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ORIGINAL ARTICLE

Quality Analysis of Chlorogenic Acid and Hyperoside in *Crataegi fructus*

Jin Bae Weon[1], Youn Sik Jung[1], Choong Je Ma[1,2]

[1]Department of Medical Biomaterials Engineering, College of Biomedical Science, Kangwon National University, [2]Department of Biomaterials Engineering, Kangwon National University, Chuncheon 200-701, Korea

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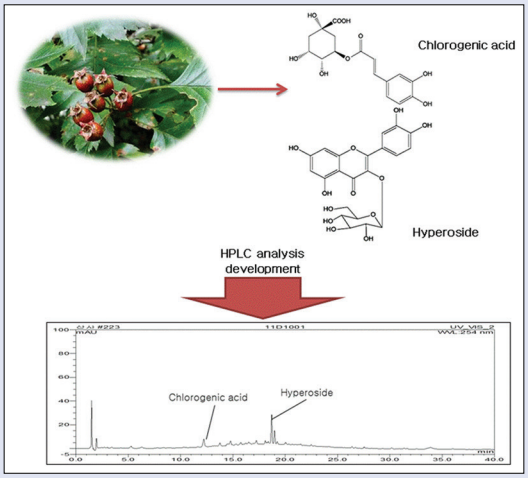
ABSTRACT

Background: *Crataegi fructus* is a herbal medicine for strong stomach, sterilization, and alcohol detoxification. Chlorogenic acid and hyperoside are the major compounds in *Crataegi fructus*. **Objective:** In this study, we established novel high-performance liquid chromatography (HPLC)-diode array detection analysis method of chlorogenic acid and hyperoside for quality control of *Crataegi fructus*. **Materials and Methods:** HPLC analysis was achieved on a reverse-phase C_{18} column (5 µm, 4.6 mm × 250 mm) using water and acetonitrile as mobile phase with gradient system. The method was validated for linearity, precision, and accuracy. About 31 batches of *Crataegi fructus* samples collected from Korea and China were analyzed by using HPLC fingerprint of developed HPLC method. Then, the contents of chlorogenic acid and hyperoside were compared for quality evaluation of *Crataegi fructus*. **Results:** The results have shown that the average contents (w/w %) of chlorogenic acid and hyperoside in *Crataegi fructus* collected from Korea were 0.0438% and 0.0416%, respectively, and the average contents (w/w %) of 0.0399% and 0.0325%, respectively. **Conclusion:** In conclusion, established HPLC analysis method was stable and could provide efficient quality evaluation for monitoring of commercial *Crataegi fructus*.

Key words: *Crataegi fructus*, chlorogenic acid, diode array detection, high performance liquid chromatography, hyperoside, quality evaluation

SUMMARY

- Quantitative analysis method of chlorogenic acid and hyperoside in *Crataegi fructus* is developed by high-performance liquid chromatography (HPLC)-diode array detection
- Established HPLC analysis method is validated with linearity, precision, and accuracy
- The developed method was successfully applied for quantitative analysis of *Crataegi fructus* sample collected from Korea and China.



Abbreviations used: HPLC: High-performance liquid chromatography, GC: Gas chromatography, MS: Mass spectrometer, LOD: Limits of detection, LOQ: Limits of quantification, RSD: Relative standard deviation, RRT: Relative retention time, RPA: Relation peak area.

Correspondence:

Prof. Choong Je Ma,
Department of Medical Biomaterials Engineering,
College of Biomedical Science,
Kangwon National University,
Hyoja-2 Dong, Chuncheon 200-701, Korea.
E-mail: cjma@kangwon.ac.kr

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INTRODUCTION

Herbal medicines have been widely used for the prevention and treatment of many diseases for 1000 of years. Herbal medicines had fewer side effects and were suitable for body. Various pharmacological effects of herbal medicines have been attributed to many bioactive compounds.[1,2]

