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Qualitative and Quantitative Analysis of Thirteen Marker Components in Traditional Korean Formula, Samryeongbaekchul-san using an Ultra-Performance Liquid Chromatography Equipped with Electrospray Ionization Tandem Mass Spectrometry

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Abstract – For efficient quality control of the Samryeongbaekchul-san decoction, a powerful and accurate an ultra-performance liquid chromatography (UPLC) coupled with electrospray ionization (ESI) tandem mass spectrometry (MS) method was developed for quantitative analysis of the thirteen constituents: allantoin (**1**), spinosin (**2**), liquiritin (**3**), ginsenoside Rg1 (**4**), liquiritigenin (**5**), platycodin D2 (**6**), platycodin D (**7**), ginsenoside Rb1 (**8**), glycyrrhizin (**9**), 6-gingerol (**10**), atractylenolide III (**11**), atractylenolide II (**12**), and atractylenolide I (**13**). Separation of the compounds **1 - 13** was performed on a UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm) at a column temperature of 40 °C with a gradient solvent system of 0.1% (v/v) formic acid aqueous-acetonitrile. The flow rate and injection volume were 0.3 mL/min and 2.0 μL. Calibration curves of all compounds were showed good linearity with values of the correlation coefficient ≥ 0.9920 within the test ranges. The values of limits of detection and quantification for all analytes were 0.04 - 4.53 ng/mL and 0.13 - 13.60 ng/mL. The result of an experiment, compounds **2**, **6**, **12**, and **13** were not detected while compounds **1**, **3 - 5**, and **7 - 11** were detected with 1,570.42, 5,239.85, 299.35, 318.88, 562.27, 340.87, 12,253.69, 73.80, and 115.01 μg/g, respectively.
Keywords – Qualitative analysis, Quantitative analysis, Samryeongbaekchul-san, UPLC, LC-MS/MS

Introduction

Traditional herbal formulas have been used for thousands of years with a long history in Korea and some East Asian countries and commonly contain a lot of herbs and chemical components. Therefore, they have been widely used to improve the immunity and treat various diseases associated with multiple targets and are suitable for long-term administration compared with chemically synthesized medicine.[1-3] Samryeongbaekchul-san (SRBCS), also known as Shenlingbaizhu-san in Chinese and Jinryobyakujutsu-san in Japanese, is one of the well-known traditional herbal formulas, consisting of the twelve herbal medicines including Ginseng Radix, Atrctylodes Rhizoma Alba, Poria Sclerotium, Dioscoreae Rhizoma, Glycyrrhizae Radix et Rhizoma, Coicis Semen, Nelumbinis Semen, Platycodonis Radix, Dolichoris Semen, Amomi Fructus, Zingiferis Rhizoma Recnes, and Zizyphi Fructus, in a ratio of 6:6:6:6:6:3:3:3:3:3:2:2:2. It has been first recorded in "Taepyeonghyeminhwajegukbang" of the Song Dynasty and used for treatment of chronic gastritis, chronic enteritis, colitis, and dyspepsia.[4,5] In addition, the biological activity of SRBCS have been investigated a protective and treatment effects of Alzheimer's disease, nonalcoholic steatohepatitis, ulcerative colitis, and inflammatory bowel disease.[4,6-9] As above, various physiological effects using the SRBCS have been reported. However, analytical method for the quality assessment of the SRBCS has not been reported. Therefore, this study was designed to improve the quality control of the SRBCS through the qualitative and quantitative analysis of the thirteen marker constituents including allantoin (**1**), spinosin (**2**), liquiritin (**3**), ginsenoside Rg1 (**4**), liquiritigenin (**5**), platycodin D2 (**6**), platycodin D (**7**), ginsenoside Rb1 (**8**), glycyrrhizin (**9**), 6-gingerol (**10**), atractylenolide III (**11**), atractylenolide II (**12**), and atractylenolide I (**13**) using an ultra-performance liquid chromatography (UPLC) coupled with electrospray ionization (ESI) tandem mass spectrometry (MS) method.

