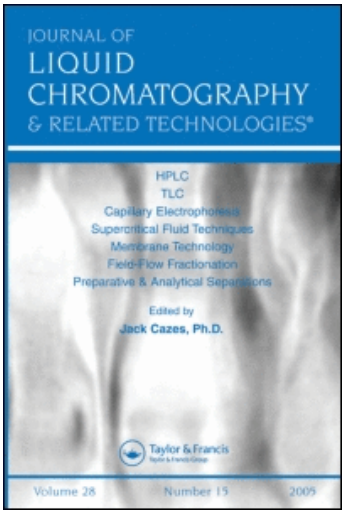

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Seung Hyun Kim[a]; Mi Kyeong Lee[b]; Mi-Jeong Ahn[c]; Ki Yong Lee[d]; Young Choong Kim[d]; Sang Hyun Sung[d]

[a] Institute for Life Science, ElcomScience Co. Ltd., Seoul, Korea [b] College of Pharmacy, Chungbuk National University, Cheongju, Korea [c] College of Life Science and Natural Resources, Jinju National University, Jinju, Korea [d] College of Pharmacy, Seoul National University, Seoul, Korea

To cite this Article Kim, Seung Hyun , Lee, Mi Kyeong , Ahn, Mi-Jeong , Lee, Ki Yong , Kim, Young Choong and Sung, Sang Hyun(2009) 'Efficient Method for Extraction and Simultaneous Determination of Active Constituents in *Cornus officinalis* by Reflux Extraction and High Performance Liquid Chromatography with Diode Array Detection', Journal of Liquid Chromatography & Related Technologies, 32: 6, 822 — 832

To link to this Article: DOI: 10.1080/10826070902768021

URL: http://dx.doi.org/10.1080/10826070902768021

Journal of Liquid Chromatography & Related Technologies®, 32: 822–832, 2009

ISSN: 1082-6076 print/1520-572X online
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Efficient Method for Extraction and Simultaneous Determination of Active Constituents in *Cornus officinalis* by Reflux Extraction and High Performance Liquid Chromatography with Diode Array Detection

Seung Hyun Kim,[1] Mi Kyeong Lee,[2] Mi-Jeong Ahn,[3] Ki Yong Lee,[4] Young Choong Kim,[4] and Sang Hyun Sung[4]

[1]Institute for Life Science, ElcomScience Co. Ltd., Seoul, Korea
[2]College of Pharmacy, Chungbuk National University, Cheongju, Korea
[3]College of Life Science and Natural Resources, Jinju National University, Jinju, Korea
[4]College of Pharmacy, Seoul National University, Seoul, Korea

Abstract: A high performance liquid chromatography diode array detector (HPLC-DAD) based method was developed for the simultaneous determination of the three active constituents, morroniside, loganin, and gallic acid in *Cornus officinalis* fruits. The separation was achieved on a C_{18} column with a gradient solvent system of acetonitrile, methanol, and 0.1% acetic acid at 260 nm. The method was validated for linearity, repeatability, limits of detection, and limits of quantification. The analytes were confirmed by electrospray ionization mass spectrometry (ESI-MS). In addition, the effect of various parameters including solvent, extraction technique, and time on the extraction of the three active constituents from *C. officinalis* fruits was examined. The highest extraction efficiency was obtained with 100% methanol using reflux extraction. The developed method could be used to effectively and comprehensively evaluate the quality of *C. officinalis.*

Keywords: *Cornus officinalis*, Extraction, Gallic acid, Loganin, Morroniside, Simultaneous determination

Correspondence: Sang Hyun Sung, College of Pharmacy, Seoul National University, San 56-1, Sillim-Dong, Gwanak-Gu, Seoul 151-742, Korea. E-mail: shsung@snu.ac.kr

