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Simultaneous Determination of Six Components in the Traditional Herbal Medicine 'Oryeongsan' by HPLC-DAD and LC-MS/MS

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Abstract – A simple high performance liquid chromatography – diode array detector (HPLC-DAD) method has been developed and validated for simultaneous determination of the six components (cinnamic acid, cinnamaldehyde, eugenol, atractylenolide I, atractylenolide III, and ergosterol) in Oryeongsan. In addition, identification of six marker compounds was conducted by a LC-MS/MS analysis. The six compounds in Oryeongsan were separated on Shishedo C_{18} column (5 μm, 4.6 × 250 mm) at a column temperature of 30 °C. The mobile phase was a mixture of 0.1% trifluoroacetic acid (TFA) water and acetonitrile employing gradient elution at a flow rate of 1.0 mL/min. The detection wavelength was set at 205, 250, 280, and 330 nm. The developed method had good linearity ($R^2 > 0.9997$) and the limit of detection (LOD) and limit of quantification (LOQ) were observed within the ranges 0.01~0.15 and 0.05~0.45 μg/mL, respectively. The relative standard deviation (RSD) values of intra- and inter-day testing were indicated that less than 3% and 90.31~103.31% of accuracy. The results of recovery test were 90.56~106.72% and RSD range was measured from 0.84 to 2.95%. In conclusion, this HPLC-DAD method has been successfully applied to the simultaneous determination of six compounds in Oryeongsan samples.
Keywords – Oryeongsan, HPLC-DAD, Simultaneous determination, LC-MS/MS

Introduction

Traditional herbal medicines such as herbal formulas and prescriptions have been used for a long time in Korea, China, Japan, and other oriental countries to prevent and treat clinical diseases (Chan, 1995; Liang, 2004). These herbal medicines have fewer side effects and exhibit multiple activities (Normile, 2003; Jiang, 2005). These facts contribute to the increase of popularity of traditional herbal medicines. Multiple constituents in traditional herbal medicines may be have synergic effect and quality of bioactive constituents in component herbs in the traditional herbal medicine products were depended on plant origins, manufacturing processes and other factors (Liang, 2004). And accurate the quantity and quality analysis data of traditional herbal medicine be used to evaluate safety and efficacy of traditional herbal medicines. Therefore, the reliable and accurate quality control method for herbal medicines is necessary.

Oryeongsan (Wulingsan) is one of the Korean traditional prescription which is first recorded in old traditional medicine book, Sanghanron (Treatise on Febrile Diseases) written by Zhang Zhong Jing in the third century. In Sanghanron, it was originally used in the symptoms of perspiration, thirsty, decreased urine volume, and flatulence. This prescription specifically used for the treatment of renal diseases including edema, dysuria, and oliguria (He *et al.*, 2008). It also exhibited therapeutic effect of urinary stone by inhibition of calcium oxalate crystal growth, aggregation, and formation, antihypertensive and antidiabetic effects (Tsai *et al*., 2008; Kiga *et al.*, 2008; Liu *et al.*, 2009).

Oryeongsan is composed of five crude drugs: *Alisma orientalis* Juzep, *Poria cocos* Wolf, *Atractylodes macrocephala* Koidez, *Polyporus umbellatus* Fries, and *Cinnamomum cassia* Presl. And these herbs are frequently contained in other prescriptions for the treatment of dysfunction of the body fluid balance (Ahn *et al.*, 2012).

Previously, analytical determination methods have been developed for quality control of the two major components,

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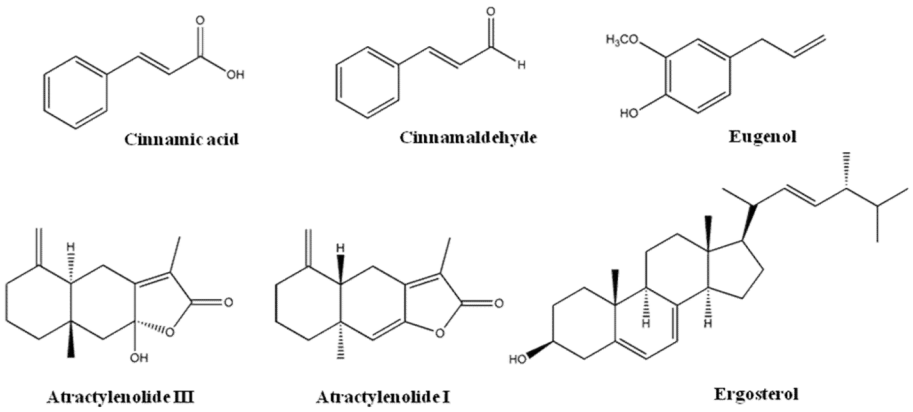