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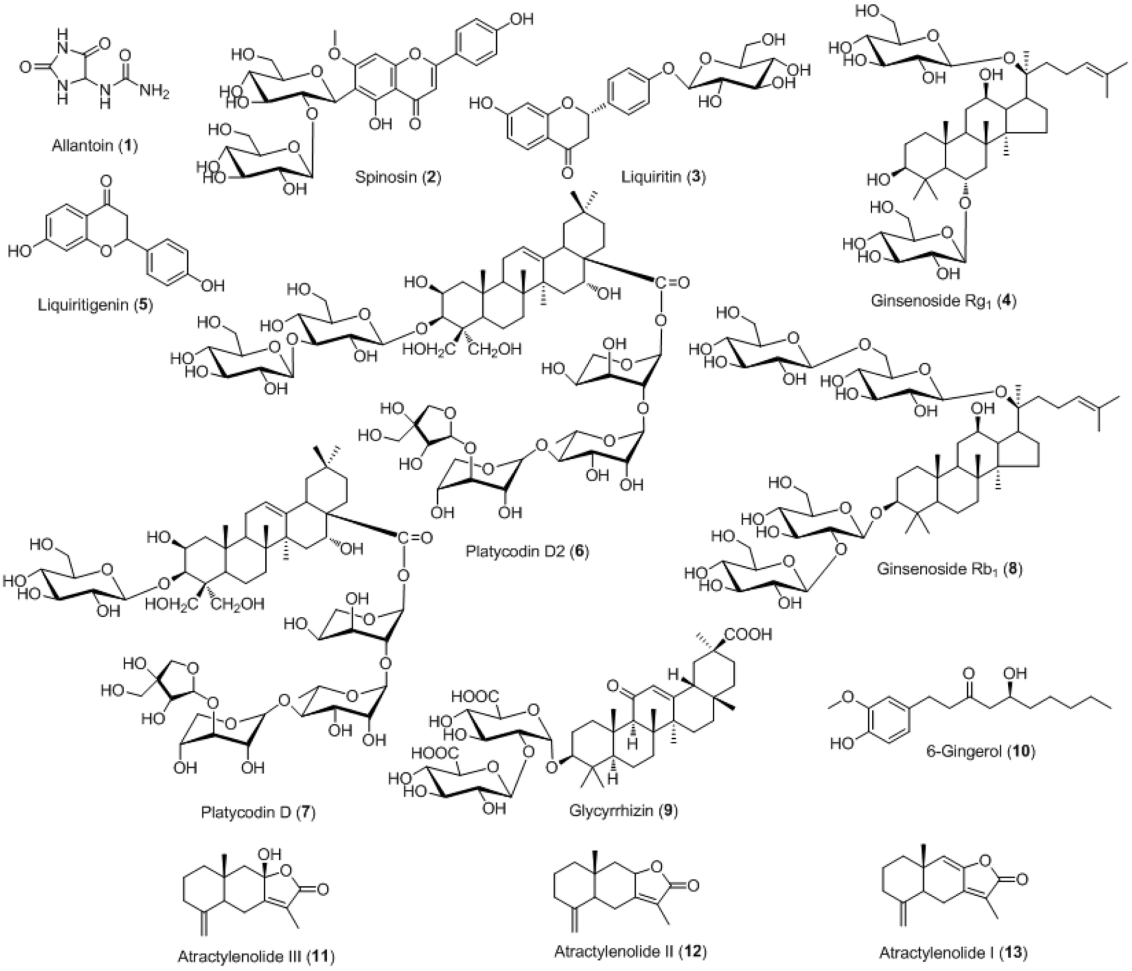


Fig. 1. Chemical structures of the compounds **1 - 13** in Samryeongbaekchul-san.

Experimental

Plant materials – The twelve crude herbal medicines forming SRBCS was purchased from the Korean herbal market, Kwangmyungdang Medicinal Herbs (Ulsan, Korea) in February 2012 and identified by Prof. Je Hyun Lee, Dongguk University, Gyeongju, Korea. Voucher specimens (2012-KE39-1 ~ KE39-12) have been deposited at the K-herb Research Center, Korea Institute of Oriental Medicine.

General experimental procedures – As the reference standards, compound **1** was purchased from Sigma-Aldrich (St. Louis, MO, USA) and compounds **2**, **4**, and **6** were obtained from Biopurify Phytochemicals (Chengdu, China). Compounds **3** and **8 - 10** were purchased from Wako Chemicals (Osaka, Japan) and compounds **5** and **7** were obtained from ChemFaces (Wuhan, China). Compounds **11 - 13** were purchased from KOC Biotec. (Daejeon, Korea). The purities of all reference standards were ≥ 98.0% (Fig. 1). For qualitative and quantitative analysis, HPLC-grade methanol, acetonitrile and water were purchased from J.T. Baker (Phillipsburg, NJ, USA) and analytical reagent-grade, formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Apparatus – The chromatographic system was used a Waters ACQUITY UPLC system (Milford, MA, USA), consisting of a pump, degasser, column oven, and auto-sampler. The chromatographic separation of the compounds **1 - 13** was carried out on an ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm). The LC-MS/MS system used was the Waters ACQUITY TQD LC-MS/MS system (Milford, MA, USA) with an electrospray ionization (ESI) source. All data was collected and analyzed using Waters MassLynx software (version 4.1, Milford, MA, USA).

Preparation of standard solutions – Reference compounds **1 - 13** were accurately weighed and dissolved in methanol at a concentration of 1.0 mg/mL. Each standard stock solution was kept at 4 °C and used after serial dilution with methanol in order to prepare the calibration curves.