Because of complex composition and variation by culture environment of the compounds in herbal medicines, quality consistency control of herbal medicines is difficult. Thus, accurate and stable analysis method of herbal medicine is required for quality evaluation. Analysis systems, such as high-performance liquid chromatography (HPLC), gas chromatography, and mass spectrometer have been widely used for qualitative and quantitative analysis of compounds in herbal medicines. Among systems, HPLC is the most common system for quality control of herbal medicines using characteristic fingerprint analysis.[3,4] HPLC chromatographic fingerprint technology is the comprehensive peak identification method and is applied to reveal bioactivity and chemical information of herbal medicines.[5] HPLC fingerprint is additionally used to approach a quality analysis of compounds in herbal medicine.[6]

Crataegi fructus is the fruit part of *Crataegus pinnatifida* Bunge var. typica Schneider (*Rosaceae* family) and is a well-known herbal medicine for strong stomach, analgesic effect, sterilization, and alcohol detoxification. Recent studies showed that *Crataegi fructus* prevents hyperlipidemia induced by alcohol and ameliorate arterial contraction via antioxidant effect.[7] *Crataegi fructus* also showed inhibition effect of sodium nitroprusside-induced cell lipid peroxidation and anti-inflammatory effect.[8]

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Chlorogenic acid and hyperoside are major bioactive compounds in *Crataegi fructus*. Chlorogenic acid, a related family of hydroxycinnamic acid, showed anti-diabetic and anti-lipidemic effects.[9] Hyperoside is one of the phenolic compounds and showed antiviral activity, anti-inflammatory effect, and protective effect on PC12 cells against cytotoxicity.[10-12]

Previous study reported simultaneous determination of eight polyphenols in the leaves of *Crataegus pinnatifida* by HPLC and HPLC analysis method of chlorogenic acid, hyperoside, and rutin for quality control of *Crataegi fructus*.[13,14]

We determinate HPLC analysis method and improve the resolution of hyperoside using modified gradient system of mobile phase based on previous studies.

In this study, quantitative determination of chlorogenic acid and hyperoside in *Crataegi fructus* was developed and analyzed in 31 *Crataegi fructus* samples collected from Korea and China.

MATERIALS AND METHODS

Chemicals and plant materials

Water and acetonitrile as HPLC grade were purchased from J.T Baker. Standards of chlorogenic acid and hyperoside are both obtained from the Ministry of Food and Drug Safety (Korea) [Figure 1].

The 4 batches of *Crataegi fructus* samples (collected from Korea, 11D2001, 2002, 2024, 2027) were obtained from the Ministry of Food and Drug Safety (Korea). 11 batches of *Crataegi fructus* samples (collected from Korea, 12D3017-3027) and 16 batches of *Crataegi fructus* samples (collected from China, 12D3001-3016) were purchased from Kyungdong mart (Seoul, Korea).

Preparation of samples and standard solution

Samples of *Crataegi fructus* powder were accurately weighed and extracted by sonication with 70% methanol for 60 min. Before HPLC analysis, extract solutions were filtered with 0.45 μm membrane filter.

Stock standard solution of chlorogenic acid and hyperoside were prepared in methanol at a concentration of 1000 μg/mL. Stock standard solutions appropriately diluted to obtain work standard solutions by methanol.

High-performance liquid chromatography-diode array detection condition

HPLC was performed on Dionex Ultimate 3000 HPLC system (Dionex, Germany) equipped with a pump (LPG 3X00), an auto sampler (ACC-3000), a column oven (TCC-3000SD), and diode array UV/VIS detector (DAD-3000(RS)). HPLC chromatogram data were processed using Dionex Chromeleon™ Chromatography Data System.

Chromatographic separation was conducted on Agilent eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 μm) at column temperature 30°C The mobile phase consisted of aqueous with 0.1% trifluoroacetic acid solution (A) acetonitrile (B) using gradient elution system of 5% (B) at 0–5 min and 5–50% (B) at 5-40 min and flow rate was 1.0 mL/min. The ultraviolet (UV) wavelength was selected and monitored at 254 nm according to the wavelength of chlorogenic acid and hyperoside.