Fig. 1. The chemical structure of six marker components of Oryeongsan.

alisol A 24-acetate and alisol B 23-acetate in *Alisma orientalis* by HPLC-ELSD and of the two compounds ergosterol and cinnamaldehyde by HPLC (Li *et al*., 2007; Li *et al.*, 2011). However, these methods analyzed two compounds in Oryeongsan and are insufficient to control the quality of Oryeongsan.

In this study, a simple and reliable HPLC-DAD method has been developed and validated for simultaneous determination of the six components, cinnamic acid, cinnamaldehyde, eugenol, atractylenolide III, atractylenolide I, and ergosterol in Oryeongsan (Fig. 1). Compounds that detected in HPLC-DAD analysis were selected as analysis compound. In addition, Identification of the six marker compounds was carried out by using the LC-MS/MS.

Experimental

Instruments – The HPLC equipment used was Dionex system (Dionex, Germany) including a pump (LPG 3X00), an auto sampler (ACC-3000), a column oven (TCC-3000SD), diode array UV/VIS detector [DAD-3000(RS)], and Dionex ChromelonTM Chromatography Data System software. HPLC analysis was conducted on Shiseido C_{18} column (4.6 × 250 mm, 5 μm pore size). The LC-MS/MS system used was TSQ Quantum Ultra (Thermo Electron Co., USA) equipped with electro-spray ionization (ESI) in positive ion source. Standard and sample were separated on an Atlantis dC_{18} column (3 μm pore size, 2.0 × 150 mm).

Reagents and solution – Cinnamic acid was purchased from NPC BioTechnology (Korea). Cinnamaldehyde and ergosterol were purchased from Sigma Aldrich Co. Ltd (USA). Eugenol, atractylenolide III, and atractylenolide I were provided by Korea Food and Drug Administration. The purities of six standards were above 95%. 12 commercial Oryeongsan samples were provided by the Korea Institute of Oriental Medicine. HPLC-grade acetonitrile, methanol, and water were purchased from J. T. Baker (USA). TFA was purchased from DAE JUNG (Korea).

Preparation of standard solutions – Standard stock solutions of cinnamic acid (156 μg/mL), cinnamaldehyde (39.3 μg/mL), eugenol (216 μg/mL), atractylenolide III (172.5 μg/mL), atractylenolide (195 μg/mL), and ergosterol (132 μg/mL) were prepared in methanol and stored below 4 ºC. Working standard solutions were prepared by appropriate dilution of the stock solution with methanol. These working solutions were mixed and used for establishment of calibration curves.

Preparation of sample solutions – 12 Oryeongsan samples were weighed and dissolved in water at 10.70 mg/mL, 10.76 mg/mL, 10.54 mg/mL, 10.80 mg/mL, 10.00 mg/mL, 10.54 mg/mL, 10.76 mg/mL, 10.44 mg/mL, 10.60 mg/mL, 10.52 mg/mL, 10.74 mg/mL, and 10.48 mg/mL respectively. All sample solutions were filtered through a 0.45 μm membrane filter before HPLC analysis and were stored at 4 ºC.

Liquid chromatographic conditions – The mobile phase was composed of 0.1% TFA aqueous solution (A) and acetonitrile (B) at a flow rate of 1.0 mL/min. The HPLC linear gradient profile was as follows: 10% B at 0~5 min, 10~90% B at 5~40 min, 100% B at 40~60 min. The sample injection volume was 20 μL. The column temperature was maintained at 30 ºC. Detection was performed at four different UV wavelengths, 205 nm, 250 nm, 280 nm, and 330 nm.