Table 1. Composition of Samryeongbaekchul-san

Herbal medicine	Scientific name	Family	Origin	Amount (g)
Ginseng Radix	*Panax ginseng* C. A. Meyer	Araliaceae	Yeongju, Korea	11.250
Atrctylodes Rhizoma Alba	*Atractylodes macrocephala* Koidzumi	Compositae	China	11.250
Poria Sclerotium	*Poria cocos* Wolf	Polyporaceae	Pyeongchang, Korea	11.250
Dioscoreae Rhizoma	*Dioscorea batatas* Decaisne	Dioscoreaceae	Andong, Korea	11.250
Glycyrrhizae Radix et Rhizoma	*Glycyrrhiza uralensis* Fischer	Leguminosae	China	11.250
Coicis Semen	*Coix lacryma-jobi* L. var. *Ma-yuen* Stapf	Gramineae	Muju, Korea	5.625
Nelumbinis Semen	*Nelumbo nucifera* Gaertner	Nymphaeaceae	Vietnam	5.625
Platycodonis Radix	*Platycodon grandiflorum* A. De Candolle	Campanulaceae	Muju, Korea	5.625
Dolichoris Semen	*Dolichos lablab* L.	Leguminosae	China	5.625
Amomi Fructus	*Amomum villosum* Loureiro	Zingiberaceae	Laos	5.625
Zingiferis Rhizoma Recnes	*Zingiber officinale* Roscoe	Zingiberaceae	Ulsan, Korea	3.750
Zizyphi Fructus	*Zizyphus jujube* Miller var. *inermis* Rehder	Rhamnaceae	Yeongcheon, Korea	3.750
Total amount				91.875

Table 2. Conditions for the LC-MS/MS analysis of Samryeongbaekchul-san

HPLC condition			
Column	ACQUITY UPLC BEH C_{18} (100 × 2.1 mm, 1.7 μm)		
Flow rate	0.3 mL/min		
Injection volume	2.0 μL		
Column temperature	45°C		
Sample temperature	5°C		
Mobile phase	Time (min)	A (%)[a]	B (%)[b]
	0	80	20
	0.1	80	20
	14.0	5	95
	15.0	0	100
	15.1	80	20
	18.1	80	20
MS condition			
Capillary voltage (kV)	3.3		
Extract voltage (V)	3.0		
Source temp. (°C)	120		
RF lens (V)	0.3		
Desolvation temp. (°C)	300		
Desolvation gas (L/h)	600		
Cone gas (L/h)	50		
Collision gas (mL/min)	0.14		

[a]0.1% (v/v) formic acid in water, [b]Acetonitrile

Preparation of SRBCS water decoction and sample solutions – The SRBCS is composed of the twelve crude herbal medicines and these herbs was mixed with a total weight of 5.0 kg as shown in Table 1 (about 54.4 times of composition of single dose). The mixed sample was extracted in a 10-fold mass of water at 100 °C for 2 h under pressure (98 kPa) using an electric extractor (COSMOS-660; Kyungseo Machine Co., Incheon, Korea). The water extract was filtered through a standard sieve (no. 270, 53 μm; Chung Gye Sang Gong Sa, Seoul, Korea) and lyophilized by freeze-drier (PVTFD10RS, IlShinBioBase, Yangju, Korea). The amount of SRBCS extract was 705.0 g (14.1%). For qualitative and quantitative analysis of the marker components by LC-MS/MS, 54.2 mg of the lyophilized SRBCS powder was dissolved in 5 mL of 70% methanol by sonication for 5 min. Subsequently, the

solution was diluted 100-fold and filtered through a 0.22 μm membrane filter before the LC-MS/MS system injection.

Liquid chromatographic conditions – The mobile phase consisting of 0.1% (v/v) formic acid in water (A) and acetonitrile (B) was flowed with gradient elution at a flow rate of 0.3 mL/min. These conditions are summarized in Table 2.

LC–MS/MS conditions – The MS conditions were as follows: capillary voltage 3.3 kV, extractor voltage 3.0 V, RF lens voltage 0.3 V, source temperature 120 °C, desolvation temperature 300 °C, desolvation gas 600 L/h, cone gas 50 L/h and collision gas 0.14 mL/min (Table 2).

Calibration curves, limits of detection (LOD), and quantification (LOQ) – Each calibration curve was calculated by plotting the peak areas (y) *versus* the corresponding concentrations (x, ng/mL) using standard solutions. For the calibration curves, five concentrations of each compound (0, 10, 50, 100, and 500 ng/mL) were prepared and measured in triplicate. The LOD and LOQ data under the present chromatographic conditions were determined at signal-to-noise (S/N) ratios of approximately 3 and 10, respectively.