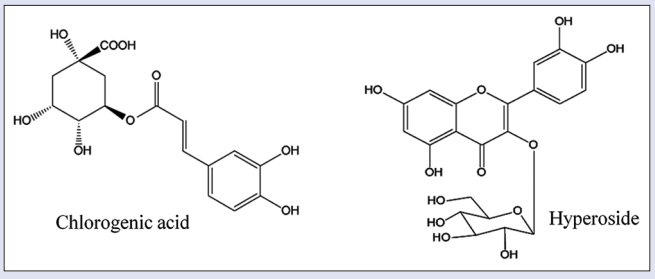

Figure 1: Chemical structures of chlorogenic acid and hyperoside

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

In the previous study, resolution of hyperoside and rutin is low. Therefore, we evaluated HPLC condition for improvement of resolution of hyperoside. Mobile phase system (acetonitrile-water, methanol-water, and acetonitrile and aqueous with 0.1% trifluoroacetic acid), gradient elution system, and column temperature (25°C, 30°C and 35°C) were tested to obtain good resolution.[15,16]

Acetonitrile and water were found to be effective for chromatographic separation, and trifluoroacetic acid was used for the inhibition of compounds and improvement of peak shape. Gradient elution system was proposed to achieve good separation. Overall, gradient elution system was varied as follows: 0–5 min, 5% acetonitrile; and 5–40 min, 5–50% acetonitrile based on previous study. Flow rate of 1.0 ml/min and column temperature of 30°C were selected. The detection UV wavelength of 254 nm was selected according to the wavelength of two compounds.

We evaluated resolution value (R) of hyperoside using the retention time (t_{R1} and t_{R2}) and the widths (W_1 and W_2) of two peaks. $R \geq 1.5$ represents good peak resolution in many cases. Resolution value of hyperoside was 1.76.

HPLC chromatograms of standard compounds and *Crataegi fructus* sample were obtained without interfering peaks on optimized separation condition [Figure 2].

Method validation

Linearity, limits of detection, and limits of quantification

The calibration curves of chlorogenic acid and hyperoside were constructed by plotting the peak area versus six different concentrations of standard solutions. Limits of detection (LOD) and limits of quantification (LOQ) of two compounds were investigated at noise ratio of 3 and 10, respectively. The linear regression equations were present as Y = ax + b (a: The slope of the calibration curve, b: The intercept of calibration curve, x: Concentration and Y: Peak area of compound). Regression equations and correlation coefficients of chlorogenic

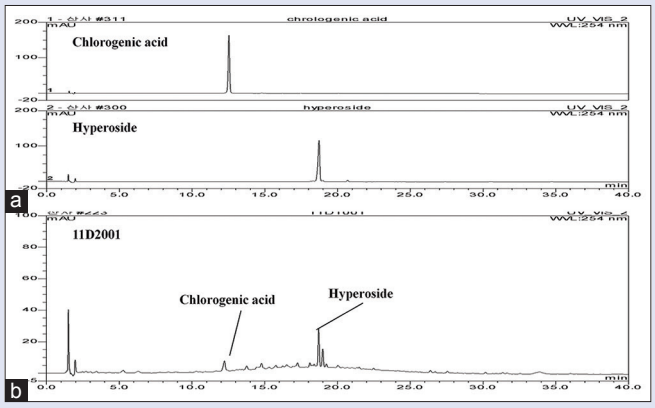

Figure 2: High-performance liquid chromatography-diode array detection chromatograms for chlorogenic acid and hyperoside in *Crataegi fructus*. (a) Standards, (b) *Crataegi fructus* sample

acid and hyperoside were Y = 0.0857x + 0.1784 (R^2 = 0.9995) and Y = 0.1669x + 0.349 (R^2 = 0.9986), respectively. The LOD of chlorogenic acid and hyperoside were 0.26 μg/ml and 0.04 μg/ml, respectively, and the LOQ were found to be 0.78 μg/ml and 0.12 μg/ml for chlorogenic acid and hyperoside, respectively [Table 1].

Results showed that this HPLC method has good linearity and high detection sensitivity for chlorogenic acid and hyperoside.

