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Ultra-High Performance Liquid Chromatography–Tandem Mass Spectrometry for Rapid Analysis of Seven Phenolic Compounds of Sparganii Rhizoma

X. WANG[1,2], Y. WU[3], Q. WU[1,*], Y. QIAN[1], W. YUE[1], AND Q. LIANG[1]

[1]Pharmaceutical School, Nanjing University of Chinese Medicine, 210023 Nanjing, P.R. China
[2]Chemical Engineering and Pharmaceutical School, Henan University of Science and Technology, 471023 Luoyang, P.R. China
[3]Pharmaceutical College, Xinxiang Medical University, Xinxiang 453003, P.R. China
*E-mail: qnwyjs@163.com

Summary. The rhizome of *Sparganium stoloniferum* Buch.-Ham has been used as a traditional Chinese folk medicine for thousands of years. Phenolic compounds are the main bioactive ingredients of the plant. In order to determine the content of phenolic compounds from different major cultivations, a reliable method has been developed using ultra-high performance liquid chromatography coupled with a triple quadrupole electrospray tandem mass spectrometry. Seven compounds, including rutin, kaempferol, *p*-hydroxybenzaldehyde, formononetin, ferulic acid, vanillic acid, and *p*-coumaric acid, were simultaneously measured in 10 min. The established approach was fully validated in terms of linearity, sensitivity, precision, repeatability as well as recovery, and successfully applied to determine seven phenolic compounds of Rhizoma Sparganii. This study may be helpful in the quality control of Rhizoma Sparganii and can offer technical support for the pharmacological and clinical study of related drugs.

Key Words: *Sparganium stoloniferum* Buch.-Ham, phenolic compounds, UPLC–MS/MS, quality control

Introduction

Sparganium stoloniferum Buch.-Ham, a perennial wet habitat herb, is widely distributed in East Asia. The dried rhizome of *S. stoloniferum* Buch.-Ham is widely used for a traditional Chinese medicine, which has antiplatelet [1], antithrombotic [2], antioxidant [3, 4], anti-inflammatory [5], and anticancer [6, 7] properties. Investigations have indicated that phenolic compounds such as biochanin A, isorhamnetin 3-O-β-D-rutinoside, 5,7,3′,5′-tetrahydroxy flavonol-3-O-β-D-glucopyranosyl glucoside-*p*-hydroxybenzaldehyde, vanillic acid, *p*-coumaric acid, ferulic acid, rutin, kaempferol, and formononetin are the main bioactive ingredients responsible for these activities.

Until now, a number of analytical techniques have been developed to analyze phenolic compounds, including high-performance liquid chromatography (HPLC) [8–10], spectrophotometry [11–12], thin-layer chromatography (TLC) [13, 14], gas chromatography (GC) [15, 16], and capillary chromatography (CE) [17, 18]. In our previous study, an RP-HPLC method was established to determine phenolic compounds in Sparganii Rhizoma [8]. However, the extraction process is complicated, and RP-HPLC method has a long chromatographic run-time. Recent application of liquid chromatography coupled with triple quadrupole mass spectrometry (LC–MS/MS) in characterizing and quantifying various compounds in complex matrix [19–21] suggests that LC–MS/MS might be a good technique for simultaneous analysis of phenolic compounds from Sparganii Rhizoma extract. In addition, tandem mass spectrometry (MS/MS) with triple quadrupole instruments operating in multiple reaction monitoring (MRM) mode is characterized with favorable duty cycles and reduced chemical noise that provides further improvement of sensitivity [22]. To the best of our knowledge, no studies have been performed to measure the phenolic compounds in Sparganii Rhizoma using ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UPLC–MS/MS).

In the present paper, an efficient and sensitive method was established for the simultaneous detection of seven analytes including *p*-hydroxybenzaldehyde, vanillic acid, *p*-coumaric acid, ferulic acid, rutin, kaempferol, and formononetin by using UPLC–MS/MS. The proposed method was successfully used to analyze eight batches from different cultivation areas in China. Hence, the developed method is suitable for the qualitative and quantitative assessment of Rhizoma Sparganii and related product.