LC-MS/MS conditions – LC-MS/MS analysis was performed under the same solvent condition and a little modified gradient with HPLC-DAD. The flow rate used was 0.2 mL/min and the injection volume was 20 μL. The

mobile phase consisted of 0.1% TFA water (A) and acetonitrile (B). The LC-MS/MS gradient used was as follows: 10% B at 0~5 min, 10~90% B at 5~40 min, 90~100% B at 40~42 min, 100% B at 42~52 min. Positive ion electro-spray ionization (ESI) was performed at 3800 V spray voltage. The vaporizer and capillary temperature was kept at 102 ºC and 265 ºC, respectively. Moreover, the sheath and aux gas pressure was set to 12 and 30 psi.

Validation of the HPLC method – The validation of established HPLC method was performed according to the International Conference on Harmonisation (ICH) guidelines. Linearity, LOD, LOQ, precision, and recovery were evaluated for the method (Xie *et al.*, 2007; Yi *et al.*, 2007; Yang *et al.*, 2011; Weon *et al.*, 2012). To establish the calibration curves, standard stock solutions containing cinnamic acid, cinnamaldehyde, eugenol, atractylenolide III, atractylenolide I, and ergosterol were prepared and diluted quantitatively with methanol to provide working stock solutions of six different concentrations. Six concentrations of working standard solutions were analyzed in triplicate. Calibration curves were constructed by plotting the peak areas versus the concentrations of each analyte and then obtained regression equations in the form of $y = ax + b$, where y and x are the peak area and the concentration of the analyte. Correlation coefficient (R^2) values of the obtained regression equations indicated linearity. LOD and LOQ values were determined at signal-to-noise (S/N) ratios of 3 and 10, respectively. The precision of developed method was determined by inter- and intra-day variation during on three different days and a single day, respectively. Variations were expressed by Relative Standard Deviation (RSD), and the RSD was calculated by following equation; RSD = (standard deviation / mean measured amount) × 100. The recovery test was utilized to evaluate the accuracy of this HPLC method. Accurate amounts of mixed standard solution were added to Oryeongsan sample, and analyzed three different concentrations in three times, respectively. The recoveries were calculated by the equation; recovery (%) = (amount found – original amount) / amount added × 100, and RSD also calculated.

Quantification of Oryeongsan samples – 12 Samples of Oryeongsan were determined by this developed method, and each sample was determined in three times. The amount of six marker compounds in each sample was calculated from its calibration curve.

Results and Discussion

Optimization of HPLC-DAD condition – The optimization of HPLC condition was performed to obtaining a better resolution of adjacent peaks. In this study, three

