Quantitative analysis of the eight major compounds in the Samsoeum using a high-performance liquid chromatography coupled with diode array detection and electrospray ionization mass spectrometer

Jin Bae Weon[1], Hye Jin Yang[1], Bohyoung Lee[1], Jin Yeul Ma[2], Choong Je Ma[1,3]

[1]Department of Biomaterials Engineering, Division of Bioscience and Biotechnology, [3]Research Institute of Biotechnology, Kangwon National University, Chuncheon 200-701, Korea, [2]TKM Converging Research Division, Korea Institute of Oriental Medicine, 483 Exporo, Yuseong-gu, Daejeon 305-811, Korea

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ABSTRACT

Background: Samsoeum was traditionally used for treatment of a respiratory disease. **Objective:** The simultaneous determination of eight major compounds, ginsenoside Rg3, caffeic acid, puerarin, costunolide, hesperidin, naringin, glycyrrhizin, and 6-gingerol in the Samsoeum using a high-performance liquid chromatography (HPLC) coupled with diode array detection (DAD) and an electrospray ionization mass spectrometer was developed for an accurate and reliable quality assessment. **Materials and Methods:** Eight compounds were qualitative identified based on their mass spectra and by comparing with standard compounds and quantitative analyzed by HPLC-DAD. Separation of eight compounds was carried out on a LUNA C_{18} column (S-5 μm, 4.6 mm i.d. ×250 mm) with gradient elution composed of acetonitrile and 0.1% trifluoroacetic acid. **Results:** The data showed good linearity (R^2 > 0.9996). The limits of detection and the limits of quantification were <0.53 μg and 1.62 μg, respectively. Inter- and Intra-day precisions (expressed as relative standard deviation values) were within 1.94% and 1.91%, respectively. The recovery of the method was in the range of 94.24–107.90%. **Conclusion:** The established method is effective and could be applied to quality control of Samsoeum.

Key words: High-performance liquid chromatography-diode array detection, high-performance liquid chromatography-mass spectrometer, marker constituents, samsoeum, simultaneous determination, validation

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INTRODUCTION

Over the years, traditional herbal medicines (THM) have considerable attraction in many countries due to their high therapeutic effects in various diseases.[1,2] Samsoeum, a THM was used for treatment of a respiratory disease such as chronic bronchitis, bronchitis, and cold. Samsoeum has been shown to have anti-allergic effect and anti-inflammatory effects.[3,4] It is composed of 13 herbs, *Panax ginseng*, *Perilla frutescens*, *Angelica decursiva*, *Pinellia ternate*, *Pueraria lobata*, *Poria cocos*, *Aucklandia lappa*, *Citrus unshiu*, *Playtcodon grandiflorum*, *Citrus aurantum*, *Glycyrrhiza uralensis*, *Zingiber officinale*, and *Zizyphus jujube*.

Address for correspondence:
Prof. Choong Je Ma, Department of Biomaterials Engineering, Division of Biotechnology and Bioengineering, Kangwon National University, Hyoja-2 Dong, Chuncheon 200-701, Republic of Korea.
E-mail: cjma@kangwon.ac.kr

Most of THM are used in the complex formulas of many herbs. The quality of THM is closely related to the amount of their bioactivity compounds, which is slightly different according to culture environment and manufacturable condition. In general, chromatography and relative techniques are used to analysis of THM and plants. High-performance liquid chromatography (HPLC) is the most frequently used separation technique. Liquid chromatography-mass spectrometer (LC-MS)/MS technique was applied to qualitative and quantitative analysis of THM as a new method.[5] Currently, many analytical techniques have been developed and reported to quality control of THM or herbs.[6-9]

In this study, reliable and accurate quantitative HPLC method for simultaneous determination of eight compounds, ginsenoside Rg3 of *P. ginseng*, caffeic acid of *P. frutescens*, puerarin of *P. lobata*, costunolide of *A.*

lappa, hesperidin and naringin of *C. unshiu*, glycyrrhizin of *G. uralensis* and 6-gingerol of *Z. officinale* in the Samsoeum was developed. Compounds that registered and reported HPLC-diode array detection (DAD) analysis method in National Standard of Traditional Medicinal (Herbal and Botanical) Materials and literature were selected as analysis compound. The quantitative analysis of the eight compounds was conducted by HPLC-DAD method at four ultraviolet (UV) wavelengths. The established method was applied to commercial Samsoeum samples. For confirmation of eight compounds in Samsoeum sample, the LC-electrospray ionization (ESI)-MS method was performed.