Result and Discussion

Linearity, range, LOD, and LOQ – The linearity of this method was evaluated from the correlation coefficient (r^2) of the calibration curves of the thirteen compounds.

Table 3. Linearities, regression equation, correlation coefficients, LOD, and LOQ for the compounds **1 - 13**

Analyte	Linear range (ng/mL)	Regression equation[a]	Correlation coefficient	LOD[b] (ng/mL)	LOQ[c] (ng/mL)
1	0–500	$y = 0.50x - 9.16$	0.9922	4.53	13.60
2	0–500	$y = 4.02x - 9.16$	0.9987	0.18	0.53
3	0–500	$y = 5.62x - 11.57$	0.9997	0.18	0.53
4	0–500	$y = 1.03x - 12.52$	0.9974	1.11	3.33
5	0–500	$y = 15.87x + 28.64$	0.9996	0.06	0.18
6	0–500	$y = 0.29x - 3.61$	0.9923	2.79	8.37
7	0–500	$y = 1.44x - 2.69$	0.9978	0.96	2.89
8	0–500	$y = 0.70x - 4.92$	0.9920	0.94	2.81
9	0–500	$y = 1.56x - 18.10$	0.9964	0.46	1.38
10	0–500	$y = 5.72x - 43.06$	0.9982	0.04	0.13
11	0–500	$y = 21.20x + 41.06$	0.9997	0.28	0.85
12	0–500	$y = 28.32x + 172.36$	0.9993	0.12	0.37
13	0–500	$y = 35.25x + 133.65$	0.9996	0.06	0.17

[a]y: peak area of compounds; x: concentration (ng/mL) of compounds, [b]LOD = 3 × signal-to-noise ratio, [c]LOQ = 10 × signal-to-noise ratio.

Table 4. Mass detection condition of the compounds **1 - 13**

Analyte	Molecular weight (Da)	Ionization mode	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
1	158.12	$[M-H]^-$	0.80	157.0	96.9	20	15
2	608.54	$[M+H]^+$	1.36	609.6	327.1	40	25
3	418.39	$[M-H]^-$	1.65	417.4	255.3	30	15
4	801.01	$[M-H]^-$	2.69	800.4	637.0	50	20
5	256.25	$[M+H]^+$	2.98	257.1	137.0	35	25
6	1387.48	$[M-H]^-$	3.29	1386.4	843.6	45	48
7	1225.32	$[M-H]^-$	3.38	1224.1	469.4	45	48
8	1109.29	$[M-H]^-$	4.34	1107.4	178.8	50	45
9	822.93	$[M-H]^-$	5.20	821.8	351.0	45	40
10	294.39	$[M+H]^+$	6.16	295.2	177.2	13	10
11	248.32	$[M+H]^+$	6.71	249.3	231.2	25	10
12	232.32	$[M+H]^+$	8.24	233.2	187.1	35	15
13	230.13	$[M+H]^+$	9.33	231.2	185.1	35	20