INTRODUCTION

Fructus Corni, the fruits of *Cornus officinalis* (Cornaceae), has been prescribed to nourish the liver and kidney, to increase vigor, and to treat chronic debility in traditional Oriental medicine.[1] Triterpenes,[2] tannins,[3] and iridoid glycosides[4] have been reported to be isolated from *C. officinalis*. Among these compounds, iridoid glycosides such as morroniside and loganin, and low molecular weight polyphenols showed an antidiabetic effect in streptozotocin induced diabetic rats.[5]

There are definitely issues regarding the quality control of herbal medicines that must be addressed to achieve reproducible phytoequivalence without undesirable effects. It is widely accepted that multiple constituents are responsible for the pharmacological and biological effects of herbal medicines.[6] Accordingly, it is necessary to quantitatively analyze the multibiocative compounds of herbal medicines. The conventional content determination of herbal medicines is to measure the known effective constituents or the index constituents. With the development of quality control techniques, the number of constituents determined in an herbal medicine is increased.[7] Wang et al. reported simultaneous determination of oleanolic acid and ursolic acid in *C. officinalis* by capillary electrophoresis.[2] Determination methods of loganin and gallic acid were also developed.[8,9] However, no method for the simultaneous analysis of the above mentioned bioactive constituents in *C. officinalis* has yet been reported.

The primary step in the qualitative and the quantitative analysis of herbal material is extraction. The solvent used provides the most obvious means of influencing the chemical composition of the extract. Extraction with low polarity solvents yields the more lipophilic components, while alcohols isolate a broader spectrum of nonpolar and polar compounds from the material. In addition to extraction solvent, the choice of extraction method may have a significant effect on the feature of the extract.

Therefore, morroniside, loanin, and gallic acid contained in the fruits of *C. officinlais* were simultaneously analyzed by a simple high performance liquid chromatography diode array detector (HPLC-DAD) in this study. The effects of extraction methods, solvents, and time were also evaluated by comparing the contents of these three compounds in various extracts.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a chromatographic pump (P680, Dionex, Germany) and an injector (7725i, Rheodyne, USA) equipped with a

photodiode array (UVD 340U, Dionex). The output signal of the detector was recorded using a Dionex Chromelon^{TM} Chromatography Data System. Chromatographic separation was achieved on a Shiseido Capcell Pak C18 MG (5 μm, 4.6 mm × 150 mm), using the mobile phase composed of acetonitrile (AcCN) – methanol (MeOH) – water with 0.1% acetic acid (10: 5: 85 v/v) at a flow rate of 1 mL/min, and monitored at 260 nm.

A Hewlett-Packard 1100 series HPLC system equipped with an autosampler, a column oven, a binary pump, and a degasser (Hewlett-Packard, Waldbronn, Germany) was used for HPLC electrospray ionization mass spectrometry (HPLC-ESI-MS). Separation was achieved at 20°C on Shiseido Capcell Pak C18 MG (5 μm, 4.6 mm × 150 mm) using the mobile phase composed of AcCN – MeOH – water with 0.1% acetic acid (10: 5: 85 v/v) at a flow rate of 0.5 mL/min. The eluent was detected with DAD at 210, 220, 250, 260, and 280 nm. The Chemstation software (Hewlett-Packard, Avondale, CA, USA) was used to operate this HPLC-DAD system. A post column microsplitter (Upchurch, WA, USA) was applied to restrict the flow to the mass spectrometer's source into 0.3 mL/min. All ESI-MS spectra were acquired using a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with a Finnigan electrospray source. The capillary temperatures were set to 250°C and 300°C for negative and positive modes, respectively.

Solvents and Chemicals

HPLC grade solvents (AcCN, MeOH, and water) and reagents were obtained from BDH chemicals (Poole, UK). Acetic acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Triply deionized water (Millipore, Bedford, MA, USA) was used for all preparations. A membrane filter (MF3-13 PTFE, diameter 13 mm, pore size 0.5 μm, Advantec, CA, USA) was used to filter each sample.

Morroniside, loganin, and gallic acid were isolated from the fruits of *C. officinalis* through extraction and several column chromatographies in our laboratory (Figure 1). The purified standard compounds were identified by comparison of the MS, ^{1}H-NMR, and ^{13}C-NMR data with the published ones. The purity was determined by HPLC-UV with two wavelengths (210 and 330 nm) and LC-MS, and was shown to be greater than 97%.