MATERIALS AND METHODS

Materials

Ginsenoside Rg3 and caffeic acid were purchased from Sigma (USA). Puerarin, hesperidin, naringin, glycyrrhizin, and 6-gingerol were purchased from the Korea Food and Drug Administration. Costunolide was purchased from Chromadex (USA). The purity of each compound was determined to be above 98%. The chemical structures of the eight marker compounds were shown in Figure 1. Acetonitrile and water were of HPLC grade and purchased from J.T. Baker (USA). Analytical grade trifluoroacetic acid (TFA) was obtained from DAE JUNG (Korea). Samsoeum sample was prepared, and other commercial Samsoeum samples were obtained from different herbal medicine companies.

Samsoeum sample preparation

In this study, Samsoeum sample was prepared by heating extraction method. Samsoeum was comprised of 4 g of *P. ginseng*, 4 g of *P. frutescens*, 4 g of *A. decursiva*, 4 g of *P. ternate*, 4 g of *P. lobata*, 4 g of *P. cocos*, 4 g of *A. lappa*, 3 g of *C. unshiu*, 3 g of *P. grandiflorum*, 3 g of *C. aurantum*, 3 g of *G. uralensis*, 1.49 g of *Z. officinale*, 2 g of *Z. jujube*. These herbs were deposited in water of 10 times the weight of herbs for 1 h and reflux water extracted at 115°C for 3 h. Extraction was powdered by freeze-drying method.

High-performance liquid chromatography-diode array detection condition

The HPLC-DAD system was a Dionex Ultimate 3000 HPLC system (Dionex, Germany) and comprised a pump (Liquefied petroleum gas 3X00), an autosampler (ACC-3000), a column oven (TCC-3000SD) and diode array UV/Visible detector (DAD-3000 [Rapid separation]). A chromatogram data equipped with Dionex ChromelonTM Chromatography Data System. Separation was performed on a LUNA C_{18} column (250 × 4.60 mm i.d., 5 μm), and the column temperature was maintained at 35°C. The mobile phase was consisted of two solvents, acetonitrile (A) and 0.1% (v/v) TFA in water (B) at a flow rate of 1.0 mL/min. A gradient elution system of mobile phase was used to achieve analysis (0–10 min, 10%→15% A; 10–20 min, 15%→30% A; 20–30 min, 30% A; 30–40 min, 30%→50% A; 40–50 min, 50%→75% A). The detect UV wavelength was set between 190 nm and 400 nm for UV maximum wavelength of various compounds. The injection volume was set 20 μL.

Liquid chromatography-electrospray ionization-mass spectrometer condition

Liquid chromatography-ESI-MS analysis was conducted using TSQ Quantum Ultra Triple Stage Quadrupole MS (Thermo). Analysis was performed at 25°C on Atlantis dC18 column (150 × 2.0 mm i.d., 3 μm). The mobile phase was the same as HPLC-DAD analysis. The linear gradient was used as follows: 0–10 min, 15%→20% A; 10–20 min, 20%→25% A; 20–30 min, 25%→50% A; 30–40 min, 50% A; 40–50 min, 50%→25% A. Eluent A was acetonitrile. The flow rate was 200 μl/min. Mass spectrometry conditions were optimized to provide the highest sensitivity. All analytes were monitored under positive ionization mode. The ion spray voltage was 4,700 V, and the vaporizer temperature was 320°C. The other conditions were as follows: Sheath gas pressure, 60 psi; aux gas pressure, 30 psi; capillary temperature, 320°C.

Standard solutions and sample preparation

Each accurately weighed standard was dissolved in 10 mL of 60% methanol. Individual stock solutions were prepared at a concentration of 200 μg/mL for puerarin, 350 μg/mL for caffeic acid, 289.5 μg/mL for naringin, 175 μg/mL for hesperidin, 400 μg/mL for glycyrrhizin, 265 μg/mL for 6-gingerol, 1000 μg/mL for ginsenoside Rg3, and 170 μg/mL for costunolide. The analytical working solutions were prepared by appropriate dilution of the stock solution and mixed before HPLC analysis.

