	$C_{15}H_{25}NO_5$	10285-07-1	300.20	94.1	138.1	− 27
16	Lycopsamine *N*-oxide	LaN	10.37	$C_{15}H_{25}NO_6$	95462-15-0	316.35	172.15	94.15	− 43
17	Monocrotaline	Mc	6.02	$C_{16}H_{23}NO_6$	315-22-0	326.15	120.1	94.1	− 27
18	Monocrotaline *N*-oxide	McN	7.96	$C_{16}H_{23}NO_7$	35337-98-5	342.15	137.1	119.1	− 20
19	Retrorsine	Re	11.47	$C_{18}H_{25}NO_6$	480-54-6	352.20	120.1	138.15	− 28
20	Retrorsine *N*-oxide	ReN	12.27	$C_{18}H_{25}NO_7$	15503-86-3	368.20	94.20	118.05	− 41
21	Senecionine	Sc	16.15	$C_{18}H_{25}NO_5$	130-01-8	336.20	120.1	94.05	− 31
22	Senecionine *N*-oxide	ScN	17.61	$C_{18}H_{25}NO_6$	13268-67-2	352.20	94.1	118.05	− 29
23	Seneciphylline	Sp	12.59	$C_{18}H_{23}NO_5$	480-81-9	334.15	120.10	94.1	− 26
24	Seneciphylline *N*-oxide	SpN	7.25	$C_{18}H_{23}NO_6$	38710-26-8	350.20	120.15	94.10	− 38
25	Senecivernine	Sv	15.42	$C_{18}H_{25}NO_5$	72755-25-0	336.20	120.1	308.3	− 28
26	Senecivernine *N*-oxide	SvN	16.60	$C_{15}H_{25}NO_6$	101687-28-9	352.20	118.1	94.15	− 19
27	Senkirkine	Sk	20.47	$C_{19}H_{27}NO_6$	2318-18-5	366.20	168.15	122.15	− 29
28	Trichodesmine	Td	11.49	$C_{18}H_{27}NO_6$	548-90-3	354.25	222.10	120.05	− 48

[a] *RT* retention time

[b] *CE* collision energy

purchased from sigma-aldrich (MO, USA) were used. Two kinds of herbal medicines, Farfarae Flos and Lithospermi Radix used for herbal medicinal products were purchased from an herbal medicinal store located in Daejeon, Korea. Standardized herbal medicines are produced by GMP facilities in Korea after herbal medicines were imported from China. Prior to carrying out any further experiments, the identities of the herbal medicine samples were confirmed through sensory tests performed by a specialist. The present study used as only genuine herbal medicines, which satisfied the appearance recommended by Korean Herbal Pharmacopoeia.

Standard solution preparation

The stock solutions of each PA standard were prepared to be a concentration of 100 μg/mL in MeOH and stored at − 20 ℃. The mixed PA standard solution was prepared by taking a part of the volume of each solution and mixing them with the solvent of 5% methanol. After the suitable concentrations in each PA were determined through a preliminary test, the mixed PA standard solution was serially diluted with the blank sample solution, which did not contain any analytes the standard solution, to be within calibration concentration range as shown on Tables 3 and 4. In the present study, most of the PAs were quantified using a matrix-matched analysis, however, the standard solution was diluted with solvent for calibration curve in 8 PAs contained in the herbal medicines (Fig. 1).

Sample preparation

Extraction using MCX-SPE cartridge

Prior to grinding the herbal medicines, the samples (600 g) were divided into four equal parts to make homogenous samples. A part of herbal medicine samples was grounded to become powders using a grinder (KSP-35, Koreamedi, Korea). After weighing 2.0 g of powder, it was mixed with 40 mL of extraction solution, 50% MeOH solution containing 50 mM sulfuric acid. After the addition of MeOH, the solutions were shaken for 30 min at room temperature to extract the PA compounds from herbal medicines using an orbital shaker (SH30t, FINEPCR, Korea). After extraction, the samples were centrifuged at 3320×*g* for 10 min and then the supernatant was collected. MCX-SPE cartridges (6 cc/150 mg, Waters Oasis, USA) were employed for purification to remove unnecessary or interfering materials contained in the extracted solution. The activation of MCX-SPE cartridges was achieved by 3 mL of MeOH and 3 mL of distilled water. After loading the supernatant (2 mL) on the cartridge, it was washed with 4 mL of distilled water and the residual solvent in the cartridge was dried under vacuum. At the next step, the target analytes were eluted

