Biotechnology and Bioprocess Engineering 2008, 13: 671-676
DOI/10.1007/s12257-008-0019-2

Simultaneous Extraction and Separation of Liquiritin, Glycyrrhizic Acid, and Glabridin from Licorice Root with Analytical and Preparative Chromatography

Minglei Tian, Hongyuan Yan, and Kyung Ho Row*

Center for Advanced Bioseparation Technology, Department of Chemical Engineering, Inha University, Incheon 402-751, Korea

Abstract Simultaneous extraction and separation of liquiritin, glycyrrhizic acid, and glabridin from licorice were developed by liquid-liquid extraction with liquid chromatography separation. By utilizing different extraction solvents, procedures, and times, the optimum extraction conditions were established. The extracts of licorice were separated and determined using a C_{18} column with a mobile phase consisting of acetonitrile-water (containing 1.0% acetic acid) with a gradient elution of 0~10 min from 20:80 to 60:40 (v/v). Preparative columns with different packing sizes were investigated to isolate the three compounds from the extracts of licorice. The 12 μm chromatographic column showed better separation for the three compounds from licorice. 0.29 mg/g for liquiritin, 1.43 mg/g for glycyrrhizic acid, and 0.07 mg/g for glabridin were obtained and the recoveries were 80.8, 89.7, and 72.5%, respectively. © KSBB

Keywords: extraction and separation, liquiritin, glycyrrhizic acid, glabridin, preparative, licorice

INTRODUCTION

Licorice, the root of the *glycyrrhiza* plant species, has been used medicinally for more than 4,000 years [1]. It is a Chinese herb commonly used as an expectorant and to arrest coughing, reduce fever, comfort the stomach, alleviate urgency, and potentiate the effects of various other herbs [2,3].

Liquiritin (LQ) (Fig. 1A) is has anti-viral properties [4,5] and antioxidative properties [6] and is the most prevalent flavonoids in licorice [7]. Glycyrrhizic acid (GA) (Fig. 1B), the most studied active constituent in licorice, is a sweet-tasting material. It is 50 times sweeter than sugar and is widely used as a sweetening additive in the food industry [8,9]. GA has anti-inflammatory, anti-ulcer, anti-hepatotoxic, and antivirus activities [10-13]. In many countries, it is used as a major therapeutic agent to treat allergic dermatitis and chronic viral hepatitis [14]. Glabridin (Fig. 1C) is another active component in licorice. It exhibits multiple pharmacological activities, such as antimicrobial activity, cytotoxic activity, and estrogenic and anti-proliferative activity against human breast cancer cells. It also effects on low-density lipoprotein oxidation, melanogenesis, and inflammation and protects mitochondrial functions from oxidative stresses [15].

Reversed-phase preparative high-performance liquid chromatography (HPLC) has been applied to the purification of LQ, GA, and glabridin from licorice. Preparative chromatography is a purification process and is employed to isolate pure substances from a mixture [16-18].

There have been some reports on the separation of LQ, GA, and glabridin, respectively [19,20]. However, a method for the simultaneous extraction of these three compounds has not yet to be established. Hence, the purpose of this study is to develop a simple method to simultaneously extract LQ, GA, and glabridin from licorice and isolate the three compounds using analytical and preparative chromatography. Compared with the previously reported results, the present method is simple and rapid and can be used to simultaneously separate the three aforementioned compounds from licorice.