Plant Materials

A total of 16 samples of *C. officinalis* were purchased from Kyungdong traditional herbal market (Seoul, Korea). Among them, 8 samples were

collected in Korea. The other 8 samples were imported from China. The samples were authenticated by Prof. J. H. Park in the College of Pharmacy, Pusan National University, Korea.

Preparation of Standard Solutions and Samples

Stock solutions for morroniside, loganin, and gallic acid were prepared in HPLC grade MeOH at the concentration of 1 mg/mL, respectively. Working calibration solutions were prepared, appropriately, by successive serial dilution of the stock solution with MeOH.

The fruits of *C. officinalis* were ground into powder and lyophilized. The finely pulverized powder was weighed (2.0 g), and 100 mL of MeOH was added, and the mixture was extracted for 2 h at 90°C, using a reflux. The extract was then filtered with filter papers (Whatman No. 40) and evaporated in vacuo, followed by adding 50 mL of 50% MeOH. This sample solution was filtered through a 0.45 μm membrane filter (Millipore, Nylon, 170 mm) and analyzed with HPLC.

Comparison of Extraction Parameters

Comparison of reflux and ultrasonic extractions was carried out using aqueous MeOH. Three kinds of solvents in the proportions of MeOH, 50%, 75%, and 100% MeOH, were used to estimate the effects of the extraction solvent. The extraction time varied from 30 min to 2 h.

RESULTS AND DISCUSSION

Selection of HPLC-DAD Conditions

Reversed phase columns have been usefully applied to analyze the compounds of natural resources. The resolutions of morroniside, loganin, and gallic acid were tested and compared with the reversed phase conditions using a variety of analytical columns including Capcell Pak C18 UG 120 (5 μm, 4.5 mm × 250 mm), Capcell Pak C18 MG (5 μm, 4.5 mm × 150 mm), XTerra RP18 (5 μm, 4.5 mm × 150 mm), Luna C18 (5 μm, 4.5 mm × 150 mm), and Motor C18 (5 μm, 4.5 mm × 150 mm). The preferred chromatographic condition was found to be using the Capcell Pak MG (5 μm, 4.5 mm × 150 mm) column. Various mixtures of AcCN, MeOH, and water, in combination with acetic acid, were tested as a mobile phase. Acid is known to achieve better separation for phenolic compounds by reducing the tailing of the peaks. Under our

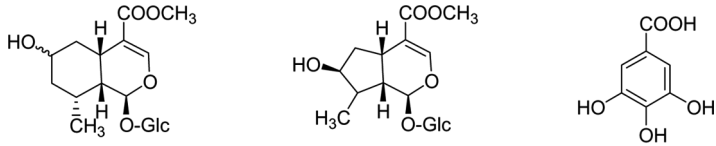


Figure 1. Chemical structures of morroniside, loganin and gallic acid.

chromatographic condition, the addition of 0.1% acetic acid in water increased the resolution of the peaks. Both morroniside and loganin showed maximum UV absorption at 240 nm. Gallic acid showed a strong UV absorption at 270 nm. For the simultaneous detection of these three compounds, the wavelength was set at 260 nm. The presence of the three compounds in the extract of *C. officinalis* was verified by comparing each retention time (Figure 2A).

