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Quantitative Analysis of Ursolic Acid and Euscaphic Acid in Chaenomelis Fructus by HPLC-Evaporative Light Scattering Detection

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A fruit of *Chaenomeles sinensis* (Chaenomelis Fructus), which is known as "Mo-Gua" in Korea, is one of the most important drugs in traditional medicines used to treat throat diseases.[1] The main chemical compositions of Chaenomelis Fructus include essential oil, flavonoids, proanthocyanidins, tannins, and triterpenes.[2,3] Among these components, ursolic acid is the most abundant triterpene acid in Chaenomelis Fructus and has various pharmacological activities including antioxidant, anti-tumor, and anti-dementia effects.[4,5] Euscaphic acid, another triterpene acid present in several plants, has been reported to exert anti-inflammatory and anti-diabetic activities.[6-8]

In general, the chemical composition of traditional herbal medicines is complex and the quantities of active or marker compounds are variable depending on multiple factors such as environmental conditions, harvest period, storage time, and processing method.[9] Thus, the quality control method is important. The quality is currently predicted by analysis of one or more selected marker compounds in herbal medicines.[10,11] Sometimes, the isolation of maker compounds from traditional herbal medicines is a prerequisite in quality control since most of these compounds are not commercially available.[12,13] In this study, ursolic acid and euscaphic acid (Figure 1) were isolated from the ethanolic extract of Chaenomelis Fructus and used as marker compounds for quality control of Chaenomelis Fructus products.

As most of the triterpenoids lack a UV chromophore, the analysis is often performed by UV detection at a very low wavelength below 210 nm or by LC-mass spectrometry.[14,15] Evaporative light scattering detection (ELSD) is an excellent option when analyzing samples having no or very low UV absorption by HPLC. Some studies on the HPLC-ELSD analysis of triterpenoids in plants including Chaenomelis Fructus have been reported.[16,17] Yang *et al.* reported HPLC-ELSD determination of seven triterpenoids from *Chaenomeles sinensis* collected in several areas of China.[16] However, there are no reports on the detection and analysis of euscaphic acid in Chaenomelis Fructus. In this study, the simultaneous determination of ursolic acid and euscaphic acid as marker compounds to ensure the quality of Chaenomelis Fructus was performed by HPLC-ELSD. The method was validated and applied to assess the quality of Chaenomelis Fructus cultivated in different sites.

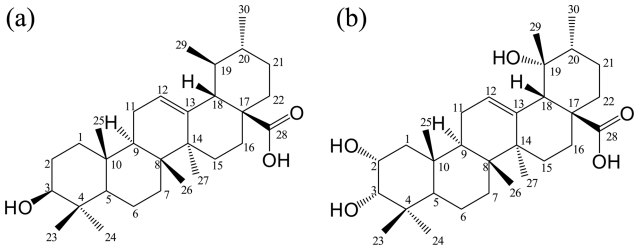

Figure 1. Chemical structures of ursolic acid (a) and euscaphic acid (b).

From the ethanolic extract of Chaenomelis Fructus, ursolic acid and euscaphic acid as marker compounds were isolated with purity over 99.0%. The purity was determined by HPLC method developed in this study, as described in Experimental Section. The structural identities of ursolic acid and euscaphic acid were verified by comparison of ^{1}H- and ^{13}C-NMR data (Table S1) with previously published spectral data.[18]

HPLC conditions for efficiently separating and detecting ursolic acid and euscaphic acid were optimized after several trials with mobile phases of acetonitrile-water or methanol-water containing trifluoroacetic acid (TFA) in various proportions. Among C-4 and C-18 reversed-phase columns, the C-4 column was chosen because ursolic acid showed strong interaction with the C-18 column. Figure 2 shows the HPLC chromatograms of euscaphic acid and ursolic acid obtained by optimized gradient elution with a mobile phase consisting of acetonitrile-water containing 0.1% TFA. In the optimal HPLC condition, euscaphic acid and ursolic acid showed retention times of 7.4 min and 17.1 min, respectively, with good resolution and satisfactory peak shape. The peaks of euscaphic acid and ursolic acid were also well separated from other peaks found in the extracts of Chaenomelis Fructus, as shown in Figure 2(c).