The Samsoeum powders were weighed accurately and added in 8 mL of 60% methanol. The sample solutions were filtered through a 0.45 μm membrane filter before analysis.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

To obtain the best separation condition, four different columns have been tested Dionex C_{18} column (150 mm × 4.6 mm i.d., 5 μm), LUNA C_{18} column (250 mm × 4.60 mm i.d., 5 μm), SHISHEDO C_{18} column (250 mm × 4.6 mm i.d., 5 μm) and XTerra™ RP18 (250 mm × 4.60 mm i.d., 5 μm). As a result of the

test, use of LUNA C_{18} column (250 mm × 4.60 mm i.d., 5 μm) resulted in a well separation of eight compounds. In mobile phase condition, buffer such as 0.1% TFA was added to improve peak shape and inhibit the ionization of compounds.[10] Colum temperature was considered of peak retention time and peak shape and set at 35°C.[11] The maximum UV wavelength of eight compounds was different. Thus, the detection UV wavelength was selected according to their maximum wavelength. 6-gingerol, ginsenoside Rg3, and costunolide were at 205 nm. Puerarin and glycyrrhizin were at 250 nm. Naringin and hesperidin were at 280 nm. Caffeic acid was at 330 nm. The chromatograms of the standard solution and Samsoeum sample were shown in Figure 2. It was appeared that a good separation was achieved under the established LC condition.

Liquid chromatography-electrospray ionization-mass spectrometer analysis

This method involved the use of LC-ESI-MS to identify the peaks of eight compounds found in HPLC chromatogram of Samsoeum. Accurate molecular mass of puerarin, caffeic acid, naringin, hesperidin, glycyrrhizin, 6-gingerol, ginsenoside Rg3, and costunolide were obtained by the LC-ESI-MS analysis. In positive ionization mode, the protonated molecular ions $[M + H]^+$ were observed at m/z 181.06, 416.37, 610.56, 822.93, 785.01, and 294.38 for caffeic acid, puerarin, naringin, hesperidin, glycyrrhizin, and 6-gingerol, respectively. Sodiated molecular ions $[M + Na]^+$ were observed at m/z 603.22 and 807.69 for naringin and ginsenoside Rg3, respectively [Table 1 and Figure 3].

Method validation

Linearity, limits of detection, and limits of quantification

Good linear correlation and high sensitivity were evaluated by the correlation coefficient, limits of detection (LOD) and limits of quantification (LOQ). The linear calibration curves were plotted with diluted six different concentrations of standard solutions. Each concentration of compounds was analyzed in triplicate. The linear regression equations were calculated in the form of $Y = ax + b$ (a is the slope of the calibration curve b is the intercept of calibration curve, x and Y are the concentration and peak area of compound, respectively). Calibration curves exhibited good linearity ($R^2 > 0.9996$) in the range of measured

Table 1: Identification of the 8 compounds

Components	Exact mass	Molecular ions (m/z)
Puerarin	416.37	$[M+H]^+$ 417.12
Caffeic acid	180.16	$[M+H]^+$ 181.06
Naringin	580.54	$[M+Na]^+$ 603.22
Hesperidin	610.56	$[M+H]^+$ 611.25
Glycyrrhizin	822.93	$[M+H]^+$ 823.54
6-Gingerol	294.38	$[M+H]^+$ 295.17
Ginsenoside Rg3	785.01	$[M+Na]^+$ 807.69
Costunolide	233.16	$[M+H]^+$ 233.16