MATERIALS AND METHODS

Chemicals

Licorice was purchased from a local market. Liquiritin

***Corresponding author**
Tel: +82-32-860-7470 Fax: +82-32-872-0959
e-mail: rowkho@inha.ac.kr

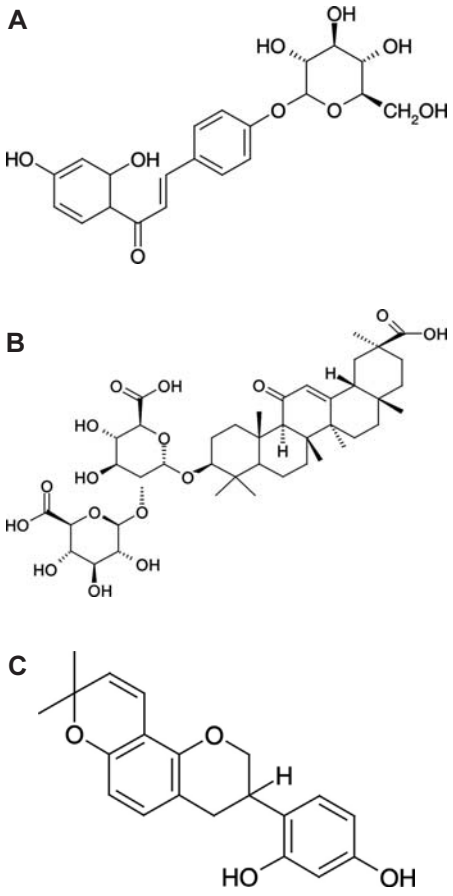

Fig. 1. Molecular structures of liquiritin (A), glycyrrhizic acid (B), and glabridin (C).

was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Glycyrrhizic acid (mono-ammonium salt hydrate) was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and glabridin was from Wako Pure Chemical Industries, Ltd. (Japan). Methanol, acetonitrile, chloroform (HPLC Grade), acetic acid, and n-hexane (extra pure reagent) were purchased from Duksan Pure Chemical. Co. Ltd. (Korea). Water was twice distilled and filtered (FH-0.45 μm, Advantec MFS, Inc., Japan) using a decompressing pump (Division of Millipore, Waters, USA).

HPLC Analysis

The HPLC system in this study is comprised of a M930 solvent delivery pump (Young Lin Co., Korea), a UV detector (M 720 absorbance detector, Young-In Scientific Co., Korea), and an integrated data system (Autochrowin. Ver. 1.42, Young Lin Co., Korea). Two reodyne injection valves with 25 and 200 μL sample loops were used. The flow rate was 0.5 mL/min and UV wavelength was set at 252 nm. All the solvents must be filtered by a disposable syringe filter unit (0.2 μm) for further HPLC analysis.

Samples and Columns Preparation

The licorice roots were oven-dried, sliced, and crushed into powder for use in the extraction experiments. The standards of LQ, GA, and glabridin were dissolved in methanol to yield a final concentration of 0.33 mg/mL. Different HPLC columns were packed by using different diameters of C_{18} particles as stationary phase. The uniform C_{18} particles (12 and 40/63 μm) purchased from YMC Co. (Kyoto, Japan) were suspended in methanol and degassed by helium. The slurries were pressed into the hollow HPLC columns (250 mm × 4.6 mm) using a pump, respectively. After then, the packed columns were washing by methanol until a stabile baseline was observed. All experiments were carried out at ambient room temperature.

Extraction and Separation

Choosing the Optimum Extraction Solvent

The different extraction solvents used in the experiment for the extraction of LQ, GA, and glabridin from licorice was water, methanol, n-hexane, and chloroform. Fifty mL of each solvent was used to extract 1.0 g of licorice using the same dipping time (60 min) under room temperature respectively.

Methanol Extraction with Different Extraction Methods

Five samples of 1.0 g licorice powder were mixed with 50 mL of methanol for 20, 30, 60, 120, 150, and 240 min under room temperature, respectively. The same 5 mixtures were then prepared again with 2, 5, 10, 20, and 30 min ultrasonic, respectively.

Extraction with Different Volumes of Methanol

Five samples of 1.0 g licorice powder were added to 20, 50, 70, and 100 mL methanol under room temperature without ultrasonication, respectively.

Separation of Extracts Using Different Particle Size Columns

In order to determine the maximum injection volume in the analytical column, injection volumes of 10, 25, 50, 75, and 100 μL extracts of licorice were assessed.