Precious and accuracy

The inter- and intra-day test with chlorogenic acid and hyperoside at three different concentrations were performed to investigate the precision of this HPLC method. Previously repeatability of this method was indicated and determined as relative standard deviation (RSD) values of inter- and intra-day. The intra-day test was continuously analyzed by injection standard solution 5 times on the same day and the inter-day test was analyzed for 3 consecutive days (1, 3, and 5 days).

The intra- and inter-day RSD values of chlorogenic acid and hyperoside were in the range from 0.05–1.02% to 0.62–2.49%, respectively [Table 2].

Accuracy of analysis method was determined as recovery test. Recovery was investigated by spike test. Three different concentrations of chlorogenic acid and hyperoside were added into the previously analyzed *Crataegi fructus* sample, and then the sample was analyzed in triplicate. Accuracy (%) was calculated by difference of spiked and unspiked sample.

The recovery value of two compounds ranged from 97.02% to 109.39% with RSD <1.17% [Table 3].

Quantitative analysis of chlorogenic acid and hyperoside in *Crataegi fructus* samples

To obtain the HPLC fingerprint of *Crataegi fructus*, 31 batches of *Crataegi fructus* from different areas were analyzed by optimized HPLC method. Peaks of chlorogenic acid and hyperoside are identified in all the samples. Among different peaks, chlorogenic acid and hyperoside have relatively high intensity peak in *Crataegi fructus* [Figure 3].

Relative retention time and relation peak area of chlorogenic acid and hyperoside peak were calculated [Table 4].

This HPLC method was applied for the quantitative determination of chlorogenic acid and hyperoside in 31 *Crataegi fructus* samples using calibration curve of two compounds. Among *Crataegi fructus* sample, 15 species were collected from Korea and 16 species were collected from China. The content results of chlorogenic acid and hyperoside were shown in Table 5.

The content (w/w %) of chlorogenic acid in *Crataegi fructus* ranges were as follows: 0.0129–0.1065% (Korea) and 0.0121–0.1125% (China). The contents of hyperoside ranged from 0.0150–0.0660% (Korea) to 0.0204–0.0560% (China). The average content of chlorogenic acid in

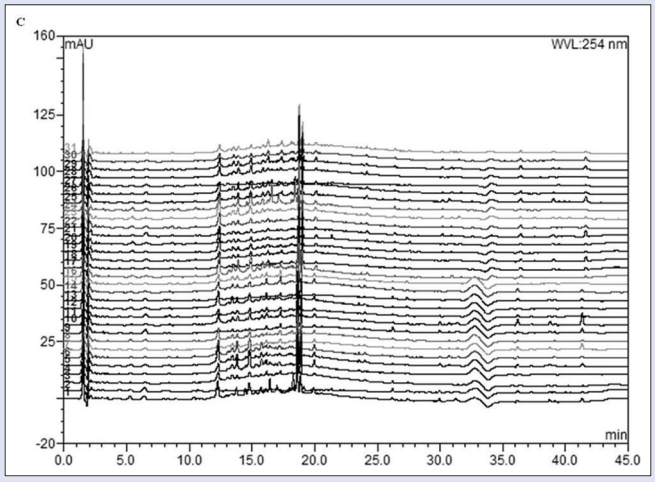

Figure 3: Chromatographic fingerprints for 31 *Crataegi fructus* samples

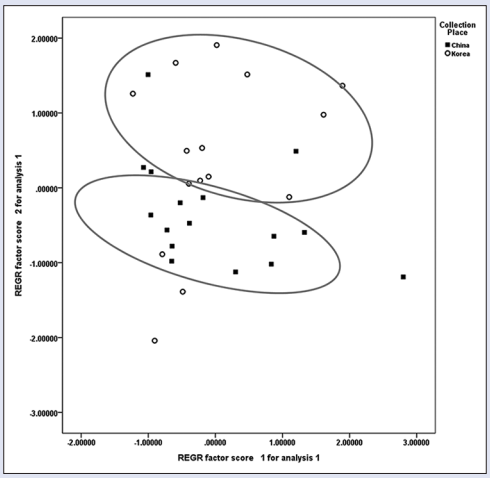

Figure 4: Principal components analysis projection of 31 *Crataegi fructus* samples. ■: *Crataegi Fructus* sample collected from China, ○: *Crataegi fructus* sample collected from Korea