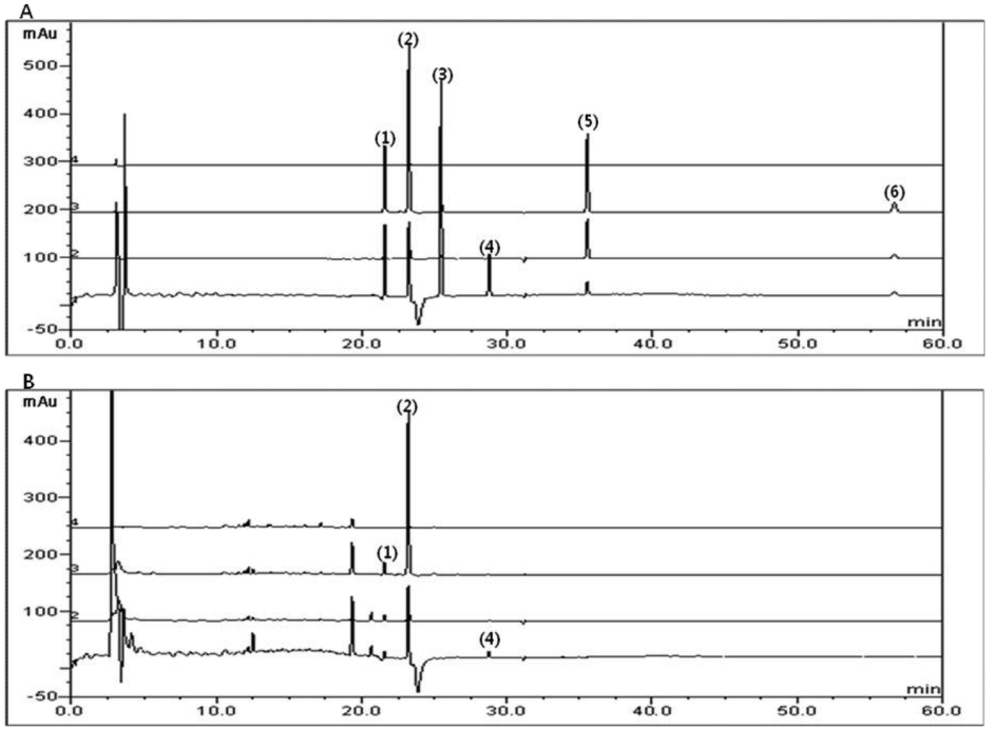

Fig. 2. HPLC chromatogram of Oryeongsan standard compounds mixture (A) and sample (B).
1. cinnamic acid, **2**. cinnamaldehyde, **3**. eugenol, **4**. atractylenolide III, **5**. atractylenolide I, and **6**. ergosterol.

different column, Dionex C_{18} column (150 mm × 4.6 mm i.d., 5 μm), LUNA C_{18} column (250 × 4.6 mm i.d., 5 μm), SHISHEDO C_{18} column (250 × 4.6 mm i.d., 5 μm), and XTerra™ RP18 (250 mm × 4.6 mm i.d., 5 μm) and two mobile phases, 0.1% TFA in water/acetonitrile and 0.1% TFA in water/methanol were compared to obtain good resolution within HPLC analysis. SHISHEDO C_{18} column (250 × 4.6 mm i.d., 5 μm) and 0.1% TFA in water/acetonitrile showed the good separation. Trifluoroacetic acid (0.1% in water) was added to obtain the improved peak shape and inhibited the peak tailing. Because of the difference in maximum UV absorption wavelength of each of the six compounds, The UV wavelength of the DAD detector was selected variously. The detection wavelength was set at 280 nm for cinnamic acid, cinnamaldehyde, atractylenolide I, and ergosterol and at 205 nm for eugenol and atractylenolide III. A HPLC chromatogram of the six standard compounds is shown in Fig. 2A. The identities of the six chromatographic peaks were confirmed by comparing the retention time and UV spectrum of each marker compound. The retention times of cinnamic acid, cinnamaldehyde, eugenol, atractylenolide III, atractylenolide I, and ergosterol were 21.56, 23.21, 25.43, 28.75, 35.50, and 56.60 min, respectively.

Linearity, LOD and LOQ – Each calibration curves and coefficients (R^2) were determined by analysis of using standard solutions. The results are shown in Table 1. All the calibration data of the six marker compounds showed good linearity ($R^2 > 0.9997$). The LOD and LOQ values were in the range 0.01~0.15 and 0.02~0.45 μg/mL, respectively.

Precision and accuracy – The RSD values of intra-

Table 1. Regression data, LODs and LOQs of the six marker compounds

Compounds	Linear range (μg/mL)	Regression equation	R^2	LOD (μg/mL)	LOQ (μg/mL)
Cinnamic acid	0.33 - 26.00	$Y = 0.628x - 0.0483$	0.9999	0.02	0.06
Cinnamaldehyde	0.04 - 6.55	$Y = 13.861x + 0.1529$	0.9997	0.01	0.02
Eugenol	0.45 - 36.00	$Y = 1.6123x + 0.0255$	0.9998	0.04	0.12
Atractylenolide III	0.36 - 28.75	$Y = 0.3998x + 0.0445$	0.9998	0.11	0.34
Atractylenolide I	0.41 - 32.50	$Y = 0.7059x - 0.0422$	0.9998	0.02	0.05
Ergosterol	0.28 - 22.00	$Y = 0.2801x - 0.0237$	0.9998	0.15	0.45