RESULTS AND DISCUSSION

Effect of Different Extraction Solvents

Table 1 shows the extracted amounts of LQ, GA, and glabridin by the different solvents, respectively. As the Fig. 1 shows, the three compounds all have several hydroxyls, which make them easily dissolved and extracted by polar solvents. GA is an organic acid among the three compounds, and it has the highest polarity and has the largest solubility in water, so using water as extractant can get more amount of GA from licorice than that of using methanol. LQ is one of

Table 1. Extracted amounts of LQ, GA, and glabridin with different solvents

Extraction solvents	Compound (mg/g)		
	LQ	GA	Glabridin
Chloroform	*	*	*
n-Hexane	*	*	0.006
Water	0.15	2.08	0.001
Methanol:water (25:75)	0.20	1.93	0.008
Methanol:water (50:50)	0.23	1.76	0.023
Methanol:water (75:25)	0.25	1.59	0.066
Methanol	0.29	1.43	0.070

*Not detected.

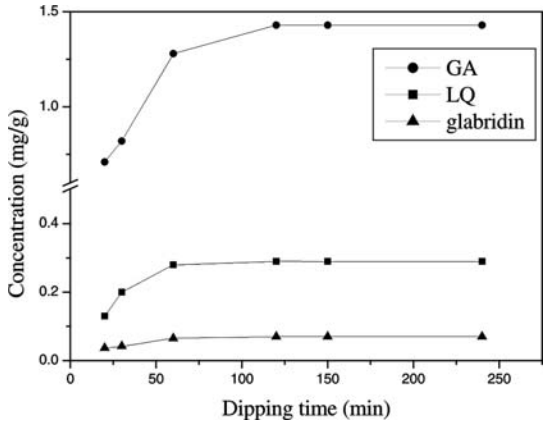

Fig. 2. Effect of different dipping times on the extracted concentration of LQ, GA, and glabridin.

the flavones and glabridin is one of the isoflavones which have middle polarity and can be easily dissolved in methanol, so the extraction amounts of LQ and glabridin clearly increased with the increasing of methanol proportion in extractant. Hence, considering the simultaneous extraction of the three compounds, methanol was used in the subsequent experiments.

Effect of Different Extraction Methods

In order to obtain the optimum extraction conditions, two methods were established. The dipping method was used first. The amounts of extracted LQ, GA, and glabridin increased as the dipping time was increasing from 20 to 120 min and there was no obvious increase after 120 min, as shown in Fig. 2. Thus, 120 min was determined to be the optimum dipping time.

Equivalent samples were then prepared by an ultrasonic method. Fig. 3 shows the extracted amounts of the three compounds. With an increasing of the ultrasonic time, the extracted amounts of GA increased obviously but for the extracted amounts of LQ and glabridin were almost same from 10 to 30 min.

However, comparing the results of the two methods, it was found that although the amounts extracted of GA via the ultrasonic method in 30 min were higher, the extracted amounts of LQ and glabridin were lower while much more energy was required in the experiments. Thus, it was determined that the ultrasonic method was not appropriate for this approach.

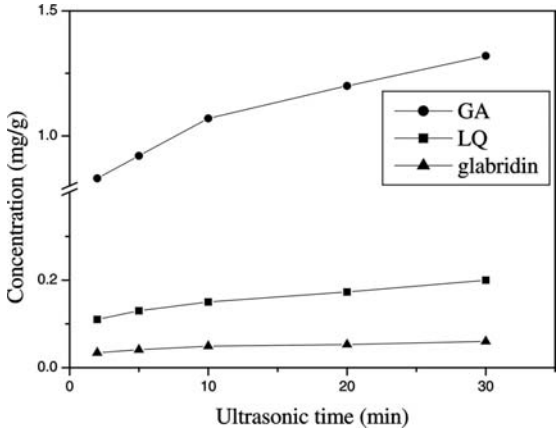

Fig. 3. Effect of different ultrasonic times on the extracted concentration of LQ, GA, and glabridin.