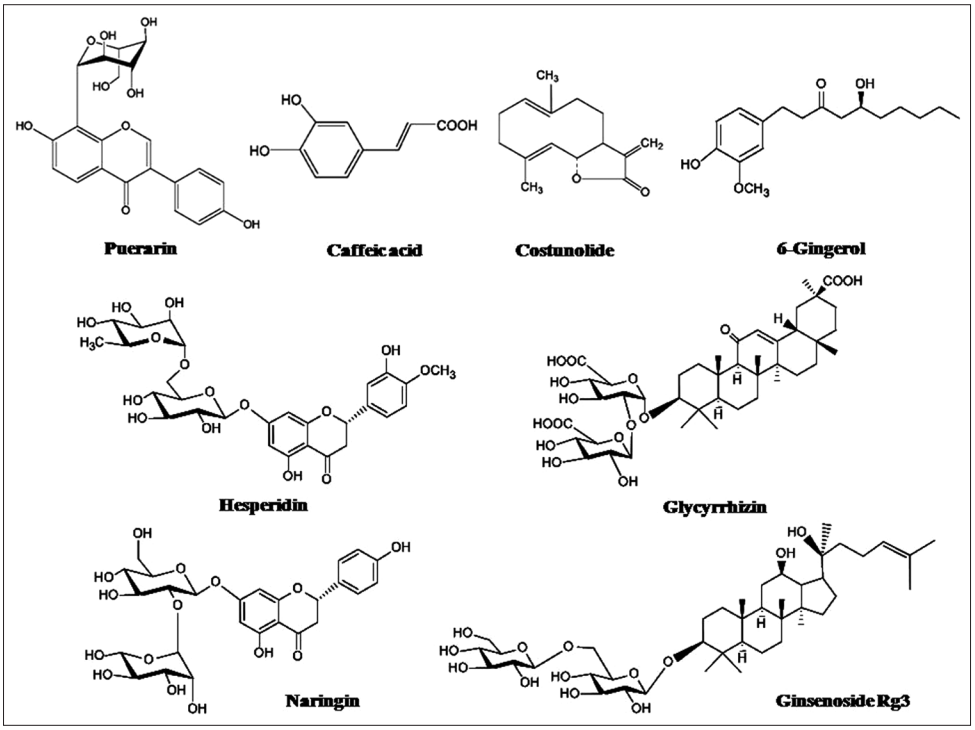

Figure 1: Chemical structures of eight major compounds

concentration for eight major compounds. LOD and LOQ were measured on the basis of the signal to noise ratio of 3 and 10, respectively. The LOD and LOQ were found to be in the range of 4.6–66.7 ng and 21.0–202.1 ng, respectively. The detailed descriptions of results were presented in Table 2.

Precision and accuracy

Precision of the method was evaluated by performing the inter- and intra-day test with eight major compounds at three different concentrations. The intra- and inter-day tests were, repetitively, conducted on the mixed standard solution five times once a day for 3 consecutive days (1, 3, 5 days) and a day, respectively. Precision was determined as relative standard deviation (RSD). RSD values of the inter- and inter-day were within 1.94% (0.34–1.94%) and 2.00 (0.56–2.00%), respectively [Table 3].

In order to confirm the accuracy, a recovery experiment was performed. Three different concentrations of the eight major compounds were added into the Samsoeum sample in triplicate. The recovery value (%) and RSD were shown in Table 4. The mean recoveries of investigated eight compounds ranged from 94.24% to 107.90% with RSD <1.92%. These results indicated that the established method had acceptable precision and accuracy.

Table 2: Regression equation, the correlation coefficient (R^2), LOD, and LOQ for the 8 compounds

Components	Linear range (μg/mL)	Regression equation[a]	R^2 (n=6)	LOD (ng)	LOQ (ng)
Puerarin	0.625-50.000	Y=1.2791x−0.2613	0.9999	14.5	44.0
Caffeic acid	0.547-43.750	Y=1.5255x−0.2570	1.0000	5.0	16.4
Naringin	0.452-36.188	Y=0.5148x−0.0384	1.0000	13.2	40.1
Hesperidin	0.273-21.875	Y=0.5248x+0.0565	0.9999	24.3	73.5
Glycyrrhizin	0.625-50.000	Y=0.1923x+0.0173	0.9999	6.9	21.0
6-Gingerol	0.414-33.125	Y=1.3554x–0.0651	1.0000	4.6	13.8
Ginsenoside Rg3	1.563-125.000	Y=0.0684x−0.0404	0.9996	44.8	135.8
Costunolide	0.266-21.250	Y=1.1565x+0.0266	0.9999	66.7	202.1

[a]Y: Peak area; x: Concentration (mg/mL). LOD: Limits of detection; LOQ: Limits of quantification