Specificity

Specificity was determined by the calculation of peak purity facilitated by DAD. The peak purity was evaluated using DAD and its corresponding computer software, which confirms the singularity of the peak

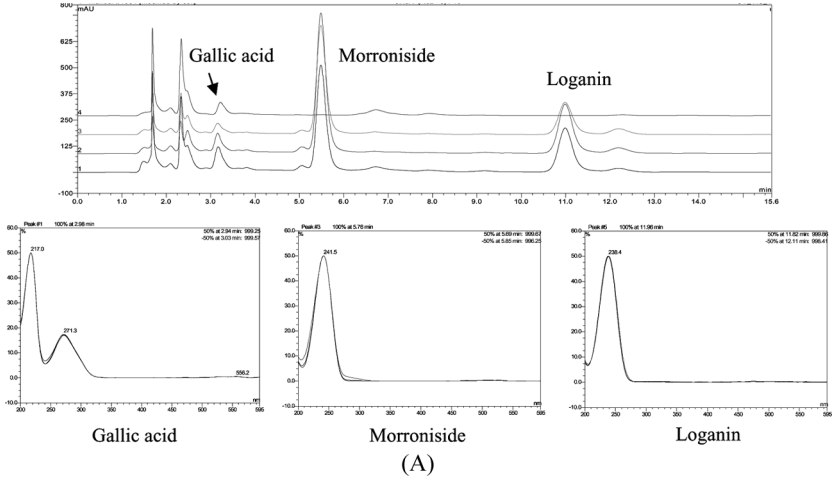
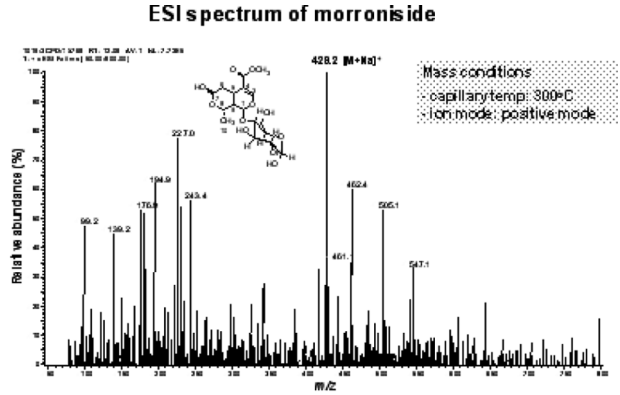
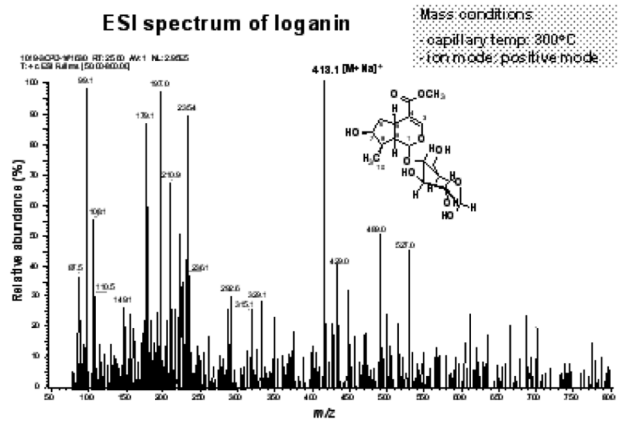


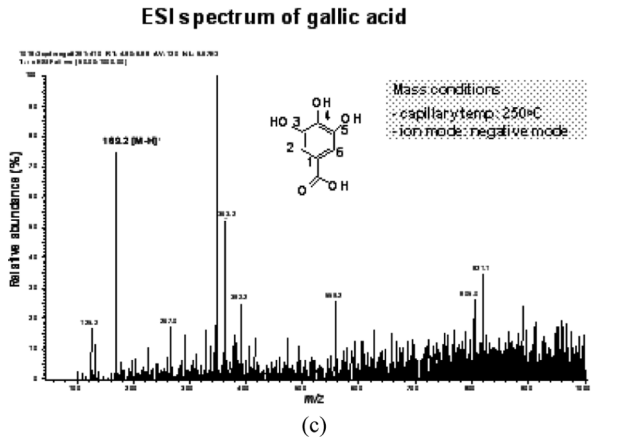
Figure 2. HPLC chromatogram of *C. officinalis* at four different wavelengths (A), and HPLC-EIS-MS spectrum of morroniside, loganin, and gallic acid in *C. officinalis* (B).