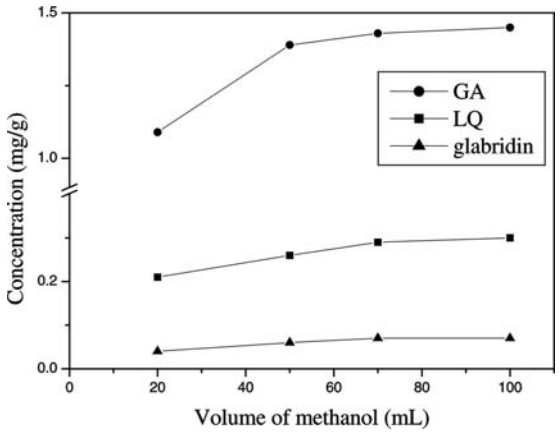

Fig. 4. Effect of volume of methanol on the extracted concentration of LQ, GA, and glabridin.

Effect of Different Volumes of Methanol

As Fig. 4 shows, the extracted amounts of LQ, GA, and glabridin increased as the volume of methanol was increased. However, beyond a volume of 70 mL, no further increase was observed. Therefore, the use of 70 mL of methanol was determined to be optimal for extraction in terms of the amount and type of solvent.

Method Validation

To ensure the specificity and selectivity of the method, concentrations of 0.2, 0.4, 0.5, 0.8, and 1.0 mg/mL were applied for standards solutions of LQ and GA respectively,

Table 2. Extracted amount of LQ, GA, and glabridin by 3 steps extraction

Methanol	Compounds (mg/g)		
	LQ	GA	Glabridin
1 step (70 mL)	0.29	1.43	0.07
2 step (70 mL)	0.001	0.03	0.0
3 step (70 mL)	0.0	0.0	0.0

and 0.01, 0.04, 0.08, 0.1, and 0.2 mg/mL concentrations were used for glabridin. Each concentration was injected 3 times in a column (C_{18}, 5 μm, 150 × 4.6 mm, RStech Corporation, Korea) with a mobile phase consisting of acetonitrile/1% (v: v) acetic acid using a gradient elution of 0~10 min from 20:80 to 60:40. The analyte peak area values were plotted against the corresponding concentrations of the analytes and the calibration curves were constructed by means of the least-square method. Calibration curves of the three compounds showed good linearity ($r^2 > 0.998$); the regression equations of LQ, GA, and glabridin were Y = 11,531x − 23.942 (x from 0.01 to 0.1 mg/mL), Y = 9,721.7x + 97.429 (x from 0. 1 to 1.0 mg/mL), and Y = 10,730x − 31.147 (x from 0.01 to 0.1 mg/mL), respectively.

In order to make sure if the three compounds can be simultaneously and fully extracted, a 3 steps experiment was established. In the first step, 1 g of licorice powder was mixed with 70 mL of methanol for 120 min and the powder was subsequently separated from the solvent and oven-dried again. The following steps were identical to the first but new 70 mL methanol was used. From Table 2, it is seen that extracted amounts of LQ and GA were extremely low in the second step, and in the third step there was no extraction from the licorice powder. Hence, it was determined that 70 mL of methanol could extract the three compounds completely and simultaneously in a period of 120 min.

Assays of repeatability calculated as relative standard deviations (RSDs) were performed by injecting standard solutions of LQ, GA, and glabridin 5 times in a 5-day period. The concentration of the standard solutions was 0.33 mg/mL, respectively, and the injection volume was set at 10 μL.

Three concentrations of LQ (0.15, 0.20, and 0.30 mg/g), GA (0.5, 0.6, and 0.8 mg/g), and glabridin (0.05, 0.06, and 0.07 mg/g) were added to 3 mL of the extracts from licorice, respectively, and to a final volume of 6 mL.

$$R = \frac{C_p - C_0}{C_m} \times 100\% \quad (1)$$

R, recovery rate; C_p, the total amount of the compound of final solvent; C_0, the amounts of the compound from licorice; C_m, the amount of the compound which was added. The measured concentration was compared with the theoretical concentration to calculate the recovery rate [21] by Eq. (1).