Table 3: Analytical results of intra-day and inter-day variability

Components	Concentration (μg/mL)	Intra-day (n=5)			Inter-day (n=5)		
		Mean±SD (μg/mL)	RSD (%)	Accuracy (%)	Mean±SD (μg/mL)	RSD (%)	Accuracy (%)
Puerarin	3.13	3.25±0.06	1.72	104.16	3.23±0.04	1.17	103.41
	6.25	6.22±0.03	0.51	99.58	6.25±0.12	1.88	100.08
	12.50	12.40±0.16	1.26	99.19	12.45±0.14	1.09	99.63
Caffeic acid	5.47	5.52±0.05	0.84	100.91	5.16±0.10	1.91	94.31
	10.94	10.72±0.11	0.99	98.03	10.58±0.21	1.99	96.70
	21.88	22.28±0.24	1.09	101.82	21.42±0.21	0.97	97.89
Naringin	4.52	4.88±0.09	1.94	107.88	4.89±0.08	1.62	108.10
	9.05	9.51±0.05	0.50	105.10	9.75±0.12	1.23	107.74
	18.09	18.06±0.28	1.55	99.81	18.60±0.18	0.99	102.81
Hesperidin	2.73	2.82±0.05	1.64	103.38	2.97±0.04	1.32	108.66
	5.47	5.76±0.03	0.59	105.37	5.95±0.07	1.10	108.78
	10.94	11.48±0.21	1.81	104.96	11.85±0.13	1.13	108.30
Glycyrrhizin	6.25	6.25±0.10	1.58	100.02	6.35±0.10	1.60	101.53
	12.50	12.34±0.08	0.63	98.74	12.68±0.19	1.51	101.47
	25.00	24.75±0.36	1.47	99.00	25.58±0.44	1.71	102.33
6-Gingerol	4.14	4.20±0.07	1.26	101.33	4.15±0.04	1.08	100.14
	8.28	8.15±0.06	1.49	98.42	8.36±0.14	1.62	100.98
	16.56	16.60±0.27	0.34	100.27	16.62±0.26	1.55	100.34
Ginsenoside Rg3	15.63	15.41±0.19	1.26	98.60	16.13±0.29	1.77	103.18
	31.25	32.86±0.49	1.49	105.14	31.74±0.17	0.55	101.58
	62.50	65.90±0.23	0.34	105.43	62.24±0.70	1.13	99.58
Costunolide	2.66	2.71±0.05	1.91	101.95	2.68±0.02	0.78	100.74
	5.31	5.70±0.11	1.89	107.26	5.42±0.11	2.00	102.01
	10.63	10.95±0.12	1.13	103.01	10.97±0.11	0.96	103.24

SD: Standard deviation; RSD: Relative standard deviation

Sample analysis

The developed HPLC method was applied to analyze eight compounds, puerarin, caffeic acid, naringin, hesperidin, glycyrrhizin, 6-gingerol, ginsenoside Rg3, and costunolide in the prepared Samsoeum sample and the seven commercial samples. Contents of the eight compounds in the samples are listed in Table 5. Table 5 shows that their contents in the samples were slightly different. Among of the compounds, puerarin was the main compound. The highest content of puerarin was 10.91 μg/mg, but the lowest was 10.27 μg/mg. The contents of caffeic acid were the lowest in Samsoeum samples and not were detected in some samples. Costunolide could not be detected in all samples. The quality of traditional medicine and the content of bioactive compounds were affected by the different processing procedures for manufacturing Samsoeum and the year of the plant cultivation, harvest time, plant origins, climate, and environment. Therefore, efficient analysis method to control the quality of traditional medicine like Samsoeum was needed. This HPLC method may be used as a protocol to evaluate the quality of Samsoeum.

CONCLUSION

Samsoeum, THM is a remedy for the treatment of respiratory disease. In the study, accurate, sensitive, and precise HPLC-DAD and LC-ESI-MS method for the

Table 4: Results of recovery of the 8 compounds

Components	Spiked amount (μg/mL)	Measured amount (μg/mL)	Recovery[a] (%)	RSD (%)
Puerarin	1.56	1.65±0.03	105.65	1.92
	3.13	3.17±0.05	101.36	1.71
	6.25	6.39±0.02	102.18	0.38
Caffeic acid	2.73	2.87±0.04	97.71	1.66
	5.47	5.40±0.05	98.74	0.99
	10.94	10.31±0.06	94.24	0.54
Naringin	2.26	2.25±0.01	99.32	0.52
	4.52	4.66±0.07	102.97	1.55
	9.05	9.16±0.13	101.20	1.44
Hesperidin	1.37	1.43±0.01	104.50	0.22
	2.73	2.80±0.02	102.58	0.61
	5.47	5.90±0.10	107.90	1.70
Glycyrrhizin	3.13	3.12±0.01	99.94	0.30
	6.25	6.44±0.04	103.07	0.63
	12.5	11.92±0.03	95.33	0.22
6-Gingerol	2.07	1.97±0.02	94.94	1.14
	4.14	4.11±0.05	99.36	1.24
	8.28	8.60±0.07	103.82	0.76
Ginsenoside Rg3	7.81	7.98±0.08	102.18	1.04
	15.63	15.00±0.05	95.98	0.33
	31.25	29.62±0.53	94.79	1.80
Costunolide	1.33	1.33±0.01	99.93	0.24
	2.66	2.69±0.04	101.29	1.41
	5.31	5.28±0.04	99.38	0.84