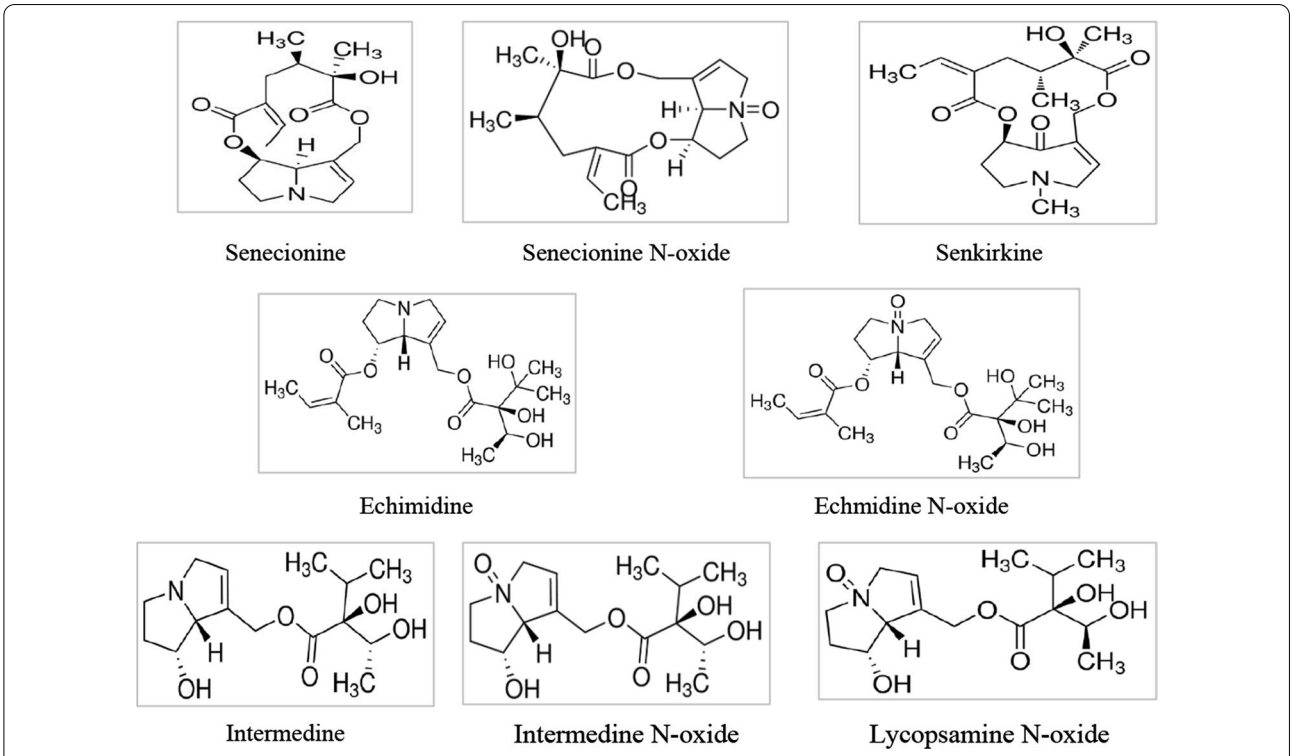

Fig. 1 Chemical structures of 8 pyrrolizidine alkaloids contained with high content in Farfarae Flos and Lithospermi Radix used in the present study

by adding the solution (4 mL) composed of NH_4OH and MeOH solution (1: 4, v/v) and the eluted solutions were evaporated (CVE3100, EYELA, Japan) at 50–55℃ under nitrogen gas (MB8-3100E, EYELA, Japan). After nitrogen concentration, the residual was reconstituted with 1 mL of methanol/water (5: 95, v/v). After filtering the solution with a syringe filter (0.22 μm), it was used as a sample solution for LC–MS/MS analysis (Fig. 2A).

Extraction using 50% MeOH solution

After weighing 0.1 g of powder, the samples were mixed with the solvent of 50% MeOH (20–200 mL). The volume of extraction solvent adding to samples was decided by depending on the PA contents possessed in the samples to become within the concentration ranges. The solutions were shaken for 30 min at room temperature to extract the PAs from the herbal medicines using an orbital shaker (SH30t, FINEPCR, Korea). After extraction, the samples were centrifuged at 3320×*g* for 10 min and then the supernatant was filtered by a syringe filter (0.22 μm). The final solution was used as a sample solution for LC–MS/MS analysis (Fig. 2B).

LC–MS/MS analysis

This study referred to the LC–MS/MS analysis procedure developed by other laboratory in MFDS, Korea (unpublished data) and BfR (German Federal Institute for Risk Assessment) method [42] to develop a more sensitive simultaneous analysis procedure of 28 pyrrolizidine alkaloids in herbal medicines. The LC–MS/MS system was coupled to a Shimadzu Nexera X2 LC-30AD, Shimadzu LCMS-8060 spectrometer in ESI positive ionization mode, and a Shimadzu lab solution system (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on a Shim-pack GIST-C18 (2.1 mm × 150 mm, 2 μm) and the column was maintained at 40 ℃. The mobile phases consisted of eluent A (0.1% formic acid in 5 mM ammonium formate) and eluent B (0.1% formic acid plus 5 mM ammonium formate in 100% methanol). A binary gradient profile was achieved as follows: 1.5 min, 1% B; 1.5–3.0 min, 1–15% B; 3.0–18.0 min, 15–30% B; 18.0–19.0 min, from 30 to 95% B with linear increasing; 19.0–21.0 min, hold at 95% B; 21.1 min, return to 1% B. Re-equilibration time between each run was given for 3.0 min. The injection volume was 5 μL and

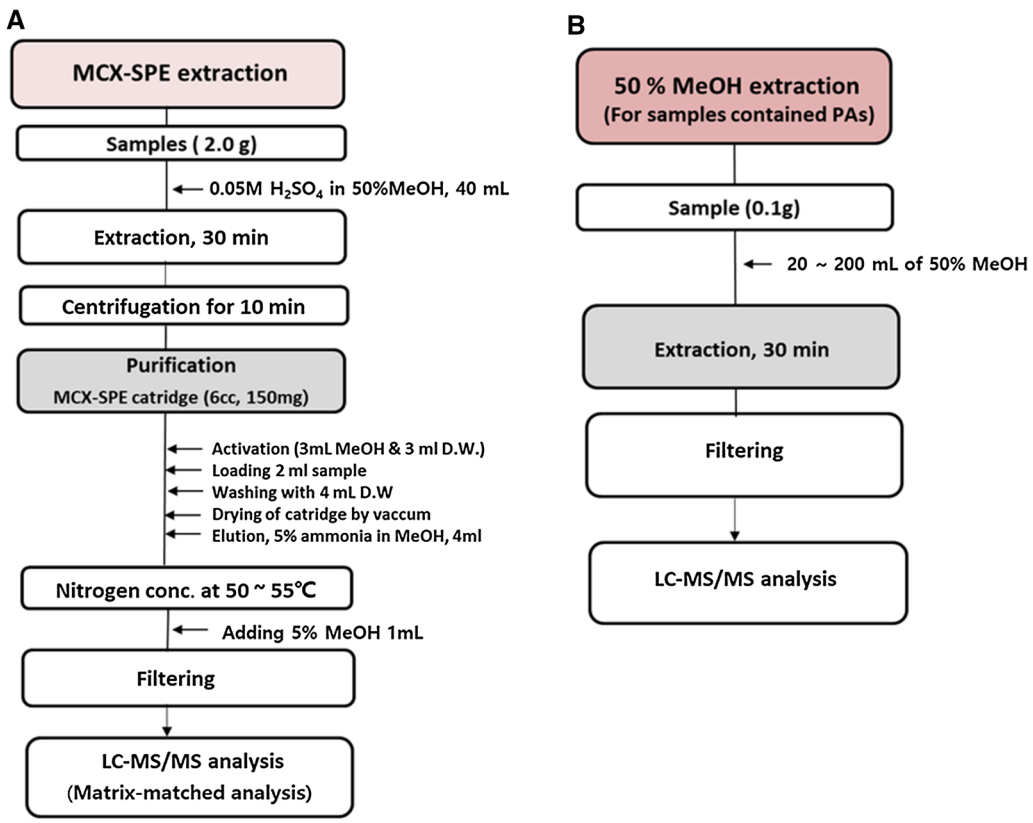

Fig. 2 Flow charts of two extraction methods developed to extract PAs from herbal medicines; MCX-SPE extraction method applied for determination of PAs that are not contained in the herbal medicines (**A**) and the new extraction method applied for determination of PAs are contained with high content in the herbal medicines (**B**)

the flow rate was maintained at 0.3 mL/min. The mass spectrometer was performed in the positive-ion mode of the ESI source using Shimadzu lab solutions and the mass spectrometer instrument and parameters were set as follows: drying gas temperature, 300 ℃; drying gas flow, 5.0 L/min; nebulizer pressure, 3 L/min; heat block temperature, 400℃; interface temperature 400 ℃, and nebulizing gas flow, 15 L/min. Nitrogen was used as the drying and nebulizing gas.