Experimental

Chemical Reagents and Materials

Chemical standards of rutin (1), kaempferol (2), *p*-hydroxybenzaldehyde (3), formononetin (4), ferulic acid (5), vanillic acid (6), and *p*-coumaric acid (7) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The chemical structures are shown in *Fig. 1*. Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Deionized water was purified by an EPED super purification system (EPED, Nanjing, China). All other reagents used in the study were analytical grade from Nanjing Chemical Reagent Co.

Sparganii Rhizoma samples were collected from eight major growing regions of China, and their botanical origin was authenticated by Professor Qi-nan Wu, Nanjing University of Chinese Medicine. The raw materials were

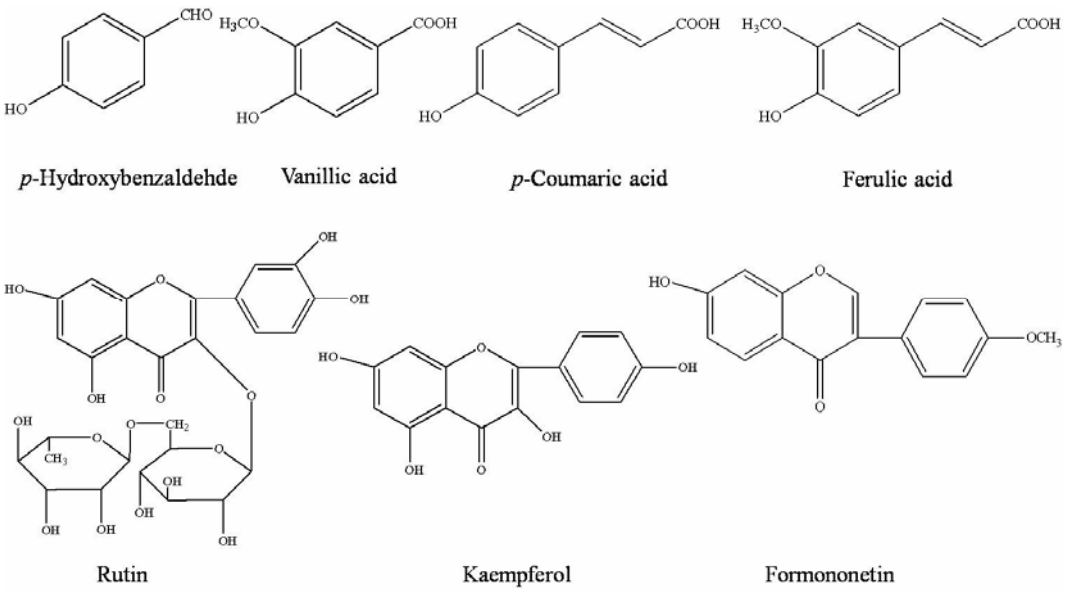

Fig. 1. The chemical structures of seven phenolic compounds

allowed to dry naturally and cut into slices, then ground to pass through a 60-mesh sieve. The powder was kept in sealed polyethylene bags at 4 °C until use.

Preparation of Standard Solutions

A mixed standard solution of rutin (94 μg/mL, 1), kaempferol (102 μg/mL, 2), *p*-hydroxybenzaldehyde (372 μg/mL, 3), formononetin (154 μg/mL, 4), ferulic acid (318 μg/mL, 5), vanillic acid (128 μg/mL, 6), and *p*-coumaric acid (166 μg/mL, 7) were prepared in methanol and stored at 4 °C. Then, the mixed stock solution was further diluted with methanol to obtain appropriate concentrations for establishing calibration curves. The standard samples were filtered through 0.22 μm membrane prior to analysis.