Table 1: Regression equation, correlation coefficient (R^2), limits of detection, and limits of quantitation

Compound	Linear range (μg/ml)	Regression Equation	R^2 (n=6)	LOD (μg/ml)	LOQ (μg/ml)
Chlorogenic acid	4.38–70.00	Y=0.0857x+0.1784	0.9995	0.26	0.78
Hyperoside	4.06–65.00	Y=0.1669x+0.349	0.9986	0.04	0.12

LOD: Limits of detection; LOQ: Limits of quantitation

Table 2: Analytical results of intra- and inter-day variability

Compound	Concentration (μg/ml)	Intra-day (n = 5)			Inter-day (n = 5)		
		Mean ± SD (μg/ml)	RSD (%)	Accuracy (%)	Mean ± SD (μg/ml)	RSD (%)	Accuracy (%)
Chlorogenic acid	35.00	32.92 ± 0.22	0.66	94.06	33.72 ± 0.79	2.35	96.35
	17.50	16.05 ± 0.16	1.02	91.70	15.85 ± 0.16	1.00	90.58
	8.75	7.89 ± 0.04	0.56	90.13	8.01 ± 0.05	0.67	91.56
Hyperoside	32.50	31.53 ± 0.05	0.05	97.02	32.23 ± 0.80	2.49	99.18
	16.25	14.93 ± 0.02	0.15	91.86	16.00 ± 0.35	2.19	98.49
	8.13	7.41 ± 0.01	0.10	91.21	7.31 ± 0.05	0.62	90.02

SD: Standard deviation; RSD: Relative standard deviation

Crataegi fructus collected from Korea (0.0477%) higher than that in China sample (0.0399%). Moreover, the average content of hyperoside in *Crataegi fructus* collected from Korea (0.0450%) was higher than that in China sample (0.0325%).

Table 3: Results of recovery test

Compound	Spiked amount (μg/ml)	Measured amount (μg/ml)	RSD (%)	Recovery (%)
Chlorogenic acid	35.00	35.53±0.19	0.53	101.52
	17.50	17.04±0.14	0.83	97.37
	8.75	8.86±0.05	0.51	101.25
Hyperoside	21.67	23.69±0.15	0.64	97.02
	10.83	11.85±0.14	1.17	109.39
	5.42	5.87±0.06	0.95	108.33

RSD: Relative standard deviation

Table 4: Relative retention time and relation peak area of the chlorogenic acid and hyperoside in *Crataegi Fructus*

Compound	Retention time (min)	RRT	RPA
Chlorogenic acid	12.36	0.967	0.997
Hyperoside	18.86	0.913	0.910

RRT: Relative retention time; RPA: Relation peak area

The difference of contents in *Crataegi fructus* was due to cultivation environment including the year of the plant cultivation, harvest time, plant origins, climate, and cultivated location.

Principal components analysis (PCA) was conducted by scatterplot for quantitative evaluation. Results of PCA showed that the contents of *Crataegi fructus* collected from Korea and China were different by two developed clusters [Figure 4].

We evaluated reproducibility of this HPLC method by cross-monitoring with other researchers. Cross-monitor was performed using some *Crataegi fructus* samples and compared to the content results of chlorogenic acid and hyperoside of other researchers. Reproducibility is determined from the difference between two test results obtained from same HPLC conditions. However, production of HPLC system and column is different. The results are listed in Table 6 and there was no difference between the contents of chlorogenic acid and hyperoside in other researchers [Figure 5].

The results of cross-monitor indicated that our HPLC method had a good reproducibility.

CONCLUSION

In this study, we developed quantitative analysis method of chlorogenic acid and hyperoside in *Crataegi fructus* and validated with linearity, precision, and accuracy. Results of validation indicated that this HPLC method was accurate and stable for the analysis of *Crataegi fructus*.