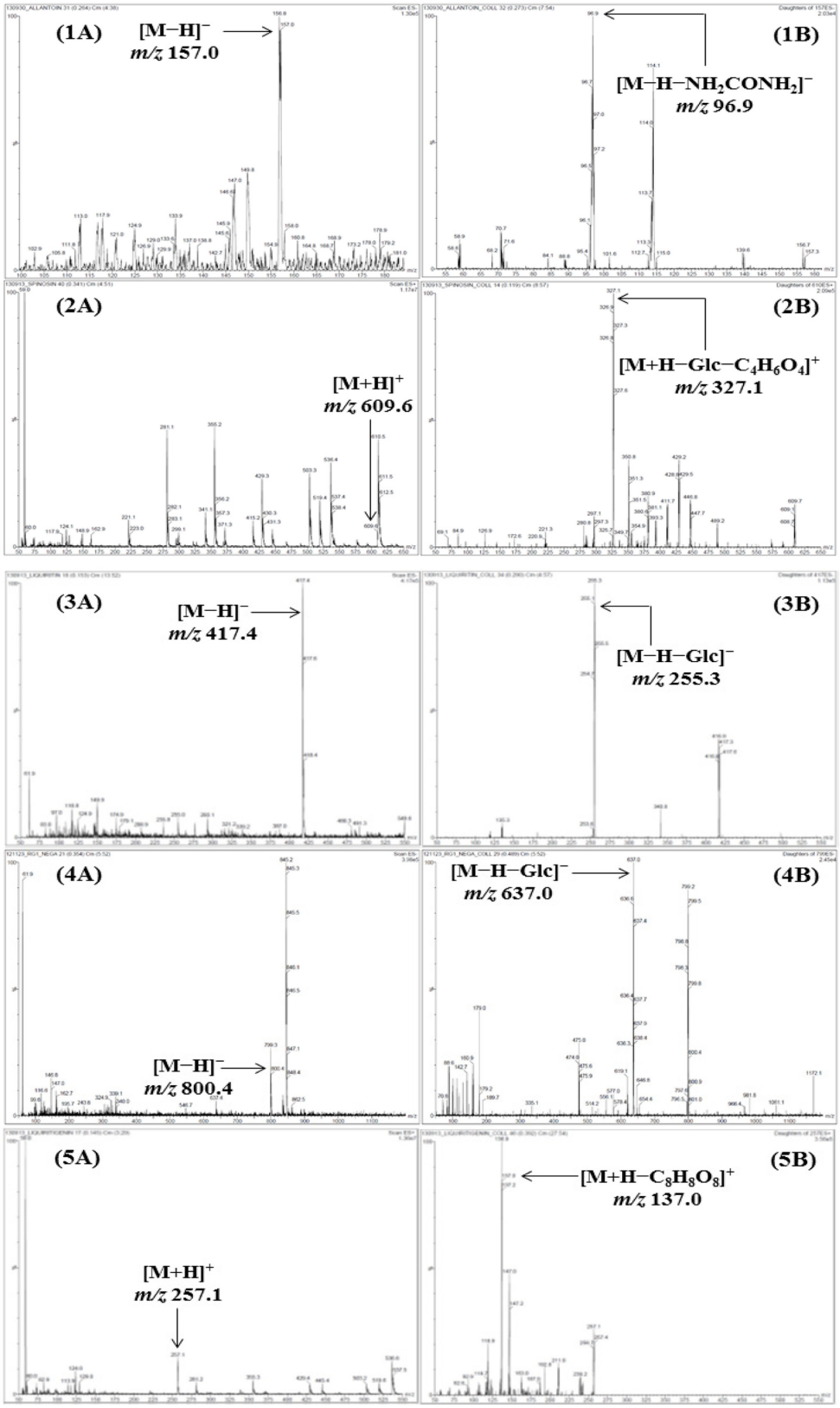
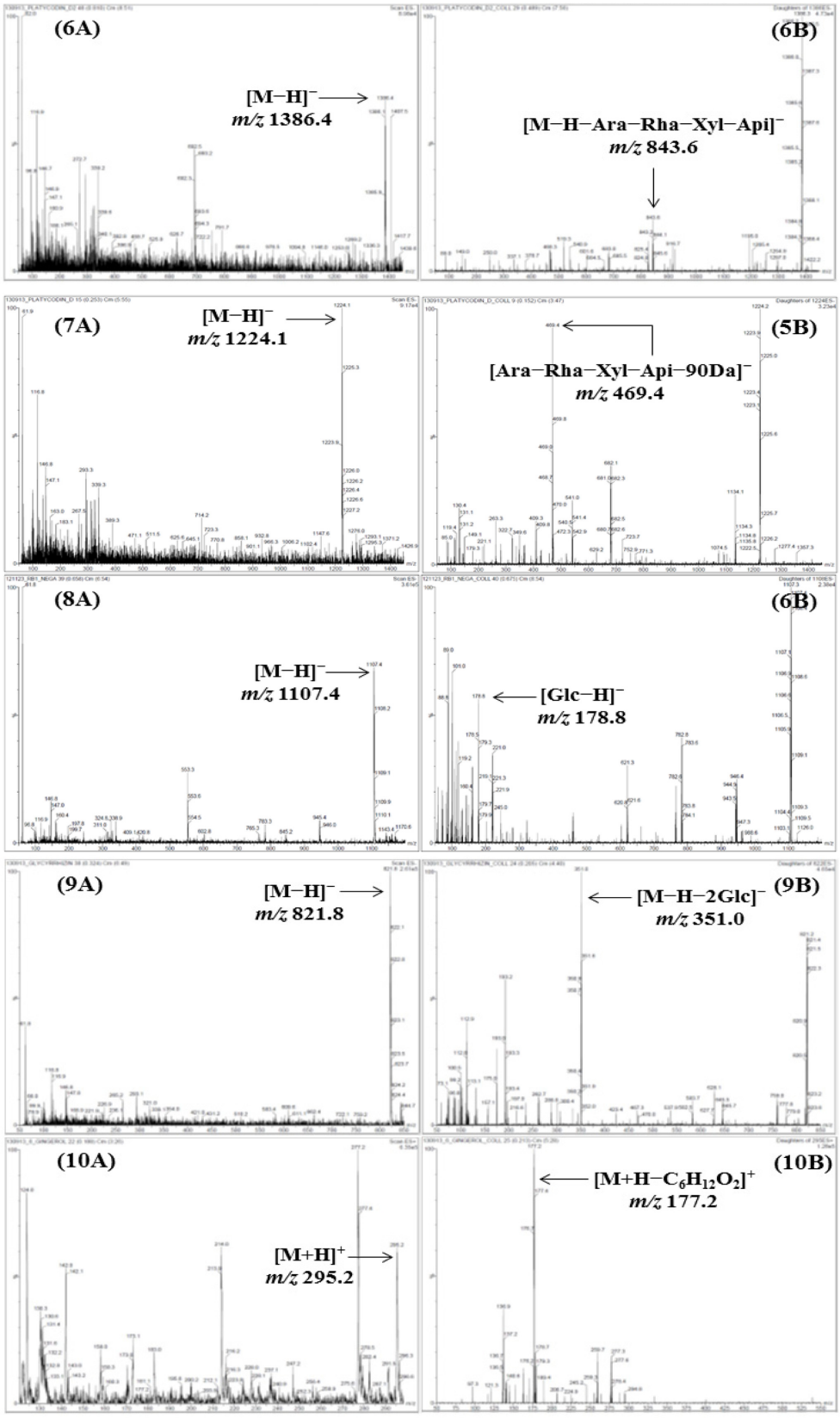