Preparation of Sample Solutions

Five grams of sample were weighed precisely and mixed with 50 mL of methanol. The extraction was carried out for 30 min using an ultrasonic water bath (KH300SP, 25 kHz, 300 W; Kunshan Ultrasonic Instrument Co., Jiangsu, China) at room temperature. The extraction was repeated twice un-

der the same conditions. The combined extracts were concentrated to dryness on a rotary evaporator at 35 °C. Experiments were performed in triplicate. The residue was dissolved in methanol to form a 5-mL solution and then filtered through 0.22 μm membrane before injection.

UPLC–MS/MS Apparatus and Conditions

Liquid Chromatography

Chromatography was performed on an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) with a binary pump solvent management system, an online degasser, and an autosampler. An ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm) was employed at a temperature of 30 °C. The mobile phase consisted of A (0.1% formic acid in deionized water) and B (acetonitrile) with a gradient elution: 0–3 min, 5–15% B; 3–5 min, 15–20% B; 5–7 min, 20–95% B; 7–8 min, 95–95%; 8–9 min, 95–5%; and 9–10 min, 5%. The flow rate and injection volume were set at 0.4 mL/min and 1 μL, respectively.

Mass Spectrometry

Mass spectrometry detection was carried out on a Xevo Triple Quadrupole MS (Waters Corp., Miford, MA, USA) with an electrospray ionization (ESI) source. The ESI–MS spectra were operated in positive ion multiple reaction monitoring (MRM) mode.

Table I. The R_t and MS/MS parameters of seven target compounds

Analyte	R_t (min)	$[M-H]^+$ (m/z)	Quantitative ion (m/z)	CV (V)	CE (eV)
1	4.67	609	300	50	36
2	6.53	285	92	46	32
3	3.34	121	91	38	20
4	6.72	268	197	22	38
5	4.64	193	144	12	16
6	3.04	167	92	18	16
7	4.05	163	90	14	24

The optimal MS conditions obtained were as follows: capillary voltage at 3.0 kV. The source temperature and desolvation temperature were 150 °C

and 550 °C, respectively. The desolvation gas and cone gas flow rate were set at 1000 l/h and 50 l/h, respectively. The cone voltage (CV) and collision energy (CE) were set to match the MRM of each marker. All data collected were automatically processed by the MassLynx software. The summary of MS/MS detection conditions is presented in *Table I*.

Validation of UPLC–MS/MS Method

Calibration Curves, Limits of Detection (LOD) and Quantification (LOQ)

A series of concentrations of standard solution was prepared for the construction of standard curves. The calibration curves were plotted by the peak area and concentrations of each marker. The LOD and LOQ for each analyte were separately determined at signal-to-noise (S/N) ratios of 3 and 10, respectively.

Precision, Repeatability, and Stability

Six samples containing seven analytes were employed to determine the intra- and inter-day (3 days) precision of the assay. The repeatability of the developed method was evaluated by the analysis of sample for six replicates. One of the sample solutions was injected into the UPLC–MS/MS system at 0, 2, 4, 8, 12, 24, and 48 h, respectively, to estimate the stability of the solution. Variations of peak area were expressed by relative standard deviations (RSD).

Recovery

To evaluate the accuracy of this method, the recoveries of seven analytes were determined by a standard addition method. Briefly, by adding the known compounds at low (80% of the known amounts), medium (100% of the known amounts), and high (120% of the known amounts) level to Sparganii Rhizoma sample, the spiked sample was extracted and analyzed using the aforementioned method.

Identification and Quantification

Identification of the target peaks was performed by comparison of UPLC retention times, and mass/charge ratios *(m/z)* with those of the known standards. The analysis of standards and samples was performed by UPLC–MS/MS to further confirm the structures of the compounds. Quantification was carried out using linear calibration.

Results and Discussion

Optimization of Extraction Conditions

Effective extraction is one of the most important steps in analyzing target compounds from plant materials [23]. In order to achieve the optimum extraction conditions, a few parameters including extraction methods, extraction solvents, extraction time, and extraction number were investigated. The results indicated that ultrasound-assisted extraction was found to be a simple, inexpensive, and efficient alternative to reflux and Soxhlet methods. The comparison of extraction solvents such as different concentrations of aqueous methanol and aqueous ethanol demonstrated that the absolute methanol was the optimal solvent for the extraction of target compounds. The extraction time and extraction number were 30 min and three, respectively.