[a]Recovery (%): (Concentration found−original concentration)/concentration spiked×100%. RSD: Relative standard deviation

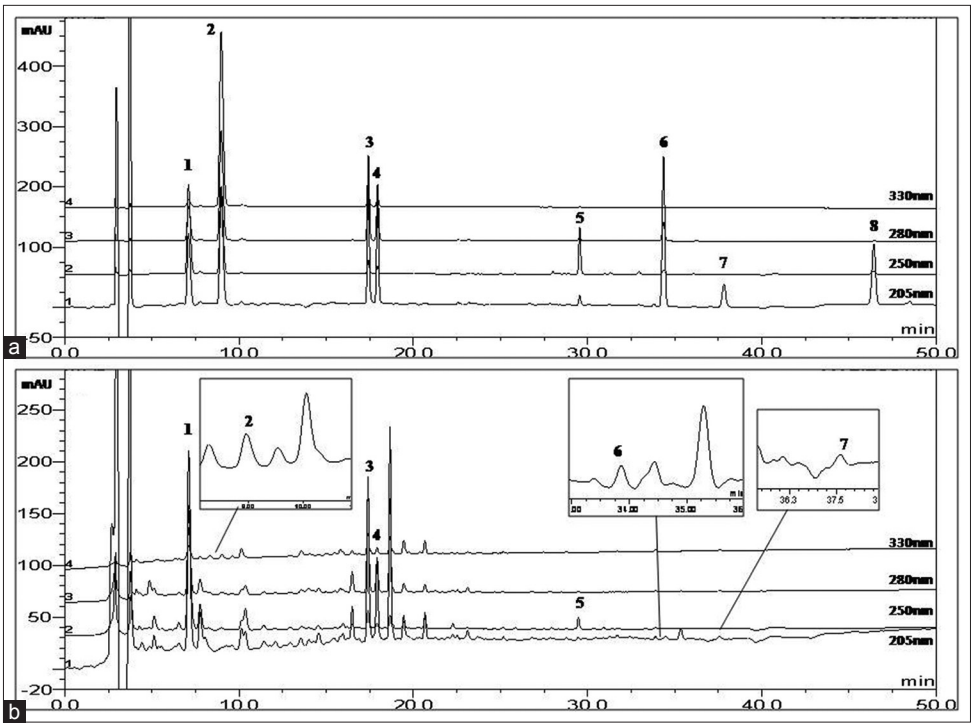

Figure 2: The high-performance liquid chromatography chromatogram of eight standard compounds (a) and Samsoeum sample (b); (1) puerarin, (2) caffeic acid, (3) naringin, (4) hesperidin, (5) glycyrrhizin, (6) 6-gingerol, (7) ginsenoside Rg3, (8) costunolide