Fig. 2. Mass spectra of the precursor ion (Q1, A) and product ion (Q3, B) for LC-MS/MS MRM mode of the compounds **1** - **13**. Allantoin (**1**), spinosin (**2**), liquiritin (**3**), ginsenoside Rg1 (**4**), liquiritigenin (**5**), platycodin D2 (**6**), platycodin D (**7**), ginsenoside Rb1 (**8**), glycyrrhizin (**9**), 6-gingerol (**10**), atractylenolide III (**11**), atractylenolide II (**12**), and atractylenolide I (**13**).



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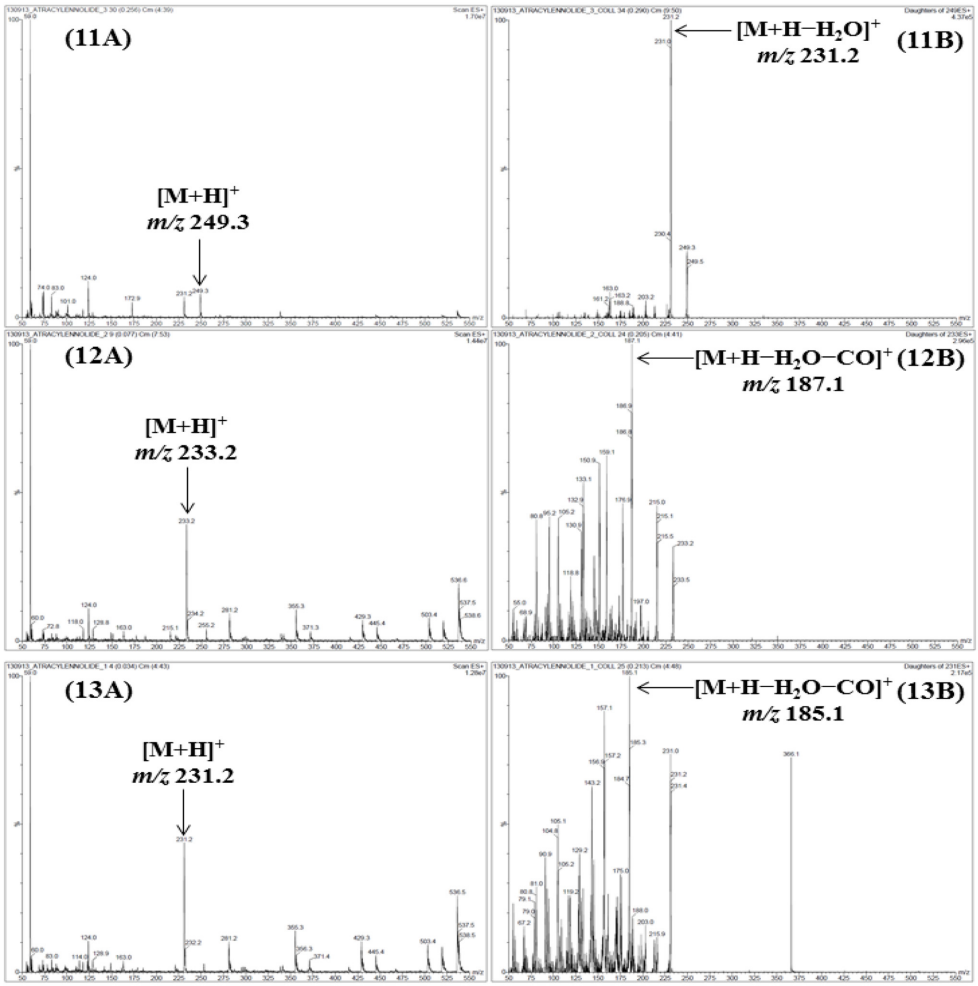

Fig. 2. contineud

Calibration curves of each compound showed good linearity with $r^2 \geq 0.9920$ in the different concentration ranges (Table 3). The LOD and LOQ values of the tested compounds **1 - 13** were in the ranges of 0.01 - 4.53 ng/mL and 0.03 - 13.60 ng/mL, respectively.