Optimization of the Chromatographic and Mass Spectrometric Conditions

In this study, chromatographic conditions including mobile phase, gradient elution program, and column temperature were optimized to obtain short retention time and good resolution for the analytes. Methanol, acetonitrile, and 0.1% formic acid water were studied as potential mobile phases. A gradient program with acetonitrile and 0.1% formic acid in water was finally selected for good peak shapes and short analytical time. In addition, it was observed that separation was better when the column temperature was set at 30 °C rather than 25 °C or 35 °C.

For mass spectra, positive and negative ion modes were both studied and negative ion mode had higher sensitivity compared to positive ion mode. The negative MS ion mode, therefore, was operated in our study. MRM detection mode was used to determine both the ion pairs of precursor/product, which made the method more specific. Then, ESI source temperature, capillary voltage, flow rate of desolvation gas and cone gas, CV, and CE were investigated to achieve optimal analysis conditions of seven analytes. Retention time (R_t), $[M-H]^+$, quantitative ions, CV, and CE are shown in *Table 1*. Under optimal conditions, the UPLC–MS/MS chromatograms of seven compounds are shown in *Fig. 2*.

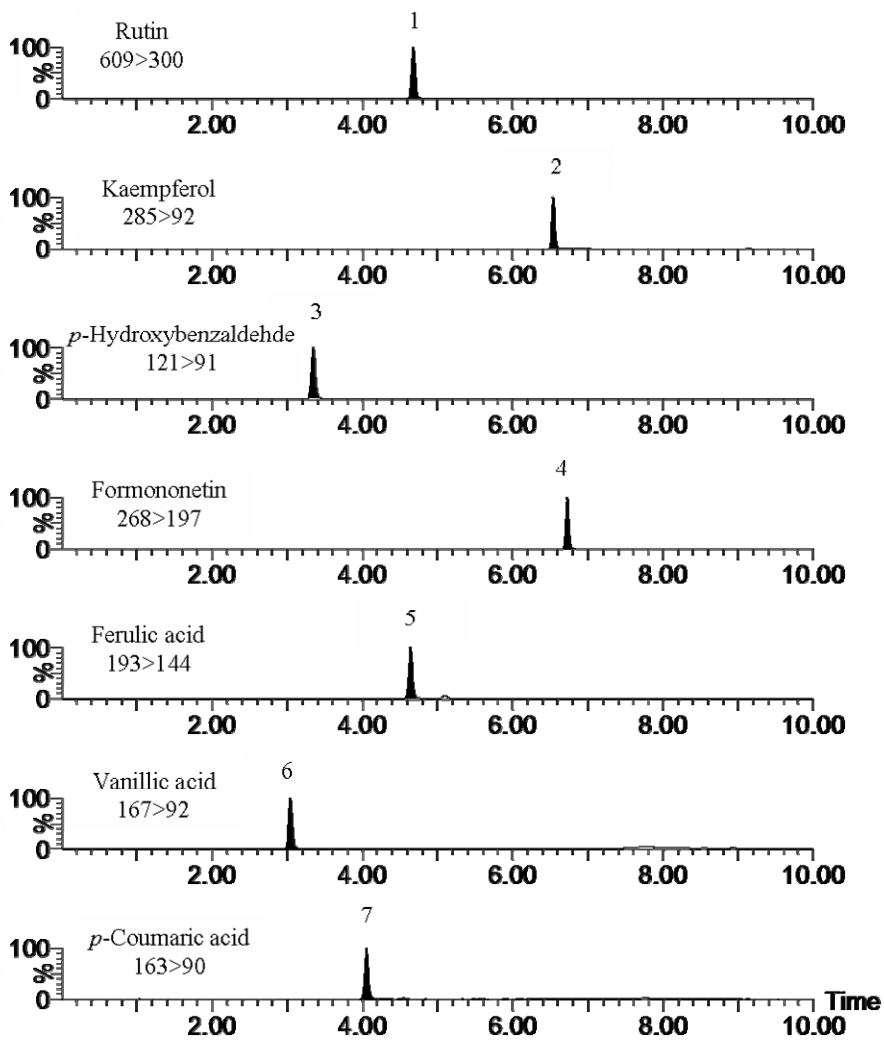

Fig. 2. UPLC–MS/MS analysis MRM chromatograms of seven phenolic compounds

Method Validation

The validation of the established UPLC–MS/MS method was estimated under optimum conditions. It was found that all calibration curves showed good regression ($r^2 > 0.9962$) within test ranges, and the LODs and LOQs were in the range of 0.73–26.66 ng/mL and 2.44–88.87 ng/mL, respectively *(Table II)*. As shown in *Table III*, the RSD values of intra-day and inter-day variations, repeatability, and stability of the seven compounds were less

than 4.18%, and the overall recovery values of analytes were between 93.81 and 100.21%. The results suggest that the developed UPLC–MS/MS method was suitable for the analysis of the investigated compounds in Sparganii Rhizoma.