Matrix effect

Prior to the performance of the validation study, this study evaluated the matrix effect of each PA compound. The matrix effect was evaluated by comparing the response of the analyte in standard solution to that of blank sample solution spiked with the analyte at the same concentration. The standard solution was prepared by dilution of the mixed stock PA standard solution with 5% MeOH, whereas the other standard solution was prepared by spiking the mixed stock standard solution to the blank sample solution. The blank sample solutions were prepared by the same pretreatment method described above. The calibration curves were achieved from five-point concentrations at the range as shown Tables 3 and 4. The matrix effect can be evaluated by the following formula:

$$\text{Matrix effect (ME, \%)} = (\text{Slope of the calibration curve in matrix}) \left(/ \textit{Slopeofcalibrationcurveinsolvent}\right)$$

Method validation

The analytical method was validated in terms of limits of detection (LOD), and LOQ, linearity, reproducibility and repeatability (precision), recovery(accuracy) recommended by the guideline of AOAC [43]. The validation study was conducted by matrix-matched analysis in most of the PAs that are contained the samples. The linearity was obtained by plotting the peak area of the analysis against the analyte concentration and the linearity was assessed by the coefficients of determination (r^2). Linearity was evaluated by calibration curves obtained in the concentration ranges of the mixed 28 PA standard solutions with five calibration points. The mixed PA standard solutions were prepared by spiking the mixed stock PA solution to blank sample solutions of Farfarae Flos or Lithospermi Radix. The measurement was carried out in triplicate. The LOD and LOQ were measured using the ratio of signal to noise obtained after the injection of the standard solution with a concentration range as shown on Tables 3 and 4 (n = 3). In addition, this study referred the LOD and LOQ values calculated based on 3.3 or 10 × standard deviations of the response and the slope of the calibration curve, respectively.

For measuring the accuracy and precision against most PAs that were not contained in Farfarae Flos or Lithospermi Radix, the mixed PA standard with three different concentration levels (low, medium, and high) were spiked to the herbal medicine powers (2.0 g) to become 2, 5, 10 times of the lowest concentration of the range as shown on Tables 3 and 4, respectively.

After conducting pretreatment, the quantification analysis using LC–MS/MS was conducted in triplicate. After the samples were extracted for 30 min using 5% ammonia in MeOH and purified by using MCX-SPE cartridge, the sample solution was analyzed by LC–MS/MS. The intra-day precision (repeatability) and accuracy (recovery rates) were assessed by analysis in triplicate on a single day (n = 3), whereas the inter-day precision (reproducibility, RSDr) and accuracy were measured by analysis in triplicate over three consecutive days.

As the 7 PAs that were found to be naturally contained with high content contained in Farfarae Flos or Lithospermi Radix, the validation of these PAs was achieved by analysis using a standard solution, instead of matrix-matched analysis. Their linearities were evaluated by measuring r^2 (coefficient of determination) values of the standard curves, which were obtained from the standard solution with the concentration ranges of 2, 5, 20, 100, 200 ng/mL by dilution of the stock standard solution (1000 ng/mL) using 50% MeOH. Their LOD and LOQ for 7 PAs could not be measured because of the high content of these PAs. Their accuracy and precision for 7 PAs were conducted as follows; A part of the mixed stock PA standard solution was spiked to small amount samples (0.1 g) to make the final concentration of the sample solution be 25, 50, 100 ng/mL. After the samples were extracted for 30 min using 50% MeOH (20–200 mL), the sample solution was analyzed by LC–MS/MS. The calibration curve obtained from the standard solution itself at the concentration ranges was used for the quantification analysis.

Monitoring of pyrrolizidine alkaloids in herbal medicines

This study attempted to measure the amount of 28 pyrrolizidine alkaloids in two herbal medicinal samples, Farfarae Flos and Lithospermi Radix. Ten samples of

each herbal medicine were used for determination of the content of 28 pyrrolizidine alkaloids. The quantitative analysis was conducted with the matrix-matched analysis. Most of the PAs were pretreated using MCX-SPE cartridge, whereas a part of PAs contained with high content in two herbal medicines were pretreated by 50% extraction method without using SPE cartridge. In the quantitative analysis, the MRM conditions of each PA established as shown on Table 1 were used. The contents of PAs in each sample were determined with triplicate samples (n = 3).

Results and discussion

Optimization of analysis method

Recently, our laboratory developed a LC–MS/MS method to determine 28 PAs in herbal medicines by referring to the BfR method [42] and LC–MS/MS methods reported previously. The developed analysis method is comprised of a combination such as extraction using 0.05 M H_2SO_4 in 50% methanol and purification by MCX-SPE cartridge and LC–MS/MS analysis (Table 2). Through a previous study, we confirmed that the MCX-SPE cartridge entirely indicated to be better recoveries for 28 PAs than those of DSC-C18 SPE cartridge, used at the BfR method. That is, when the DSC-C18 SPE cartridge was used, 3 PAs such as Europin *N*-oxide, Intermedine *N*-oxide and Lycopsamine *N*-oxide indicated very low recovery rates (< 40%) even if the most PAs showed ~ 80% of recoveries. Also, Senkirkin revealed a high recovery rate, displaying ~ 150%. However, the MCX-SPE cartridge showed entirely satisfactory recovery in 28 PAs. Based on these results, the proposed analysis method selected the cation-exchange MCX-SPE cartridge to remove unnecessary components, which could interfere with the precise quantitative analysis, instead of DSC-C18 SPE cartridge used in BfR method. However, the developed method employed the same mobile phase used at BfR method, which was consisted of eluent A (0.1% formic acid in 5 mM ammonium formate) and eluent B (0.1% formic acid plus 5 mM ammonium formate in 100% methanol). The developed LC–MS/MS analysis method used the MRM conditions as shown in Table 1. The quantitative ions and confirmation ions in some PAs were selected as different ions values compared with those of the BfR method. However, the MRM conditions established in our laboratory showed great selectivity against 28 PAs in the mixed standard PA solution (Fig. 3).