The HPLC method for determination of ursolic acid and euscaphic acid from the extracts of Chaenomelis Fructus was validated for linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ). Linear calib-

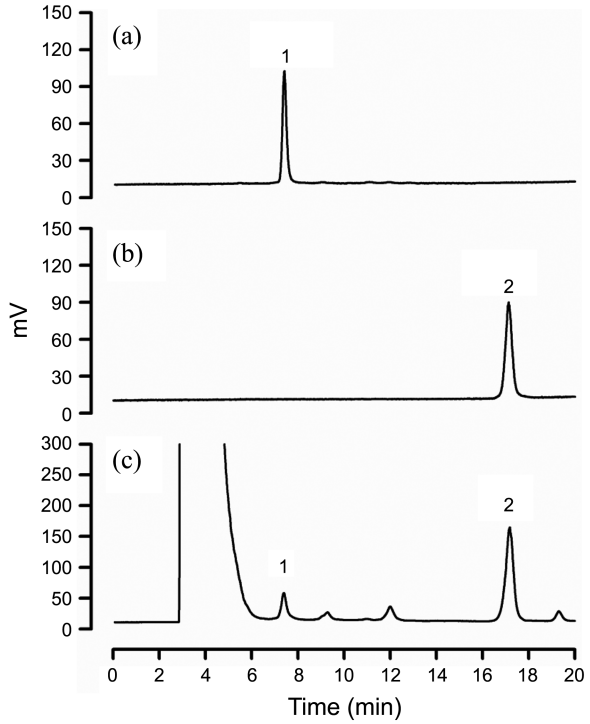

Figure 2. HPLC chromatograms of euscaphic acid standard (a), ursolic acid standard (b) and Chaenomelis Fructus extract (c). Peak 1: euscaphic acid, 2: ursolic acid.

ration curves were constructed from three assays for each standard compound (ursolic acid and euscaphic acid). The regression equation was calculated in the form of y = ax + b, in where y and x correspond to the logarithms of peak area and concentration of each standard compound, respectively (Table 1). Each correlation coefficient (r^2) was > 0.998, as determined by least square linear regression method, suggesting good linearity between the peak areas and the compound concentrations. The LOD values of ursolic acid and euscaphic acid were 0.5 and 0.6 μg/mL, respectively. With ursolic acid at a concentration of 10 μg/mL, the intra- and inter-day precisions were 0.90% and 1.67%, respectively, and the intra- and inter-day accuracies were 99.5% and 98.4%, respectively. With euscaphic acid at a concentration of 10 μg/mL, the intra- and inter-day precisions were 0.80% and 6.13%, respectively, and the intra- and inter-day accuracies were 101.5% and 96.8%, respectively. These results indicate that the developed HPLC method is accurate and precise for the determination of ursolic acid and euscaphic acid in the extracts of Chaenomelis Fructus.

The validated HPLC method was used to analyze two marker compounds, ursolic acid and euscaphic acid, in ten samples collected from different locations in Korea (Table 2). In all ten samples, the contents of ursolic acid were higher than those of euscaphic acid. While the mean contents of ursolic acid in dried Chaenomelis Fructus were 0.364-0.557%, the contents of euscaphic acid were 0.083-0.162%. Total contents of the two marker compounds ranged from 0.481% to 0.679%. As both ursolic acid and euscaphic acid were detected in sufficient quantity, this study indicates that these two marker compounds can be utilized for the quality control of Chaenomelis Fructus. In the previous studies, ursolic acid and oleanolic acid have been used as major marker compounds of Chaenomelis Fructus.[14-16] Although euscaphic acid has been identified as one of components in Chaenomelis Fructus, there are no reports on the use of euscaphic acid as a marker compound in this plant.[2,19] To the best of our knowledge, this study is the first report on the use of euscaphic acid as a marker for quality control of Chaenomelis Fructus products.

Table 2. Contents of ursolic acid and euscaphic acid in the samples of Chaenomelis Fructus collected from different cultivation sites in Korea

Cultivation area	Ursolic acid (%)[a]	Euscaphic acid (%)[a]
Jeongseon	0.528±0.001[b]	0.139±0.002
Goryeong	0.492±0.001	0.149±0.001
Gyeongsan	0.398±0.001	0.083±0.000
Hamyang	0.376±0.004	0.117±0.000
Jecheon	0.557±0.001	0.122±0.001
Uiseong	0.460±0.002	0.117±0.001
Yeongcheon 1	0.402±0.002	0.112±0.001
Yeongcheon 2	0.481±0.002	0.162±0.001
Yeongju	0.494±0.002	0.129±0.000
Yeosu	0.364±0.001	0.152±0.000

[a]% of dried Chaenomelis Fructus. [b]Mean ± standard deviation of triplicate experiments.