Table II. Regression equations, correlation coefficients, linearity ranges, LOD, and LOQ of the investigated compounds

Analyte	Regression equation	r^2	Linearity ranges (μg mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
1	$y = 33.346x + 1850$	0.9998	0.1469–5.875	5.15	17.16
2	$y = 10.347x - 429$	0.9986	0.1594–6.375	3.52	11.74
3	$y = 10.786x + 21340$	0.9976	0.5813–23.25	26.66	88.87
4	$y = 145.816x + 318$	0.9998	0.2406–9.625	0.73	2.44
5	$y = 34.565x + 20205$	0.9962	0.4969–19.875	15.72	52.41
6	$y = 41.047x + 5004$	0.9993	0.2–8	9.01	30.03
7	$y = 26.988x + 5731$	0.9985	0.2594–10.375	11.65	38.83

Table III. Results of precision, repeatability, stability, and recovery

Analyte	Precision (n = 6, RSD, %)		Repeatability (n = 6, RSD, %)	Stability (RSD, %)	Recovery (n = 3)	
	Intra-day	Inter-day			Mean (%)	RSD (%)
1	2.04	1.80	4.18	4.18	99.86	3.98
2	3.05	5.01	3.08	2.50	97.68	4.01
3	1.73	1.06	1.29	2.68	93.81	1.80
4	2.67	0.99	4.11	4.18	98.31	4.81
5	1.54	0.94	0.62	4.08	100.21	2.74
6	2.33	1.27	2.28	3.75	94.46	3.90
7	1.27	1.22	0.98	2.79	95.89	2.86

Quantitative Analysis of Samples

The analytical approach developed here was employed for determination of seven phenolic compounds in the Sparganii Rhizoma collected from the major production areas in China. *Table IV* summarizes the contents of seven chemical markers in eight batches of samples. The results indicated that the contents of seven phenolic compounds were different from our previous

study [8]. This is due to the fact that the extraction process and the sources of samples were significantly different in the two studies. In order to evaluate variations of different cultivation regions, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed using SPSS software; the results are shown in *Fig. 3*. The HCA results showed that eight samples were classified into five clusters according to their contents, which suggests an evident effect of growing region on the contents of target markers. As can be seen from *Fig. 3*, the PCA results are quite similar to those obtained with the HCA method. In comparison with RP-HPLC–UV method, the developed UPLC–MS/MS method could be used as a time-saving, efficient, and reliable approach for the quality control of Sparganii Rhizoma.