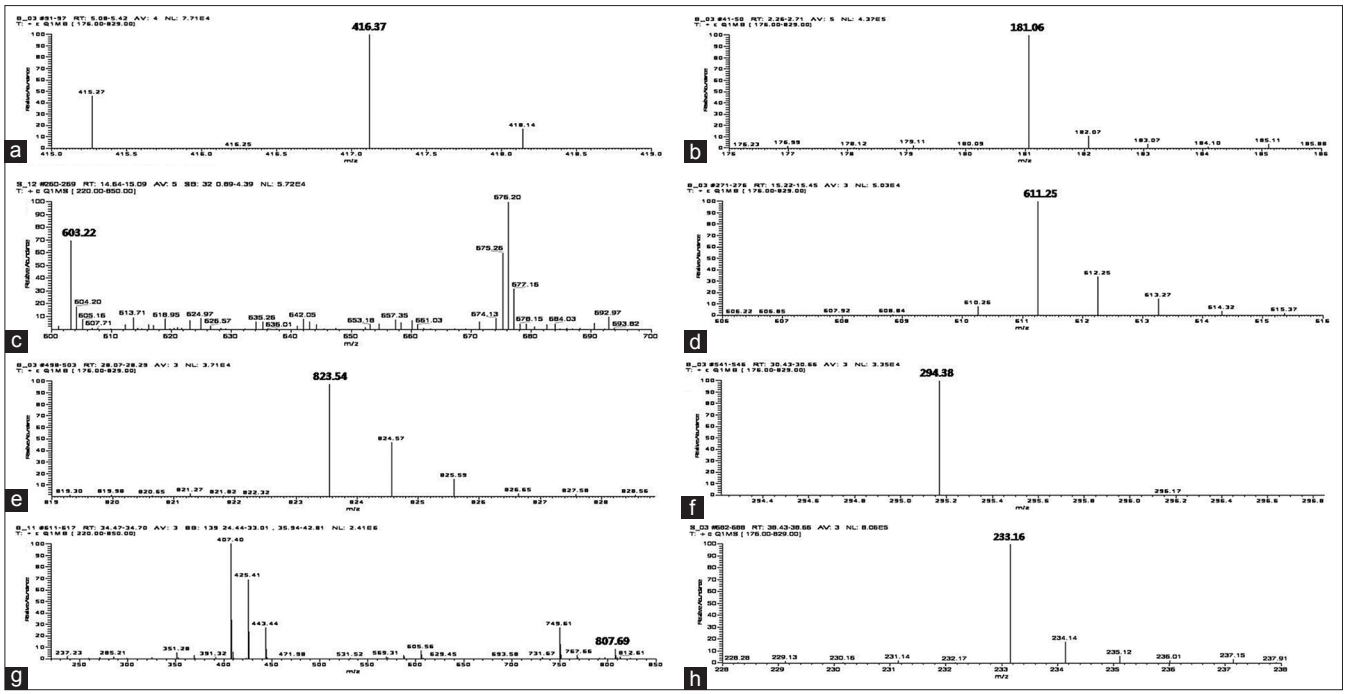

Figure 3: The mass spectra of eight major compounds; (a) puerarin, (b) caffeic acid, (c) naringin, (d) hesperidin, (e) glycyrrhizin, (f) 6-gingerol, (g) ginsenoside Rg3, (h) costunolide

Table 5: Contents (μg/mg) of the 8 compounds in the prepared Samsoeum sample and 7 commercial samples

Sample	Content (μg/mg)							
	Puerarin	Caffeic acid	Naringin	Hesperidin	Glycyrrhizin	6-Gingerol	Ginsenoside Rg3	Costunolide
Samsoeum[a]	10.27±0.11	0.15±0.01	10.30±0.01	3.42±0.04	2.89±0.02	0.18±0.01	3.10±0.14	nd[c]
S-1[b]	10.52±0.04	0.13±0.01	10.05±0.01	3.16±0.01	2.94±0.01	0.57±0.03	4.44±0.51	nd
S-2	10.48±0.18	0.18±0.01	10.01±0.11	3.14±0.05	3.00±0.06	0.67±0.06	4.45±0.07	nd
S-3	10.53±0.13	nd	10.13±0.08	3.18±0.02	2.69±0.04	0.66±0.03	4.78±0.24	nd
S-4	10.53±0.13	0.19±0.01	10.09±0.05	3.15±0.02	2.57±0.01	0.61±0.04	5.41±0.38	nd
S-5	10.60±0.07	0.21±0.02	10.19±0.01	3.18±0.01	2.84±0.10	0.63±0.02	4.36±0.26	nd
S-6	10.63±0.17	nd	10.22±0.16	3.25±0.06	2.68±0.13	0.66±0.02	4.69±0.13	nd
S-7	11.59±0.05	0.19±0.01	11.40±0.02	3.57±0.01	3.11±0.04	0.70±0.04	4.74±0.08	nd

[a]Samsoeum: Prepared Samsoeum sample; [b]S-1–7: Seven commercial Samsoeum sample; [c]nd: Not detect

quantitative analysis and identification of the eight major compounds, puerarin, caffeic acid, naringin, hesperidin, glycyrrhizin, 6-gingerol, ginsenoside Rg3, and costunolide in Samsoeum. Up to now, simultaneous determination of the seven compounds (Puerarin, daidzin, liquiritin, naringin, hesperidin, neohesperidin, and glycyrrhizin) in the Samsoeum was reported.[12] We analyzed more compounds of various herbs, and LC-ESI-MS method was additionally employed to verify the compounds in comparison to previous reported analysis method of Samsoeum. The developed method was successfully applied for simultaneous determination in the eight compounds in Samsoeum sample. Such results can form the basic method for improvement of quality of Samsoeum.

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