(a)



(b)



(c)

(B)

Figure 2. Continued.

component. The absorption spectrum of a single component remained invariable at each time point on the peak, which supported the specificity of each peak. In HPLC-ESI-MS spectra, the $[M + Na]^+$ or the $[M - H]^-$ molecular ions and fragmentation patterns of each compound were well matched with each chemical structure (Figure 2B). These results clearly showed the specificity of each peak for morroniside, loganin, and gallic acid, respectively.

Linearity and Range

Calibration curves were linear in a relatively wide range of concentrations from 1 μg/mL to 1000 μg/mL, and all showed good linear regressions with high correlation coefficient values between peak area (y) and amounts of each compound (x, μg) (Table 1).

Limits of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ were determined by means of serial dilution based on a signal to noise (S/N) ratio of 3:1 and 10:1, respectively. The LODs of morroniside, loganin, and gallic acid were 8.4, 41.7, and 0.4 ng, respectively, which showed a high sensitivity under this chromatographic condition (Table 1).

Precision

The precision test was carried out by the intraday and interday variability for morroniside, loganin, and gallic acid. The intraday variability was assayed at three concentrations on the same day and interday variability at three concentrations on three sequential days (1, 3, 5, days). The relative standard deviation (RSD) of intraday and interday variability

Table 1. Linear ranges and correlation coefficients of calibration curves

Compound	Range (μg/mL)	Regression equation[a]	Regression (r^2)	LOD (ng)	LOQ (ng)
Morroniside	1–1000	$y = 11.774x - 0.420$	0.9998	8.4	25.3
Loganin	1–1000	$y = 8.646x - 0.447$	0.9995	41.7	126.3
Gallic acid	1–1000	$y = 54.433x + 7.678$	0.9944	0.4	1.2

[a]y = peak area, x = amount (μg).

Table 2. Analytical results of precision

	Interday		Intraday	
Compound	Amount (μg)	RSD (%)[a]	Amount (μg)	RSD (%)[a]
Morroniside	1.0	1.98	2.0	1.82
	0.2	0.61	1.0	1.85
	0.1	2.21	0.2	1.30
Loganin	10.0	1.24	2.0	2.03
	5.0	1.46	1.0	2.33
	2.0	1.67	0.1	2.75
Gallic acid	10.0	0.06	2.0	1.13
	5.0	0.06	1.0	0.66
	2.0	0.36	0.2	2.43

[a]RSD (%) = (SD of amount detected/mean of amount detected) × 100 (n = 3).

was less than 3.0%, which demonstrated the good precision of this method (Table 2).

Effect of Extraction Parameters

Among various solvent extraction methods, reflux extraction is used widely in the extraction of herbal materials because of its convenience. The main advantages of reflux extraction are that it is an automatic and continuous method that does not require much manipulation. It has also been shown to be very effective in terms of extraction yield and, therefore, often used as a reference method for newer extraction methods. In ultrasonic extraction, ultrasound is used to induce a mechanical stress on the cells through the production of cavitations in herbal material. The cellular breakdown increases the solubilization of compounds in the solvent and improves extraction yields.[10] In our experimental conditions, the contents of iridoids, morroniside, and loganin, showed similar trends in the tested three extraction parameters. In ultrasonic extraction, the increases of time and MeOH proportion caused the rise in the contents of these two compounds. However, the yield of gallic acid was not significantly influenced by the MeOH proportion. Longer extraction time increased the contents of morroniside, loganin, and gallic acid in both ultrasonic extraction and reflux extraction. The reflux method more efficiently extracted, in a shorter time than with the ultrasonic method (Figure 3). Taken together, reflux extraction for 2 h using 100% MeOH was chosen for efficient extraction of *C. officinalis* in our experimental parameters.