Peak identification of the compounds 1 - 13 – The mass spectral data for peak identification of the marker components using the established MS conditions were measured in the negative and positive ion modes. Compounds **1**, **3**, **4**, and **6 - 9** were detected in the negative ion mode ($[M–H]^-$) at *m/z* 157.0, 417.4, 799.2, 1386.4, 1224.3, 1107.5, and 821.9, respectively, and compounds **2**, **5**, and **10 - 13** were detected in the positive ion mode ($[M+H]^+$) at *m/z* 609.5, 257.2, 295.3, 249.3, 233.3, and 231.2, respectively (Table 4 and Fig. 2). LC-MS/MS MRM conditions including precursor ion (Q1), product ion (Q3), cone voltage, and collision energy for quantitative determination were set as Table 4 and Fig. 2. Compound **1** was detected at *m/z* 157.0 (Q1) and tended to eliminate NH_2CONH_2 to produce the fragmentation peaks *m/z* 96.9 (Q3) $[M–H–NH_2CONH_2]^-$.[10] Q3 peak of compound **2** (Q1 *m/z* 609.6) was detected at *m/z* 327.1 $[M+H–Glc–C_4H_6O_4]^+$, which is lost the one glucose and $C_4H_6O_4$ group in precursor ion.[11] Compounds **3** (Q1 *m/z* 417.4) and **4** (Q1 *m/z* 800.4) are apt to lose the glucose to produce aglycone ions of *m/z* 255.3 and 637.0 $[M–H–Glu]^-$, respectively.[12,13] The Q3 peaks of compounds **5** and **10** were detected at *m/z* 137.0 $[M+H–C_8H_8O_8]^+$ and 115.1 $[M+H–C_6H_{12}O_2]^+$, respectively, by eliminating $C_8H_8O_8$ and $C_6H_{12}O_2$ group from the each precursor ion.[12-14] Compounds **6** and **7** were detected at at *m/z* 843.6 $[M–H–Ara–Rha–Xyl–Api]^-$ and 469.4 $[Ara–Rha–Xyl–Api–90Da]^-$, these ions were formed by loss of Ara–Rha–Xyl–Api and Ara–Rha–Xyl–Api–90Da bonded to a 28-carbon from each precursor ion.[15] The Q1 peak ion of compound 8 exhibited an $[M–H]^-$ at *m/z* 1107.4 and the characteristic MS fragmentation was observed at m/z 178.8 $[Glc–H]^-$.[13] Compounds **9** and **11** were detected at