As the LC–MS/MS analysis method characterized by a high specificity and sufficient sensitivity can measure PAs of the concentration range (1 µg/kg to 3 mg/kg) [30]. As the tandem mass spectrometry based on the MRM technique (multiple reaction monitoring transitions) is suitable analysis technology for determination of trace amount the analytes.

Employment of new extraction using 50% MeOH

After developing the LC–MS/MS analysis method for determination in our laboratory, we validated the LC–MS/MS method against the herbal medicines such as Atractylodis Rhizoma Alba, Chrysanthmi Flos, Leonuri Herba, Gastrodiae Rhizoma, and Glycyrrhizae Radix. This study was to validate the developed LC–MS/MS method against Farfarae Flos and Lithospermi Radix (Fig. 2). However, unlike othr herbal medicines, there was difficulty in performance of the validation study of the two herbal medicines. That is, we found that Farfarae

Table 2 Comparison between the BfR method and the proposed PA method

Pretreatment processing	BfR-PA-Tea (BfR, 2014)	Current PA method
Samples (g)	2.0	2.0
Extraction	0.05 M H_2SO_4 20 mL	50% MeOH included 0.05 M H_2SO_4 40 mL
	Sonication for 15 min	Shaking for 30 min,
Centrifugation	10 min, (4 ℃, 3800 × *g*)	10 min, (4 ℃, 3230 × *g*)
Filtering etc	Repeat 2 times, neutralization of supernatant with ammonia soln, pH 7	Filtering supernatant
Cartridge purification	DSC-C18 (500 mg, 6 cc)	SPE-MCX (150 mg, 6 cc)
Activation	MeOH 5 mL, D.W 5 mL	MeOH 3 mL, D.W 3 mL
Loading	Supernatant 10 mL	Supernatant 2 mL
washing (using pump)	D.W 10 mL	D.W 4 mL
Elution	MeOH soln included 2.5% ammonia 10 mL, or MeOH 10 mL	MeOH soln included 5% ammonia 4 mL
Nitrogen concentration	50–55 ℃	
Dissolving and filtering	5% MeOH, 1 mL, with 0.2 µm syringe range filter	
LC–MS/MS analysis		

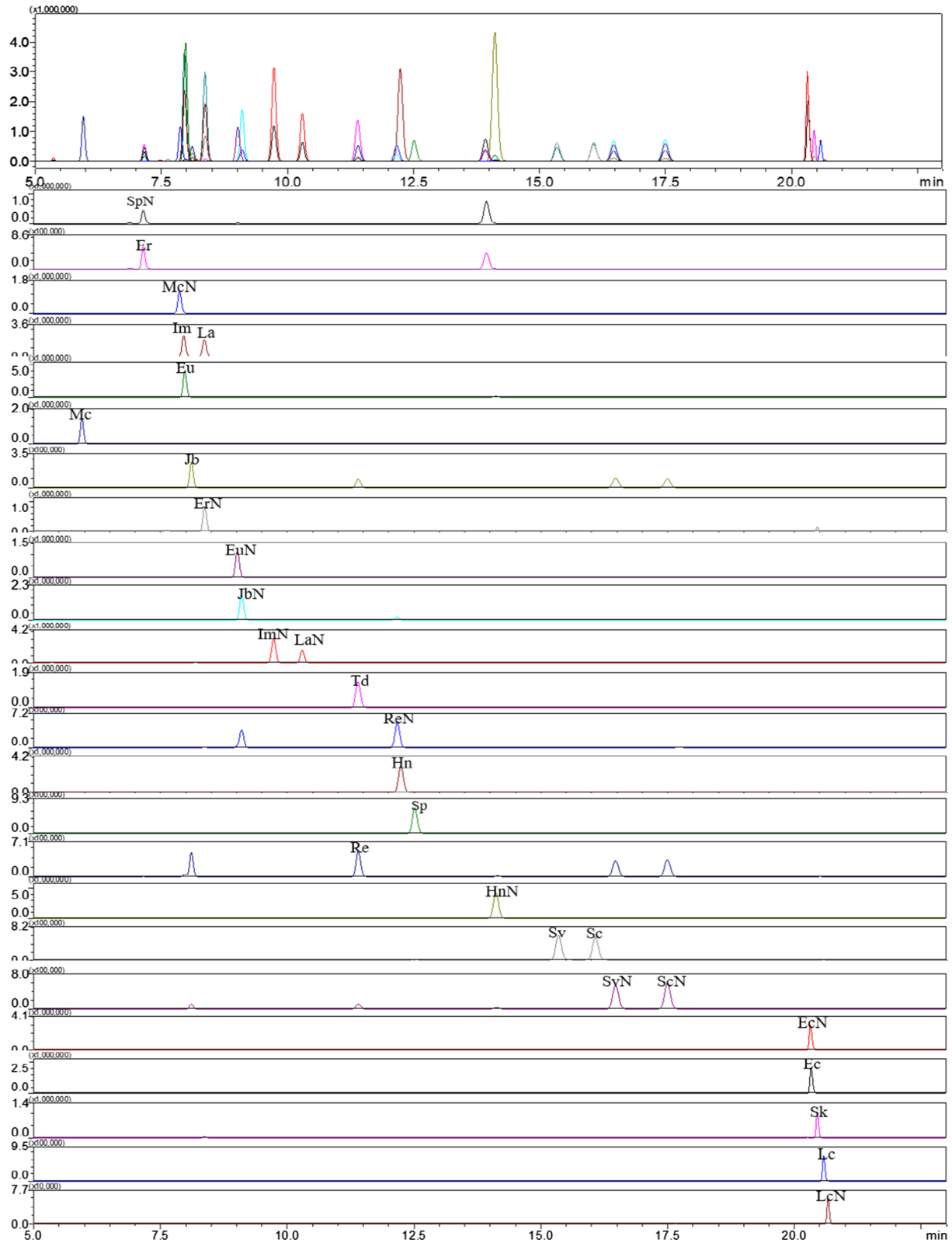

Fig. 3 The MRM chromatograms of 28 pyrrolizidine alkaloids obtained from LC–MS/MS analysis in the mixed standard solution. Each pair the molecular mass and compound name abbreviations were shown Table 1

Flos contained 3 PAs including Senecionine, Senecionine *N*-oxide, Senkirkine, whereas Lithospermi Radix had 5 PAs such as Echimedine, Echimedine *N*-oxide, Intermedine, and Intermedine *N*-oxide, Lycopsamine *N*-oxide through the preliminary test.