In conclusion, a HPLC-ELSD method for simultaneous determination of two marker compounds, ursolic acid and euscaphic acid, in the extracts of Chaenomelis Fructus was successfully validated with respect to specificity, precision and accuracy. The contents of these two marker compounds determined by the validated HPLC method could be used to assess the quality of Chaenomelis Fructus.

Experimental Section

Materials and Reagents. Dried Chaenomelis Fructus

Table 1. Linear regression, precision and accuracy for the determination of ursolic acid and euscaphic acid

Analyte	Regression equation	Correlation coefficient (r^2)	LOD[a] (μg/mL)	LOQ[b] (μg/mL)	Precision (%)		Accuracy (%)	
					Intra-day	Inter-day	Intra-day	Inter-day
Ursolic acid	y = 1.3651x − 0.223	0.9981	0.5	2.0	0.90	1.67	99.5	98.4
Euscaphic acid	y = 1.344x − 0.4745	0.9985	0.6	2.0	0.80	6.13	101.5	96.8

[a]LOD: Limit of detection. [b]LOQ: Limit of quantitation.

samples from ten different habitats were obtained from herbal medicine markets in Daegu and Seoul, Korea. A voucher specimen was deposited at the Laboratory of Natural Products Medicine, College of Pharmacy, Kyungpook National University. Ethanol, dichloromethane, ethyl acetate, *n*-butanol, and methanol were obtained from Duksan Chemical (Anseong, Korea). Acetonitrile (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA). TFA was supplied from Sigma-Aldrich (St. Louis, MO, USA). Column chromatography was carried out using Kieselgel 60 (Merck, NJ, USA) and C-18 (Yamazen, Ultra Pack ODS-S-50B, Japan). ^{1}H- and ^{13}C-NMR analyses were performed with a Brucker Avance Digital 400 NMR spectrometer (Karlsruhe, Germany) at 400 and 100 MHz, respectively. Chemical shifts were given in δ (ppm) from tetramethylsilane. Purified water was obtained by the Millipore-Q Water system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade and used as obtained commercially.

Isolation of Marker Compounds. Dried Chaenomelis Fructus (2.7 kg) was extracted with 95% ethanol for 3 h in three replicates. After filtration, ethanolic extract (195.0 g) was dispersed into 2 L of water and extracted by dichloromethane. The dichloromethane-soluble fraction (29.2 g) was loaded onto silica gel column chromatography (5.5 × 45 cm) using a mixed solvent of dichloromethane-acetone-methanol (200:1:1→1:1:1, gradient elution) to afford fractions of I-IV. Fraction II was precipitated using a mixed solvent of dichloromethane-methanol to yield ursolic acid as a white powder (1.02 g). Fraction IV (2 g) was chromatographed on reversed-phase C-18 (2.6 × 30 cm) with 60-100% methanol and the obtained subfraction IV-II (195 mg) was chromatographed on silica gel column chromatography (3.5 × 50 cm) using a mixed solvent of dichloromethane and acetone (30:1→6:1, gradient elution) to afford euscaphic acid (72.8 mg). The structures of ursolic acid and euscaphic acid were identified by ^{1}H- and ^{13}C-NMR (Table S1).

Preparation of Standard Solutions. Each standard of ursolic acid and euscaphic acid was accurately weighed, dissolved in methanol, and diluted to the appropriate concentration. Stock solution of standard containing ursolic acid or euscaphic acid at 1 mg/mL was prepared. A set of each standard solution was prepared by diluting the stock solution with methanol to concentrations ranging from 2 to 20 μg/mL. All solutions were filtered through a 0.45-μm syringe filter and stored in the refrigerator at 4 °C before analysis.

Preparation of Samples. Each 50 g sample of dried Chaenomelis Fructus cultivated in ten different locations in Korea was refluxed with 100 mL of 95% ethanol for 3 h. After filtration through a 0.45-μm membrane filter, the filtrate was injected onto the HPLC for the quantitative determination of ursolic acid or euscaphic acid in the extracts.