Table 2. Precisions for the six analytes

Compounds	Concentration (μg/mL)	Intra-day (n = 5)			Inter-day (n = 5)		
		Mean ± SD (μg/mL)	RSD (%)	Accuracy (%)	Mean ± SD (μg/mL)	RSD (%)	Accuracy (%)
Cinnamic acid	13.00	13.06 ± 0.11	0.81	100.48	12.77 ± 0.22	1.76	98.23
	6.50	6.25 ± 0.15	2.41	96.16	6.44 ± 0.03	0.44	99.04
	3.25	3.04 ± 0.03	0.89	93.49	3.17 ± 0.09	2.70	97.47
Cinnamaldehyde	1.64	1.67 ± 0.04	2.54	102.17	1.64 ± 0.03	2.04	99.86
	0.82	0.79 ± 0.02	2.62	96.44	0.82 ± 0.01	0.35	99.77
	0.41	0.37 ± 0.01	2.28	90.31	0.39 ± 0.01	2.89	95.95
Eugenol	18.00	18.60 ± 0.18	0.98	103.31	18.27 ± 0.12	0.68	101.50
	9.00	8.90 ± 0.26	2.90	98.84	9.17 ± 0.05	0.51	101.85
	4.50	4.37 ± 0.05	1.25	97.03	4.48 ± 0.07	1.53	99.47
Atractylenolide III	14.38	14.49 ± 0.13	0.89	100.80	14.05 ± 0.03	0.24	97.76
	7.19	6.90 ± 0.11	1.55	95.94	6.95 ± 0.01	0.15	96.75
	3.59	3.31 ± 0.02	0.51	92.09	3.49 ± 0.01	0.41	97.19
Atractylenolide I	16.25	16.62 ± 0.15	0.92	102.25	16.29 ± 0.38	2.34	100.25
	8.13	7.94 ± 0.21	2.69	97.67	8.23 ± 0.02	0.27	101.28
	4.06	3.77 ± 0.04	1.19	92.85	4.02 ± 0.12	2.94	98.91
Ergosterol	11.00	11.10 ± 0.04	0.37	100.90	10.85 ± 0.20	1.80	98.62
	5.50	5.30 ± 0.06	1.06	96.34	5.48 ± 0.04	0.64	99.72
	2.75	2.54 ± 0.01	0.54	92.44	2.70 ± 0.07	2.46	98.14

Table 3. Recoveries of the six marker compounds

Compounds	Spiked Amount (μg/mL)	Measured Amount (μg/mL)	Recovery (%)	RSD (%)
Cinnamic acid	6.50	6.05 ± 0.05	93.07	0.84
	3.25	3.01 ± 0.06	92.56	2.02
	1.63	1.51 ± 0.04	92.79	2.55
Cinnamaldehyde	0.82	0.83 ± 0.02	101.19	2.37
	0.41	0.40 ± 0.01	97.56	2.22
	0.21	0.19 ± 0.01	95.30	2.62
Eugenol	9.00	8.81 ± 0.25	97.88	2.88
	4.50	4.50 ± 0.12	99.99	2.58
	2.25	2.40 ± 0.03	106.72	1.31
Atractylenolide III	7.19	7.50 ± 0.17	104.29	2.25
	3.59	3.66 ± 0.10	101.87	2.78
	1.80	1.74 ± 0.03	96.67	1.95
Atractylenolide I	8.13	7.78 ± 0.22	95.67	2.81
	4.06	4.01 ± 0.05	98.66	1.28
	2.03	2.08 ± 0.05	102.58	2.19
Ergosterol	5.50	5.08 ± 0.15	92.33	2.95
	2.75	2.53 ± 0.07	91.87	2.61
	1.38	1.25 ± 0.03	90.56	2.27

Table 4. Contents of marker compounds in Oryeongsan samples

Samples	Contents (μg/mg)					
	Cinnamic acid	Cinnamaldehyde	Eugenol	Atractylenolide III	Atractylenolide I	Ergosterol
ORS[a]-1	0.383	0.365	ND	0.419	ND	ND
ORS-2	0.400	0.166	ND	0.359	ND	ND
ORS-3	0.394	0.061	ND	0.337	ND	ND
ORS-4	0.407	0.082	ND	0.347	ND	ND
ORS-5	0.495	0.048	ND	0.348	ND	ND
ORS-6	ND[b]	0.031	ND	0.362	ND	ND
ORS-7	0.055	0.037	ND	0.355	ND	ND
ORS-8	ND	0.003	ND	0.364	ND	ND
ORS-9	ND	ND	ND	0.360	ND	ND
ORS-10	ND	ND	ND	0.374	ND	ND
ORS-11	ND	0.011	ND	0.348	ND	ND
ORS-12	ND	0.003	ND	0.367	ND	ND

a) ORS : commercial Oryeongsan sample
b) ND : not detected

and inter-day variation used to evaluating of precision. The RSD values of intra- and inter-day were 0.37~2.90% and 0.15~2.94%, respectively. The intra-day accuracy was in the range of 90.31~103.31% and the inter-day accuracy was 95.95~101.85%. The results of inter- and intra-day test which were performed to estimate of precision are shown in Table 2. The recovery test of six marker compounds was applied to investigate accuracy. The average recovery percentage of the investigated six standard targets ranged from 90.56 to 106.72%, and RSD values were calculated from 0.84 to 2.95% (Table 3). The results of intra-, inter-day, and recovery test demonstrated that the established method was reliable and accurate for simultaneous determination of Oryeongsan.