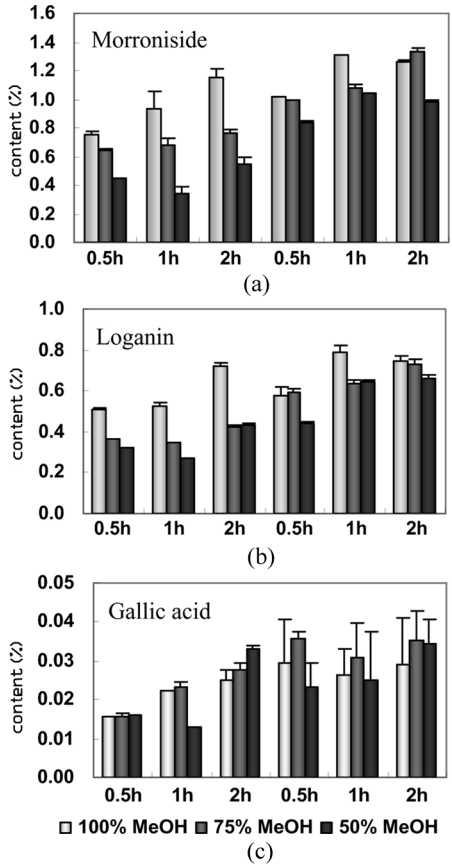


Figure 3. Contents of morroniside, loganin, and gallic acid at different extraction conditions.

Analysis of Commercial Samples by HPLC-DAD

The established method has been applied to the determination of morroniside, loganin, and gallic acid in the extraction of *C. officinalis*. Eight samples of Korean and Chinese *C. officinalis*, respectively, purchased from Kyungdong traditional herbal market were used for the determination of the three compounds. Samples were extracted with 100% MeOH for 2 h by the reflux method. There were significant differences in the contents of morroniside, loganin, and gallic acid among all sixteen samples. Furthermore, even the samples from the same countries, both Korea and China, showed big dissimilarities in the contents of the three active compounds (Table 3).

Table 3. Quantification of the contents (%) of morroniside, loganin and gallic acid in *C. officinalis* (n = 3)

Sample name	Morroniside	Loganin	Gallic acid
Korea			
K1	7.905 ± 0.072	5.975 ± 0.246	–[a]
K2	7.267 ± 0.077	5.716 ± 0.012	0.111 ± 0.004
K3	7.967 ± 0.084	4.385 ± 0.204	–[a]
K4	7.869 ± 0.016	4.015 ± 0.008	0.039 ± 0.005
K5	5.047 ± 0.036	1.091 ± 0.069	0.212 ± 0.002
K6	6.390 ± 0.283	2.018 ± 0.112	–[a]
K7	7.928 ± 0.117	4.691 ± 0.060	0.126 ± 0.009
K8	7.842 ± 0.009	2.172 ± 0.049	0.028 ± 0.013
China			
C1	9.192 ± 0.081	4.435 ± 0.055	0.039 ± 0.007
C2	8.049 ± 0.120	3.028 ± 0.135	0.094 ± 0.019
C3	7.922 ± 0.054	4.108 ± 0.187	–[a]
C4	7.087 ± 0.087	3.794 ± 0.087	0.225 ± 0.006
C5	5.420 ± 0.013	3.459 ± 0.074	0.122 ± 0.013
C6	7.822 ± 0.155	5.854 ± 0.403	0.018 ± 0.015
C7	7.714 ± 0.221	5.375 ± 0.055	0.112 ± 0.022
C8	8.510 ± 0.039	4.555 ± 0.207	–[a]

[a](–): The content was below detection limit.

CONCLUSIONS

In this paper, a rapid and reliable isocratic HPLC method for simultaneous determination of three active constituents of *C. officinalis*, morroniside, loganin, and gallic acid, has been developed and validated. The method fulfilled all the requirements to be identified as a reliable and feasible method, showing good specificity, precision, and linearity. Moreover, the effect of solvent, extraction technique, and time on the efficiency of the extraction of *C. officinalis* was examined. Therefore, this established method is useful for the quality control of *C. officinalis* by simultaneous quantitative analysis of these constituents.

ACKNOWLEDGMENT

This research was supported by a grant of the Korea Food & Drug Administration.

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Received August 1, 2008
Accepted October 27, 2008
Manuscript 6377