The blank samples that do not contain the analytes are required for measuring the precise recovery in the validation study. Due to their extremely high content, they occurred easily saturation at LC–MS/MS analysis that quantifies the trace amount in samples to deviate quantitative range so that their recoveries could not be quantified simultaneously with those of the most PAs. To overcome such problem, this study developed a new extraction method for these PAs contained with high content in the herbal medicine samples. The new extraction was to a strategy inducing dilution effect. That is, it was a simple extraction that small amount sample (0.1 g) was extracted with a large amount (20–200 mL) of 50% MeOH without the purification using cation-ion cartridge (Fig. 2B).

Lebada et al. [36] explain that the best method to extract senkirkine and senecionine from *Tussilago farfara* was a combination comprised of refluxing with 50% methanol acidified and purification using solid-phase extraction on diol-phase cartridges. The content of senkirkine in coltsfoot leaves was measured using capillary zone electrophoresis and its concentration was 2.5–11.2 ppm. Such a large variation of the amount of PAs seems to be ascribed to a different origin of plant materials, its preparation (raw material or dried samples), extraction methods (with various solvents), and quantification analytical methods (capillary zone electrophoresis, gas, or liquid chromatography) [36, 44]. To quantify the PAs in herbal medicines, the selection of appropriate solvent for extraction of PAs and analytical method is considered to be an important work. In addition, as PAs contained with a large amount in herbal medicines can occur easily saturation, the analysis methods used in practice should be validated to ensure its reliability prior to quantification analysis.

Matrix effects

As most herbal medicines are natural plants that contain various chemical compounds, they may influence the quantification analysis of pyrrolizidine alkaloids. Therefore, this study evaluated the matrix effects of 28 PAs in Farfarae Flos and Lithospermi Radix. The matrix effects (ME) were evaluated by the following categories: (i) high signal suppression ($-50\% > ME$) and moderate signal suppression ($-50\% < ME > -20\%$); (ii) no matrix effect ($-20\% < ME > 20\%$); (iii) moderate signal enhancement ($20\% < ME > 50\%$), and high signal enhancement ($ME > 50\%$) [45]. As Farfarae Flow contained 3 PAs such as Senecionine, Senecionine *N*-oxide, and Senkirkine and Lithospermi Radix included Intermedine, and Intermedine *N*-oxide with high content. Matrix effects of these PAs could not be successfully evaluated because the coefficient deviations (r^2) value of standard in these PAs curve could been not acquired as more than 0.99 so that their quantification analysis could not be accomplished.

As shown in Fig. 4 the matrix effects of the other PAs indicated as follows; In Farfarae Flow, 5 PAs such as Echimidine-*N*-oxide (EmN), Europine (Eu), Lasiocarpine (Lc), Lasiocarpine *N*-oxide (LcN), and Retrorsine *N*-oxide (ReN) indicated strong ion enhancement, whereas 4 PAs including Echimidine (Em), Erucifoline *N*-oxide (ErN), Jacobine (Jb), Lycopsamine *N*-oxide (LcN) occurred strong ion suppression. In Lithospermi Radix, 4 PAs such as Echimidine-*N*-oxide (EmN), Erucifoline *N*-oxide (ErN), Heliotrine *N*-oxide (HnN), Lycopsamine (La) showed strong ion enhancement, while 7 PAs like Erucifoline occurred ion suppression. Considering these results, quantification analysis against most of the analytes seemed to be influenced by various components originated from herbal medicine samples so that this study conducted the matrix-matched analysis to avoid or reduce the interfering of various materials contained in the herbal medicine samples.

Linearity and sensitivity

Because of matrix effects in herbal medicines, our validation study was performed by matrix-matched analysis. The results of linearity in most of the PAs revealed to have good linearity, indicating $r^2 \geq 0.997$ in Farfarae Flos or Lithospermi Radix (Tables 3 and 4). The linearity of 3 PAs (Senecionine, Senecionine *N*-oxide, Senkirkine) contained in Farfarae Flos and 2 PAs (Intermedine and Intermedine *N*-oxide) included in Lithospermi Radix revealed to have good linearities, indicating $r^2 = 0.999$. In Farfarae Flos, LOQs of 25 PAs included in the original samples were revealed to have the range of 0.1–24.2 μg/kg (Table 3). In Lithospermi Radix, LOQs of 24 PAs contained in Lithospermi Radix indicated to be the range 0.2–7.2 μg/kg. The LOQs of Seneciphylline and Retrorsin *N*-oxide indicated to have 23.0 μg/kg, and 17.4 μg/kg in Lithospermi Radix respectively, indicating comparatively higher LOQ than that of the other PAs (Table 4). The improved LC–MS/MS method indicated that Lycopsamine *N*-oxide had 1.1 μg/kg of LOQ in Farfarae Flos. Through such results, the matrix components seemed to influence LOQs of the PA.