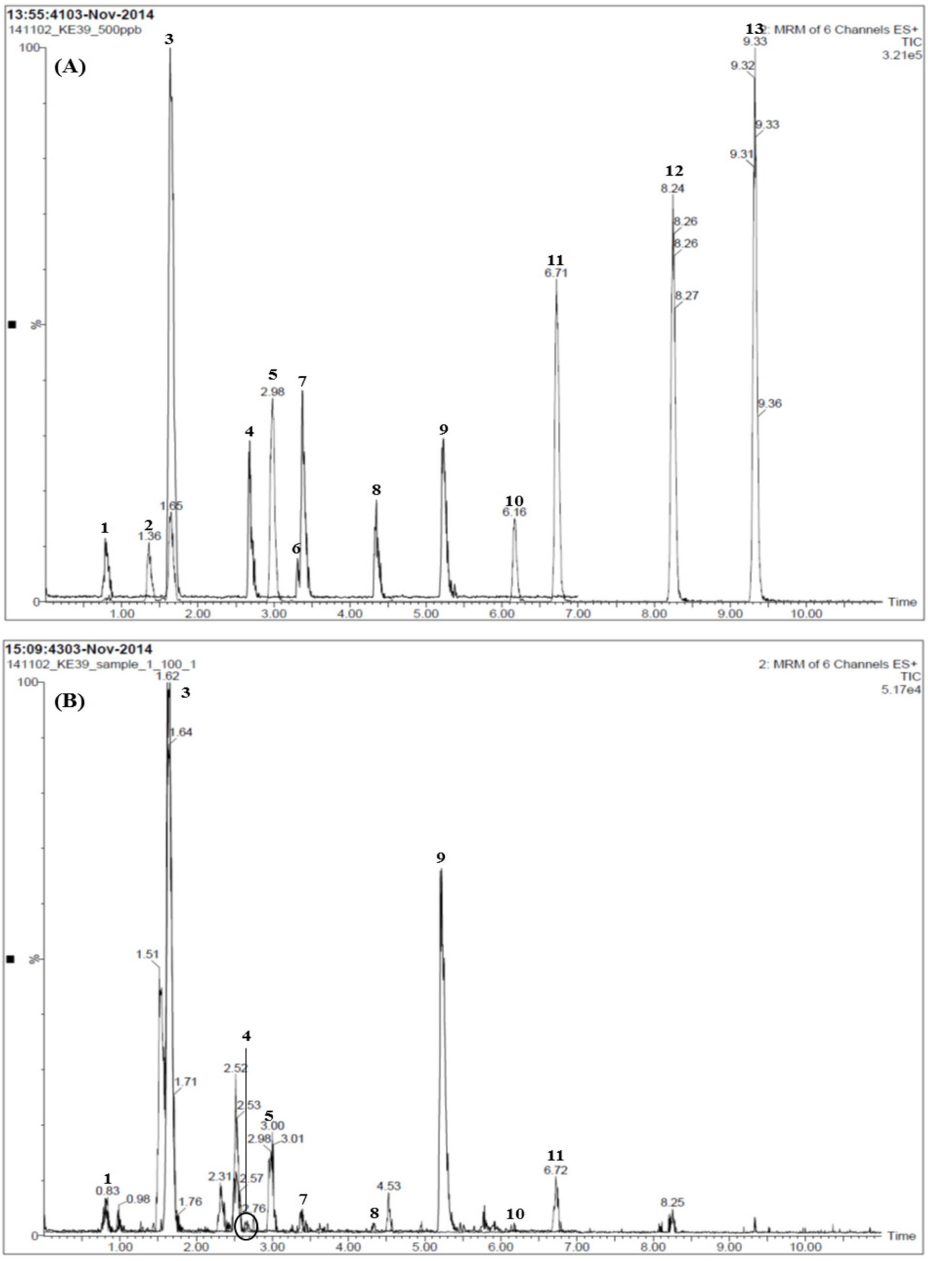

Fig. 3. Total ion chromatograms of the standard solution (A) and Samryeongbaekchul-san sample (B) by LC-MS/MS MRM mode. Allantoin (**1**), spinosin (**2**), liquiritin (**3**), ginsenoside Rg1 (**4**), liquiritigenin (**5**), platycodin D2 (**6**), platycodin D (**7**), ginsenoside Rb1 (**8**), glycyrrhizin (**9**), 6-gingerol (**10**), atractylenolide III (**11**), atractylenolide II (**12**), and atractylenolide I (**13**).

m/z 351.0 $[M–H–2Glc]^-$ and 231.2 $[M+H–H_2O]^+$ by eliminating two glucose and one H_2O molecule, respectively from the each precursor ion.[12-16] The MS fragmentations of compounds **12** and **13** were detected at *m/z* 187.1 $[M+H–H_2O–CO]^+$, and 185.1 $[M+H–H_2O–CO]^+$, with the loss of both one H_2O molecule and CO group from the parent ion $[M+H]^+$ at *m/z* 233.2 and 231.2.[16]

Simultaneous determination of the compounds 1–13 in SRBCS – The developed analytical method using LC–MS/MS was applied to the simultaneous determination of the compounds **1 - 13** in the SRBCS sample (Fig. 3). Consequently, the concentration of the compounds **1 - 13** in the SRBCS decoction were detected up to 12,253.69 μg/g (Table 5). Furthermore, compounds **9**, which are major component of *G. uralensis* was found in concentrations of 12,253.69 μg/g and the most abundant compounds in the SRBCS extract. And then compounds **3** and **1** were detected 5,239.85 and 1,570.42 μg/g. These

Table 5. Contents of the compounds **1** - **13** in Samryeongbaekchul-san (n = 3)

Compound	Amount (μg/g)			Source
	Mean	SD	RSD (%)	
1	1,570.42	94.55	6.02	*D. batatas*
2	N.D.	–	–	*Z. jujube*
3	5,239.85	87.79	1.68	*G. uralensis*
4	299.35	9.78	3.27	*P. ginseng*
5	318.88	25.30	7.94	*G. uralensis*
6	ND	–	–	*P. grandiflorum*
7	562.27	22.83	4.06	*P. grandiflorum*
8	340.87	115.46	33.87	*P. ginseng*
9	12,253.69	437.80	3.57	*G. uralensis*
10	73.80	10.44	14.14	*Z. officinale*
11	115.01	4.16	3.62	*A. macrocephala*
12	N.D.	–	–	*A. macrocephala*
13	N.D.	–	–	*A. macrocephala*

[a]N.D. means not detected.

results are summarized in Table 5.

In conclusion, a rapid, precise, and reliable LC–MS/MS method for the simultaneous determination of the thirteen marker constituents in the herbal prescription, SRBCS was developed and successfully applied to the quantitative analysis of SRBCS. These results could be helpful for the quality control of SRBCS.

Acknowledgments

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