Application to Oryeongsan sample analysis – The established HPLC-DAD method was applied to the simultaneous determination of cinnamic acid, cinnamaldehyde, eugenol, atractylenolide III, atractylenolide I, and

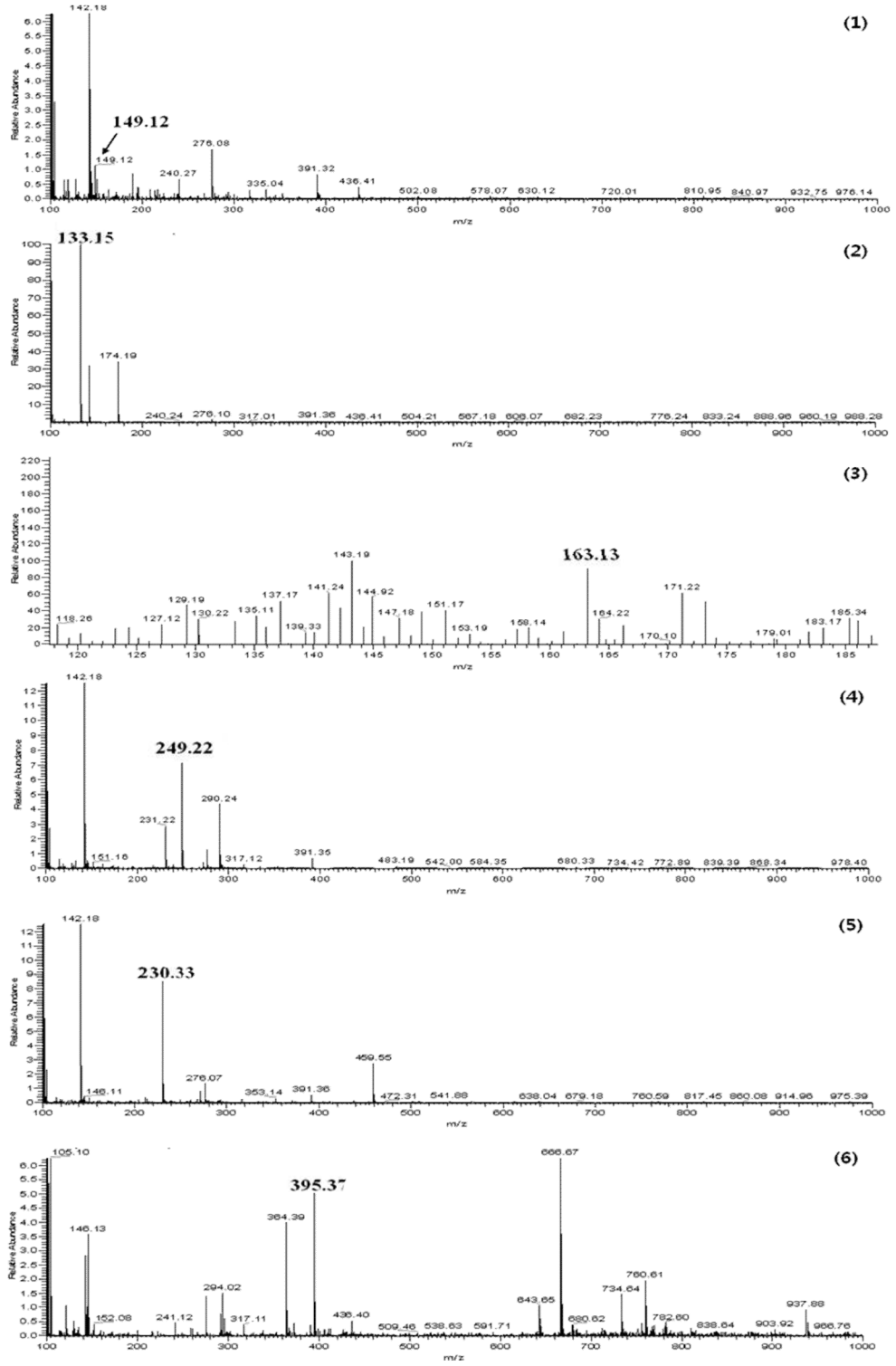

Fig. 3. LC-MS/MS spectrum of six marker compounds in Oryeongsan.
1. cinnamic acid, **2**. cinnamaldehyde, **3**. eugenol, **4**. atractylenolide III, **5**. atractylenolide I, and **6**. ergosterol.

ergosterol in 12 commercial Oryeongsan preparations. The developed method displayed good separation in Oryeongsan samples without any effect of the other component peaks (Fig. 2B). The identities of the component peaks from the commercial samples were confirmed by comparing the retention time and UV

Table 5. Identification of six marker compounds by LC-MS/MS analysis

Compounds	t_R (min)	Formula	Molecular weight	Selected ion	Experimental mass (*m/z*)
Cinnamic acid	18.74	$C_9H_8O_2$	148.16	$[M + H]^+$	149.12
Cinnamaldehyde	19.75	C_9H_8O	132.16	$[M + H]^+$	133.15
Eugenol	22.03	$C_{10}H_{12}O_2$	164.20	$[M - H]^+$	163.13
Atractylenolide III	25.68	$C_{15}H_{20}O_3$	248.32	$[M + H]^+$	249.22
Atractylenolide I	28.87	$C_{15}H_{18}O_2$	230.30	$[M]^+$	230.33
Ergosterol	45.68	$C_{28}H_{44}O$	396.65	$[M - H]^+$	395.37

spectrum of each peak with those of the standard compounds before measured. The contents of the six marker components were calculated using calibration curve of standard compounds and are described in Table 4. The amounts of the six marker components in the 12 commercial Oryeongsan preparations were found to be significantly different. The concentrations of cinnamic acid, cinnamaldehyde, and atractylenolide III were in the ranges 0.055~0.495, 0.003~0.365, and 0.337~0.419 μg/mg, respectively. Cinnamic acid and cinamaldehyde were not detected in some samples, but eugenol, atractylenolide