HPLC Conditions. HPLC analysis was performed using a HPLC system consisting of Dionex Ultimate 3000 binary pump, automated sample injector, thermostatted column compartment, and Agilent 380-ELSD with Alltech Prosphere 300 C-4 column (250 × 4.6 mm id, 5 μm). The mobile phase was composed of deionized water containing 0.1% TFA (mobile phase A) and acetonitrile containing 0.1% TFA (mobile phase B). A linear gradient elution was performed from 40% to 70% B for 20 min at a flow rate of 1.0 mL/min. The evaporator temperature for the ELSD was set at 60 °C with the nebulizing gas flow-rate of 1.2 L/min. The sample injection volume was 20 μL.

Validation of HPLC Method. Calibration curves of ursolic acid and euscaphic acid were prepared with standards at concentrations ranging from 2 to 20 μg/mL. The regression equations were calculated in the form of $y = ax + b$, where y and x correspond to the peak area and concentration, respectively. The precision and accuracy were determined by analyzing each standard sample at a concentration of 10 μg/mL. Intra-day precision was determined by repeating the analysis of each standard sample five times in a single day, and inter-day precision and accuracy were determined by repeating the analysis on three consecutive days. LOD and LOQ were defined as the minimum concentration at the signal-to-noise ratio (S/N) = 3 and 10, respectively.

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References

1. Sawai, R.; Kuroda, K.; Shibata, T.; Gomyou, R.; Osawa, K.; Shimizu, K. *J. Ethnopharmacol.* **2008**, *118*, 108.
2. Roh, S. B.; Chang, E. H.; Im, K. S. *Yakhak Hoeji* **1995**, *39*, 610.
3. Du, H.; Wu, J.; Li, H.; Zhong, P. X.; Xu, Y. J.; Li, C. H.; Ji, K. X.; Wang, L. S. *Food Chem.* **2013**, *141*, 4260.
4. Aggarwal, B. B.; Shishodia, S.; Majumdar, S.; Banerjee, S. *Cancer Res.* **2003**, *63*, 4375.
5. Tsai, S. J.; Yin, M. C. *J. Food Sci.* **2008**, *73*, 174.
6. Jung, H. J.; Nam, J. H.; Choi, J.; Lee, K. T.; Park, H. J. *Biol. Pharm. Bull.* **2005**, *28*, 101.
7. Chen, J.; Li, W. L.; Wu, J. L.; Ren, B. R.; Zhang, H. Q. *Pharmazie* **2008**, *63*, 765.
8. Kim, I. T.; Ryu, S.; Shin, J. S.; Choi, J. H.; Park, H. J.; Lee, K. T. *J. Cell Biochem.* **2012**, *113*, 1936.
9. Hendriks, M. M. W. B.; Cruz-Juarez, L.; De Bont, D.; Hall, R. D. *Anal. Chim. Acta* **2005**, *545*, 53.
10. Zhao, B. T.; Jeong, S. Y.; Moon, D. C.; Son, K. H.; Son, J. K.; Woo, M. H. *Arch. Pharm. Res.* **2012**, *35*, 1449.
11. Li, Y.; Piao, D.; Zhang, H.; Woo, M. H.; Lee, J. H.; Moon, D. C.; Lee, S. H.; Chang, H. W.; Son, J. K. *Arch. Pharm. Res.* **2013**, *36*, 335.
12. Lee, M. K.; Ahn, Y. M.; Lee, K. R.; Jung, J. H.; Jung, O. S.; Hong, J. *Anal. Chim. Acta* **2009**, *633*, 271.
13. Goo, H. R.; Choi, J. S.; Na, D. H. *Arch. Pharm. Res.* **2010**, *33*, 539.
14. Fang, X.; Wang, J.; Yu, X.; Zhang, G.; Zhao, J. *J. Sep. Sci.* **2010**, *33*, 1147.
15. Chen, Q.; Zhang, Y.; Zhang, W.; Chen, Z. *Biomed. Chromatogr.* **2011**, *25*, 1381.
16. Yang, G.; Fen, W.; Lei, C.; Xiao, W.; Sun, H. *J. Chromatogr. Sci.* **2009**, *47*, 718.
17. Lesellier, E.; Destandau, E.; Grigoras, C.; Fougère, L.; Elfakir, C. *J. Chromatogr. A* **2012**, *1268*, 157.
18. Seebacher, W.; Simic, N.; Weis, R.; Saf, R.; Kunert, O. *Magn. Reson. Chem.* **2003**, *41*, 636.
19. Gao, H.; Wu, L.; Kuroyanagi, M.; Harada, K.; Kawahara, N.; Nakane, T.; Umehara, K.; Hirasawa, A.; Nakamura, Y. *Chem. Pharm. Bull.* **2003**, *51*, 1318.