Table 5: Contents (μg/mg) of the chlorogenic acid and hyperoside in the 30 *Crataegi Fructus*

Number	Species	Collection Place	Content (w/w%)			
			Chlorogenic acid		Hyperoside	
			Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
11D2001	*C. pinnatifida*	Korea	0.0575 ± 0.0001	0.1509	0.0660 ± 0.0002	0.3374
11D2002	*C. pinnatifida*	Korea	0.0129 ± 0.0002	1.3330	0.0212 ± 0.0001	0.3349
11D2024	*C. pinnatifida*	Korea	0.0407 ± 0.0001	0.2565	0.0447 ± 0.0001	0.0752
11D2027	*C. pinnatifida*	Korea	0.0008 ± 0.0001	12.8378	0.0036 ± 0.0001	0.8172
12D3017	*C. pinnatifida*	Korea	0.0339 ± 0.0008	2.2902	0.0432 ± 0.0002	0.4058
12D3018	*C. pinnatifida*	Korea	0.0177 ± 0.0008	4.3347	0.0150 ± 0.0004	2.3626
12D3019	*C. pinnatifida*	Korea	0.0365 ± 0.0016	4.4143	0.0381 ± 0.0032	8.3023
12D3020	*C. pinnatifida*	Korea	0.0955 ± 0.0034	3.5614	0.0586 ± 0.0025	4.2608
12D3021	*C. pinnatifida*	Korea	0.0170 ± 0.0003	2.0472	0.0513 ± 0.0002	0.3165
12D3022	*C. pinnatifida*	Korea	0.1065 ± 0.0002	0.1930	0.0655 ± 0.0001	0.0958
12D3023	*C. pinnatifida*	Korea	0.0725 ± 0.0008	1.1144	0.0402 ± 0.0000	0.0837
12D3024	*C. pinnatifida*	Korea	0.0674 ± 0.0080	11.8288	0.0620 ± 0.0082	13.2434
12D3025	*C. pinnatifida*	Korea	0.0383 ± 0.0079	20.5923	0.0600 ± 0.0114	18.9926
12D3026	*C. pinnatifida*	Korea	0.0314 ± 0.0019	6.0345	0.0368 ± 0.0006	1.6618
12D3027	*C. pinnatifida*	Korea	0.0405 ± 0.0004	1.0013	0.0394 ± 0.0001	0.2681
Mean			0.0477 ± 0.0017	4.4836	0.0450 ± 0.0012	2.3878
12D3001	*C. pinnatifida*	China	0.0753 ± 0.0009	1.2617	0.0341 ± 0.0002	0.6131
12D3002	*C. pinnatifida*	China	0.0121 ± 0.0012	10.1521	0.0283 ± 0.0021	7.4884
12D3003	*C. pinnatifida*	China	0.0139 ± 0.0006	4.3536	0.0373 ± 0.0002	0.5682
12D3004	*C. pinnatifida*	China	0.0276 ± 0.0007	2.6444	0.0290 ± 0.0004	1.3112
12D3005	*C. pinnatifida*	China	0.0258 ± 0.0016	6.2941	0.0325 ± 0.0012	3.8237
12D3006	*C. pinnatifida*	China	0.0162 ± 0.0014	8.5103	0.0204 ± 0.0021	10.4409
12D3007	*C. pinnatifida*	China	0.0167 ± 0.0003	1.9890	0.0369 ± 0.0008	2.1406
12D3008	*C. pinnatifida*	China	0.0801 ± 0.0036	4.5450	0.0497 ± 0.0028	5.5924
12D3009	*C. pinnatifida*	China	0.1125 ± 0.0004	0.3935	0.0312 ± 0.0001	0.4089
12D3010	*C. pinnatifida*	China	0.0360 ± 0.0017	4.8581	0.0349 ± 0.0019	5.3955
12D3011	*C. pinnatifida*	China	0.0254 ± 0.0004	1.4704	0.0560 ± 0.0002	0.2687
12D3012	*C. pinnatifida*	China	0.0174 ± 0.0006	3.4547	0.0263 ± 0.0002	0.8685
12D3013	*C. pinnatifida*	China	0.0620 ± 0.0004	0.7211	0.0315 ± 0.0002	0.5474
12D3014	*C. pinnatifida*	China	0.0179 ± 0.0002	0.9555	0.0234 ± 0.0007	2.8732
12D3015	*C. pinnatifida*	China	0.0421 ± 0.0005	1.0901	0.0221 ± 0.0010	4.5483
12D3016	*C. pinnatifida*	China	0.0580 ± 0.0009	1.6060	0.0258 ± 0.0003	1.0983
Mean			0.0399 ± 0.0010	3.3937	0.0325 ± 0.0009	2.9992