I, and ergosterol were not detected in all samples. Peaks of these compounds were detected in chromatogram but their amount was lower than LOD. Consequently, we decided to not detect. These variations could be due to different culture environment of herbs and the manufacturing process of Oryeongsan.

LC-MS/MS – Identification of the six marker compounds in Oryeongsan was carried out by using the LC-MS/MS technique. In positive ion mode, all marker compounds (cinnamic acid, cinnamaldehyde, eugenol, atractylenolide III, atractylenolide I, and ergosterol) were detected with sufficient sensitivity. As shown in Fig. 3 (1), a peak at *m/z* 149.12 was observed. This was found to correspond to the $[M + H]^+$ of cinnamic acid ($C_9H_8O_2$, molecular weight 148.16 g/mol). In addition, cinnamaldehyde (C_9H_8O, MW 132.16 g/mol), eugenol ($C_{10}H_{12}O_2$, MW 164.20 g/mol), atractylenolide III ($C_{15}H_{20}O_3$, MW 248.32 g/mol), atractylenolide I ($C_{15}H_{18}O_2$, MW 230.30 g/mol), and ergosterol ($C_{28}H_{44}O$, MW 396.65 g/mol) obtained protonated adducts at *m/z* 133.15, 163.13, 249.22, 230.33, and 395.37, respectively. These results are summarized in Table 5.

Conclusion

In this study, reliable and accurate HPLC-DAD method was successfully established for simultaneous determination of six marker compounds (cinnamic acid, cinnamaldehyde, eugenol, atractylenolide III, atractylenolide I, and ergosterol) in Oryeongsan. Additionally, identification of each peak was confirmed by LC-MS/MS analysis. The developed method has been validated and indicated good linearity, precision and accuracy. The method was also successfully applied to quantitative analysis of six marker components in commercial Oryeongsan samples. The developed method could be used to improve the quality control of Oryeongsan.

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