Also, we assumed that certain components that contained Lithospermi Radix might occur strong suppression of Lycopasamine *N*-oxide. As 3 PAs such as Senecione, Senecione *N*-oxide, and Senkerkine in Farfarae Flos and 5 PAs intermedine, inter medine *N*-oxide,

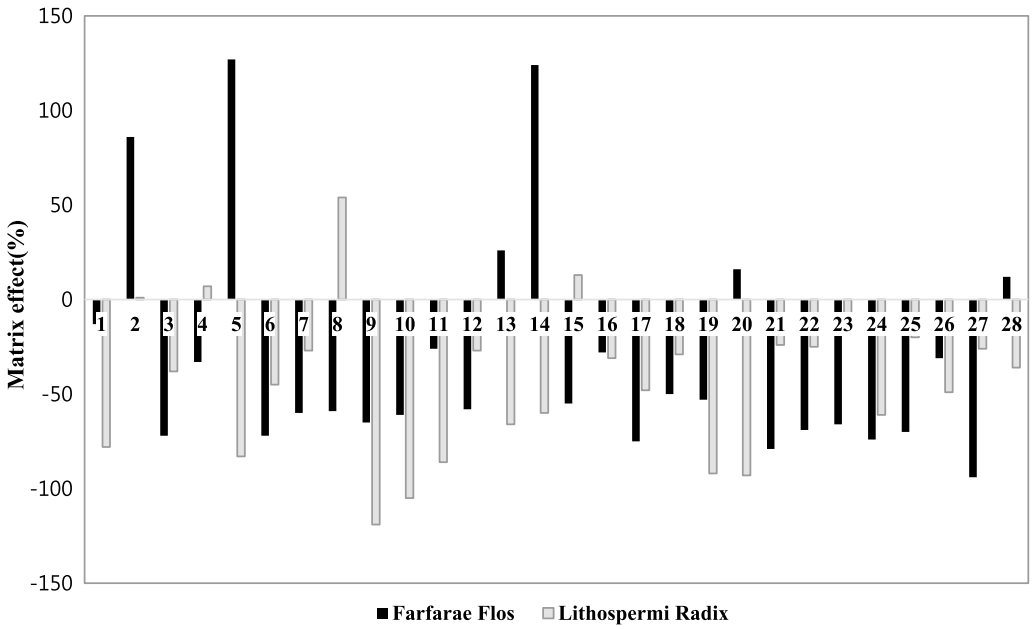

Fig. 4 Matrix effects of 28 pyrrolizidine alkaloids in Farfarae Flos and Lithospermi Radix; 1: Echimidine, 2: Echimidine-*N*-oxide, 3: Erucifoline, 4: Erucifoline-*N*-oxide, 5: Europine, 6: Europine-*N*-oxide, 7: Heliotrine, 8: Heliotrine-*N*-oxide, 9: Intermedine, 10: Intermedine-*N*-oxide, 11: Jacobine, 12: Jacobine-*N*-oxide, 13: Lasiocarpine, 14: Lasiocarpine-*N*-oxide, 15: Lycopsamine, 16: Lycopsamine-*N*-oxide, 17: Monocrotaline, 18: Monocrotaline-*N*-oxide, 19: Retrorsine, 20: Retrorsine-*N*-oxide, 21: Senecionine, 22: Senecionine-*N*-oxide, 23: Seneciphylline, 24: Seneciphylline-*N*-oxide, 25: Senecivernine, 26: Senecivernine-*N*-oxide, 27: Senkirkine, 28: Trichodesmine

Echimidine, and Echimidine *N*-oxide, and Lycopsamine *N*-oxide in Lithospermi Radix were contained with a large amount in herbal medicines so that the saturation occurs, their LOD and LOQ could not be measured. BfR-PA-Tea method indicated to have 0.5–1.7 μg/kg of LOD and 1.7–6.4 μg/kg of LOQ for 28 PAs. The proposed method revealed to have LOQ values less than 10 μg/kg in most PAs, except of a few PAs. The LOQ values obtained by the proposed method was not largely different from those of BfR method.

Accuracy and precision

The recoveries and relative standard deviations (RSDs) of PAs were evaluated through the matrix-matched analysis. The test solutions were prepared as follows; after the mixed standard solutions were spiked into the samples to be three different concentration levels and then pretreated by the method described above. However, These PAs that contained a large amount in Farfarae Flos or Lithospermi Radix, the recoveries and RSDs were measured by quantification method using standard solution instead of matrix-matched analysis. In test method validation, AOAC guideline requires the reference criteria such as recovery of 70–125%, repeatability precision (intra-day RSD) < 15%, and the reproducibility precision (inter-day RSD) < 32%.

In Farfarae Flos, most PAs that were not contained in Farfarae Flos showed satisfactory recovery as the ranges of 70.4–118.0%. In precision evaluation of Farfarae Flos, most analytes indicated satisfactory intra-day RSD values, however, a few analytes showed to be beyond the criteria in intra-day RSD as follows; Echimidine(18.8%), Retrorsine (20.1%), Seneciphylline (17.6%), Seneciphylline *N*-oxide (16.9%), Seneciverine (17.8%). In inter-day RSDs, 25 PAs indicated to be less than the criteria recommended at AOAC guideline (Table 3).

In Lithospermi Radix, most of the PAs that were not contained in the Lithospermi Radix indicted the ranges of 70.2–116.5%. Their intra-day RSDs of all analytes except 2 PAs, Europine *N*-oxide (24.2%) and Jacobine (18.5%), showed to be less than 15% and the inter-day RSDs of 24 PAs indicated to have values less than 32% (Table 3).

Three PAs including senecionine, senecionine *N*-oxide and senkirkine showed satisfactory recovery in Farfarae Flos, indicating the ranges of 92.8–119.0% and their intra-and inter-day RSDs also indicated to satisfy the recommended criteria. Also, in Lithospermi Radix, 5 PAs considered to have high content in Lithospermi Radix revealed recovery ranges of 108.5–130.3%, even though only Echimidine *N*-oxide did not satisfy the criteria, indicating 130.2% of recovery. Their intra-and

Table 3 Linearities, LODs, LOQs, recoveries, and RSDs obtained by LC–MS/MS analysis method in Farfarae Flos