SD: Standard deviation; RSD: Relative standard deviation; *C. pinnatifida*: *Crataegus pinnatifida*

Table 6: Results of cross-monitoring

Number	Lab A - content (w/w, %)		Number	Lab B - content (w/w, %)	
	Chlorogenic acid	Hyperoside		Chlorogenic acid	Hyperoside
11D2001	0.0575	0.0660	11D2001-1	0.0664	0.0672
11D2002	0.0129	0.0212	11D2002-1	0.0148	0.0235
11D2003	0.0231	0.0406	11D2003-1	0.0253	0.0435
11D2004	0.0209	0.0394	11D2004-1	0.0241	0.0391
11D2005	0.0674	0.0226	11D2005-1	0.0613	0.0237
11D2006	0.0488	0.0263	11D2006-1	0.0546	0.0278
11D2007	0.0160	0.0201	11D2007-1	0.0184	0.0230
11D2008	0.0371	0.0175	11D2008-1	0.0416	0.0189
11D2009	0.0042	0.0299	11D2009-1	0.0061	0.0322
11D2010	0.0257	0.0391	11D2010-1	0.0267	0.0431
12D3001	0.0752	0.0341	12D3001-1	0.0646	0.0357
12D3002	0.0339	0.0432	12D3002-1	0.0316	0.0403
12D3003	0.0177	0.0150	12D3003-1	0.0181	0.0137
12D3004	0.0121	0.0283	12D3004-1	0.0155	0.0293
12D3005	0.0365	0.0381	12D3005-1	0.0368	0.0420
12D3006	0.0955	0.0586	12D3006-1	0.0989	0.0607
12D3007	0.0139	0.0373	12D3007-1	0.0153	0.0360
12D3008	0.0170	0.0513	12D3008-1	0.0200	0.0482
12D3009	0.1065	0.0655	12D3009-1	0.0959	0.0642
12D3010	0.0275	0.0290	12D3010-1	0.0248	0.0280

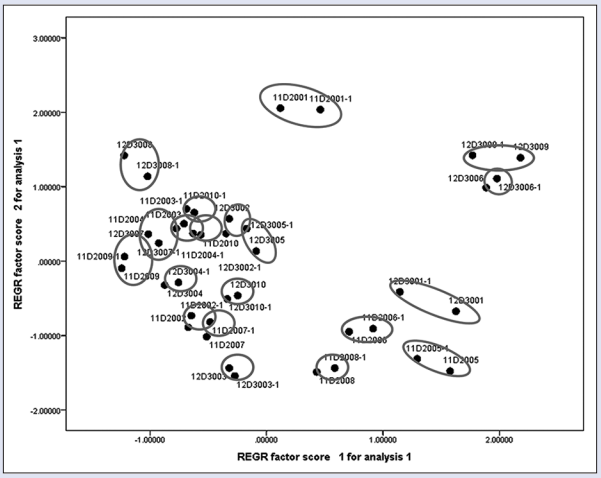

Figure 5: Principal components analysis projection of cross-monitoring of *Crataegi Fructus* samples

Moreover, the developed method was successfully applied for quantitative analysis of *Crataegi fructus* sample collected from Korea and China.

The results of validation and *Crataegi fructus* sample analysis could be used as a reference for monitoring and quality control of *Crataegi fructus*.

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Nil.

Conflicts of Interest

The authors declare no conflicts of interest.

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Choong Je Ma

ABOUT AUTHOR

Choong Je Ma, has completed his PhD at the age of 32 years from Seoul National University and postdoctoral studies from the University of Michigan. He is the professor of Department of Medical Biomaterials Engineering, College of Biomedical science, Kangwon National University, Korea. He has published more than 20 papers in reputed journals.