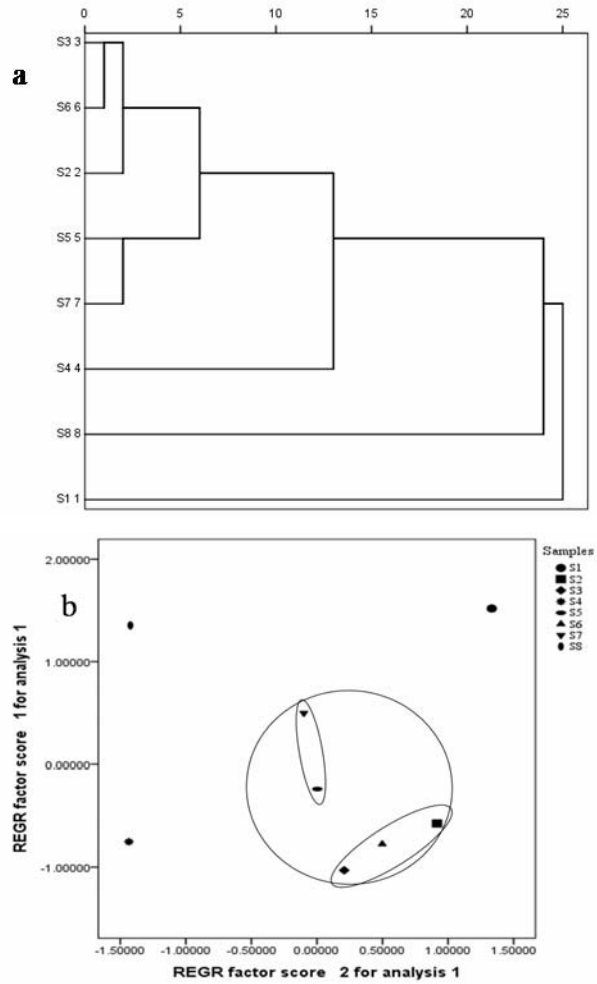

Fig. 3. Dendrograms of HCA (a); scatter plot of PCA (b)

Table IV. Contents of seven analytes in samples of Sparganii Rhizoma (μg g^{-1}, n = 3)

Samples	Analytes						
	1	2	3	4	5	6	7
S1, He county, Anhui	3.392 ± 0.233	0.054 ± 0.013	2.363 ± 0.232	ND[a]	5.163 ± 0.145	8.149 ± 0.154	12.143 ± 0.305
S2, Huangshan, Anhui	2.976 ± 0.197	0.046 ± 0.001	1.897 ± 0.239	ND	5.903 ± 0.234	7.076 ± 0.224	10.061 ± 0.284
S3, Nanjing, Jiangsu	2.638 ± 0.152	0.045 ± 0.001	2.759 ± 0.151	ND	3.507 ± 0.091	4.344 ± 0.136	8.484 ± 0.189
S4, Hurong, Hunan	0.591 ± 0.025	0.046 ± 0.001	3.108 ± 0.122	0.00177 ± 0.00036	4.926 ± 0.201	4.926 ± 0.201	10.373 ± 0.246
S5, Xinwo, Zhejiang	2.489 ± 0.091	0.046 ± 0.001	2.369 ± 0.075	ND	8.043 ± 0.105	9.914 ± 0.166	10.849 ± 0.238
S6, Xinyu, Jiangxi	2.587 ± 0.213	0.045	1.438 ± 0.133	ND	4.815 ± 0.220	5.125 ± 0.154	10.114 ± 0.364
S7, Chuangyuan, Henan	2.210 ± 0.028	0.048 ± 0.001	2.686 ± 0.096	ND	7.745 ± 0.097	9.554 ± 0.146	13.932 ± 0.355
S8, Shishou, Hubei	0.917 ± 0.041	0.051 ± 0.003	3.919 ± 0.080	0.00241 ± 0.0007	3.537 ± 0.025	13.371 ± 0.320	14.749 ± 0.142

[a]ND = not detected.

Conclusion

In this work, a fast, simple, and reliable UPLC–MS/MS method was established for the simultaneous qualification and quantification of seven phenolic compounds in Sparganii Rhizoma within 10 min, which provides a general analytical technique for the quality control of Sparganii Rhizoma.

Acknowledgments

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