No.	Pyrrolizidine alkaloids	Range (ng/mL)	Linearity (r^2)	LOD (μg/kg)	LOQ (μg/kg)	Recovery (%) mean ± SD, n = 3			Intra-day RSD[b] (%)	Inter-day RSD (%), n = 9
						Low level	Medium level	High level		
1	Echimidine	1.0–40	0.999	0.1	0.2	76.1 ± 2.8	78.8 ± 7.5	91.3 ± 3.5	18.8	11.6
2	Echimidine *N*-oxide	1.0–40	0.999	1.7	5.1	94.9 ± 1.6	97.6 ± 8.5	107.1 ± 1.8	0.9	2.7
3	Erucifoline	0.5–20	0.999	0.3	1.0	73.1 ± 5.9	78.1 ± 13.0	78.7 ± 4.2	12.0	7.4
4	Erucifoline *N*-oxide	0.5–20	0.999	0.2	0.6	84.4 ± 5.9	93.9 ± 12.3	89.8 ± 5.8	3.8	3.4
5	Europine	0.5–20	0.999	0.1	0.3	82.1 ± 3.7	77.8 ± 7.5	70.8 ± 5.1	8.6	7.5
6	Europine *N*-oxide	0.5–20	0.999	0.03	0.1	92.1 ± 2.5	84.9 ± 5.1	74.6 ± 11.6	7.2	7.7
7	Heliotrine	0.5 –20	0.999	0.03	0.1	88.7 ± 1.4	89.3 ± 8.3	91.7 ± 2.7	8.8	4.6
8	Heliotrine *N*-oxide	0.5–20	0.999	0.1	0.3	99.3 ± 1.7	99.4 ± 8.5	105.4 ± 2.9	5.1	4.8
9	Intermedine	0.5–20	0.999	0.1	0.2	82.3 ± 2.3	83.4 ± 10.0	84.5 ± 1.5	8.1	6.8
10	Intermedine *N*-oxide	0.5 –20	0.999	0.3	0.8	95.8 ± 1.6	93.5 ± 8.7	95.1 ± 3.8	1.6	1.0
11	Jacobine	2.0 –80	0.999	3.5	10.4	70.4 ± 3.0	73.1 ± 10.5	72.0 ± 3.5	10.6	12.9
12	Jacobine *N*-oxide	0.5–20	0.999	0.1	0.3	118.0 ± 1.6	117.5 ± 8.0	100.9 ± 15.6	3.5	4.6
13	Lasiocarpine	2.0–80	0.999	0.1	0.3	88.7 ± 2.4	92.6 ± 8.6	107.8 ± 4.5	14.3	10.0
14	Lasiocarpine *N*-oxide	2.0–80	0.997	8.1	24.2	108.9 ± 1.8	112.5 ± 9.7	117.0 ± 3.2	6.2	8.5
15	Lycopsamine	0.5–20	0.999	0.1	0.3	82.5 ± 2.1	83.0 ± 7.9	87.3 ± 0.8	14.3	6.2
16	Lycopsamine *N*-oxide	1.0–40	0.999	0.4	1.1	95.1 ± 3.1	98.8 ± 9.8	92.8 ± 4.3	12.4	7.0
17	Monocrotaline	0.5–20	0.999	0.3	0.8	88.0 ± 3.5	85.9 ± 9.2	92.6 ± 1.8	8.4	5.1
18	Monocrotaline *N*-oxide	0.5–20	0.999	0.2	0.6	101.0 ± 2.4	99.9 ± 10.3	100.0 ± 3.3	7.2	5.6
19	Retrorsine	1.0–40	0.999	3.1	9.3	79.8 ± 7.7	94.7 ± 11.1	81.0 ± 5.5	20.1	10.1
20	Retrorsine *N*-oxide	1.0–40	0.999	1.5	4.4	110.1 ± 3.5	103.2 ± 10.4	102.8 ± 3.6	3.8	6.6
21	Senecionine	2.0–200	0.999	–[a]	–	106.6 ± 5.7	108.9 ± 4.7	111.2 ± 5.4	4.8	3.7
22	Senecionine *N*-oxide	2.0–200	0.999	–	–	113.1 ± 1.2	116.7 ± 3.9	119.0 ± 5.8	3.1	2.0
23	Seneciphylline	0.5–20	0.999	0.5	1.6	71.0 ± 3.6	76.1 ± 10.2	79.3 ± 6.9	17.6	6.5
24	Seneciphylline *N*-oxide	0.5–20	0.999	0.5	1.6	78.6 ± 6.3	86.2 ± 14.8	82.0 ± 2.1	16.9	6.3
25	Senecivernine	1.0–40	0.999	3.0	9.0	74.0 ± 2.8	81.6 ± 9.7	84.4 ± 7.8	17.8	7.2
26	Senecivernine *N*-oxide	1.0–40	0.999	0.8	2.3	95.3 ± 2.8	101.2 ± 10.0	112.3 ± 1.9	1.6	5.0
27	Senkirkine	2.0–200	0.999	–	–	92.8 ± 1.2	111.4 ± 12.8	114.3 ± 8.7	6.8	5.1
28	Trichodesmine	1.0–40	0.999	0.3	0.9	81.8 ± 2.1	87.0 ± 7.9	94.2 ± 2.8	8.0	2.5

[a] Not available because saturation occurred by high content of the PA contained in the Farfarae Flos

[b] *RSD* relative standard deviation

Table 4 Linearities, LODs, LOQs, recoveries, and RSDs obtained by LC–MS/MS analysis method in Lithospermi Radix

No.	Pyrrolizidine alkaloids	Range (ng/mL)	Linearity (r^2)	LOD (μg/kg)	LOQ (μg/kg)	Recovery (%) mean ± SD, n = 3			Intra-day RSD[b] (%)	Inter-day RSD (%), n = 9
						Low level	Medium level	High level		
1	Echimidine	2.0–200	0.999	–	–	108.9 ± 3.3	108.5 ± 7.2	115.2 ± 3.6	4.2	2.6
2	Echimidine *N*-oxide	2.0–200	0.999	–	–	123.1 ± 5.4	118.6 ± 4.8	130.3 ± 4.4	3.9	4.2
3	Erucifoline	1.0–40	0.999	0.3	0.8	81.4 ± 4.3	83.6 ± 6.9	87.0 ± 4.6	9.5	4.2
4	Erucifoline *N*-oxide	1.0–40	0.999	0.5	1.5	102.0 ± 6.0	102.8 ± 14.6	116.5 ± 3.6	4.1	3.6
5	Europine	0.5–20	0.999	0.4	1.1	97.3 ± 7.2	95.1 ± 8.2	97.5 ± 7.4	6.4	9.0
6	Europine *N*-oxide	0.5–20	0.999	0.6	1.7	104.5 ± 7.0	98.5 ± 9.7	108.2 ± 5.5	24.2	13.8
7	Heliotrine	0.5–20	0.999	1.0	3.0	92.4 ± 5.0	92.0 ± 3.8	91.6 ± 6.0	9.0	25.2
8	Heliotrine *N*-oxide	0.5–20	0.999	0.2	0.7	110.9 ± 5.8	101.5 ± 13.6	112.5 ± 6.3	4.4	9.0
9	Intermedine	2.0–200	0.999	–[a]	–	111.1 ± 3.7	111.7 ± 14.4	116.6 ± 5.7	7.0	7.6
10	Intermedine *N*-oxide	2.0–200	0.999	–	–	119.7 ± 6.2	118.3 ± 16.2	119.6 ± 4.7	7.6	9.4
11	Jacobine	1.0–40	0.999	0.7	2.2	71.4 ± 4.0	72.3 ± 4.7	74.9 ± 3.9	18.5	9.4
12	Jacobine *N*-oxide	1.0–40	0.999	0.1	0.4	107.0 ± 6.2	106.3 ± 7.1	116.2 ± 5.9	2.6	2.6
13	Lasiocarpine	0.5–20	0.999	0.2	0.6	79.6 ± 5.4	84.6 ± 3.2	79.4 ± 3.8	9.9	10.7
14	Lasiocarpine *N*-oxide	1.0–40	0.999	0.8	2.3	106.7 ± 5.8	95.4 ± 12.7	105.4 ± 6.3	7.4	5.8
15	Lycopsamine	0.5–20	0.999	2.4	7.2	109.2 ± 7.7	102.4 ± 6.3	97.5 ± 5.3	11.1	11.7
16	Lycopsamine *N*-oxide	2.0–80	0.999	–	–	93.0 ± 8.0	82.7 ± 16.1	112.2 ± 8.4	11.4	21.0
17	Monocrotaline	0.5–20	0.999	0.6	1.8	95.8 ± 5.4	104.3 ± 4.8	82.4 ± 3.1	4.3	7.9
18	Monocrotaline *N*-oxide	1.0–40	0.999	0.6	1.8	104.4 ± 5.9	95.0 ± 14.7	108.0 ± 5.8	8.7	11.0
19	Retrorsine	1.0–40	0.999	0.7	2.1	80.8 ± 6.5	81.0 ± 4.3	80.8 ± 6.1	6.0	11.3
20	Retrorsine *N*-oxide	2.0–80	0.999	5.8	17.4	110.1 ± 5.6	105.0 ± 12.2	115.8 ± 6.1	7.8	18.3
21	Senecionine	0.5–20	0.999	0.4	1.1	73.8 ± 3.3	70.2 ± 3.8	77.2 ± 5.8	5.0	9.0

Table 4 (continued)

No.	Pyrrolizidine alkaloids	Range (ng/mL)	Linearity (r^2)	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%) mean ± SD, n = 3			Intra-day RSD[b] (%)	Inter-day RSD (%), n = 9
						Low level	Medium level	High level		
22	Senecionine *N*-oxide	1.0–40	0.999	0.9	2.8	107.8 ± 4.4	105.1 ± 10.8	114.8 ± 6.5	7.8	13.4
23	Seneciphylline	2.0–80	0.999	7.7	23.0	70.4 ± 2.9	73.0 ± 4.5	75.0 ± 4.7	9.0	19.8
24	Seneciphylline *N*-oxide	1.0–40	0.999	0.4	1.2	82.0 ± 4.9	81.9 ± 7.4	86.1 ± 5.5	8.2	2.6
25	Senecivernine	0.5–20	0.999	0.4	1.2	73.0 ± 3.4	71.0 ± 3.5	70.6 ± 4.3	8.3	18.0
26	Senecivernine *N*-oxide	1.0–40	0.999	0.4	1.1	101.9 ± 4.0	103.1 ± 5.2	105.8 ± 7.2	3.6	2.0
27	Senkirkine	0.5–20	0.999	0.1	0.2	94.8 ± 6.6	102.0 ± 3.9	100.3 ± 7.5	10.8	15.5
28	Trichodesmine	0.5–20	0.999	0.3	1.0	83.2 ± 4.6	85.9 ± 3.8	80.6 ± 6.2	4.3	5.4

[a] Not available because saturation occurred by high content of the PA contained in in the Lithospermi Radix

[b] *RSD* relative standard deviation

inter-day RSDs had satisfactory values in in Lithospermi Radix (Table 4). On the basis of these results, the proposed method was considered as a great quantification analysis that can determine some PAs with high content such as 3 PAs in Farfare Flos and 5 PAs in Lithospermi Radix as well as most of the PAs used in this study.

Monitoring of PAs

The developed LC–MS/MS method was applied to determine the content of 28 PAs in 20 herbal medicine samples purchased from Korean herbal medicine markets imported from China. Ten herbal medicine samples per matrix were used for the monitoring study. Most PAs among 28 PAs were not detected or indicated to have contents of less than the LOQ in two herbal medicine samples, Farfarae Flos and Lithospermi Radix. However, they revealed to possess a part of PAs with high concentration in. In Farfarae Flos, all samples contained three PAs such as senecionine (6.9–9.5 μg/g), senecionine *N*-oxide (6.2–13.7 μg/g), and senkirkine (97.4–175.1 μg/g), whereas in Lithospermi Radix, all samples showed to have four PAs such as intermedine (2.3–30.9 μg/g), intermedine *N*-oxide (27.1–78.5 μg/g), echimedine (0.4–1.6 μg/g), and echimedine *N*-oxide (0.7–5.5 μg/g) and only one sample contained Lycopsamine (1.73 μg/g) and Lycopsamine *N*-oxide (7.82 μg/g).

According to the literature, Adamczak et al. [35] quantified the alkaloid content (Senkirkine and Senecionine) in *T. farfara* using HPLC analysis method. The method employs two-time extraction by using 50% (v/v) methanol acidified with citric acid and dichloromethane and diethyl ether. After evaporating the solvent, the dissolved residual solution was purified by SPE extraction. As a result, their content indicated 0.02–0.58 of Senecionine and 0.02–0.47 μg/g of Senkirkine. Also, *Tussilago farfara* are reported to contain toxic PAs with various ranges from 0.1 to 368 μg/g [36, 44, 46–48]. Also, some researchers claim that dry drugs of *Tussilago farfara* (Farfarae Flos) contain senkirkine from 0.1 to 150 ppm [49, 50] and senecionine ranging from 0.1 to 10 ppm [50]. In comparing these results, the contents of senkirkine and senecionine obtained by the proposed method seemed to be not largely different from their content reported previously. Therefore, the proposed method was considered to determine well such PAs in herbal medicinal samples. In addition, this study suggests that it is are necessary to monitor their residual amount in the final herbal medicinal products used Farfarae Flos or Lithospermi Radix as well as the herbal medicines through a further study.

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Declarations

Competing interests
All authors declare no conflict of interest.

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