The standard solutions of LQ, GA, and glabridin were diluted and injected until the limit of detection (LOD) was obtained at a signal/noise ratio of 3. The RSD of precision tests, the limit of detections (LOD) on standard solutions and the recovery rates are presented in Table 3. Comparison with the real sample analysis verified that the values noted above were of acceptable precision and accuracy.

Table 3. RSDs, recovery rates, and LODs of the three compounds from licorice

Compounds	RSD (%)		Recovery rate			LOD (ng/mL)
	Intra-day	Inter-day	Added (mg/g)	Recovery (%)	RSD (%)	
LQ	0.34	0.37	0.15	81.3	0.38	365
			0.20	80.0	0.37	
			0.30	81.0	0.39	
GA	0.54	0.59	0.5	88.7	0.66	464
			0.6	90.1	0.65	
			0.8	90.3	0.70	
Glabridin	0.83	0.90	0.05	74.5	0.77	229
			0.06	69.9	0.76	
			0.07	73.2	0.74	

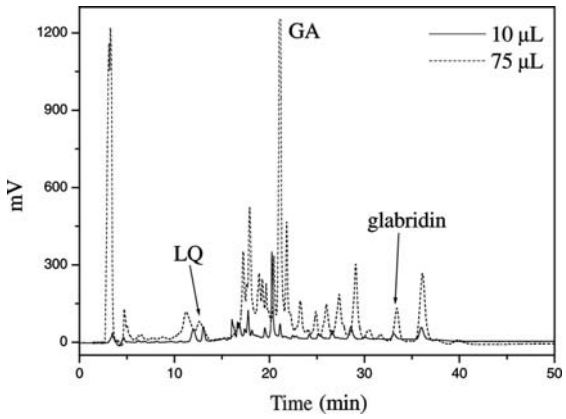

Fig. 5. Chromatogram of the extracts of licorice by different injection volume (Column: C_{18}, 5 μm, 4.6 × 150 mm, 10 and 75 μL; FR: 0.5 mL/min, 252 nm).

Preparative Separation on Different Particles Sized Columns

Larger injection volumes were used to determine the effect of injection volumes in the analytical column. The three compounds all showed good purity when the injection volume was 10 μL, as shown in Fig. 5. With an increase of the injection volume, the area of the peaks increased. GA and glabridin could be separated well, but LQ could not be separated from interference when the injection volume was larger than 50 μL.

Figs. 6 and 7 show the chromatograms at the particle sizes of 40/63 and 12 μm, respectively. Comparing the results obtained at 10 μL injection volume, GA could be separated well in both preparative columns, but the peaks of LQ and glabridin connected with interference in the 40/63 μm column. With an increase of the injection volume to 100 μL, as presented in Table 4, GA was not purely separated and the other two compounds could hardly be detected in the 40/63 μm column. When the injection volume was 200 μL, the

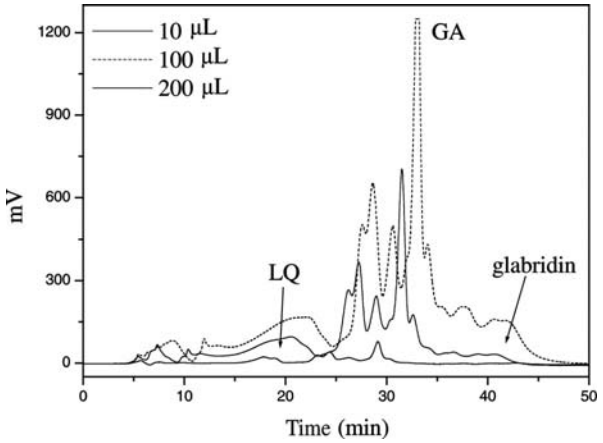

Fig. 6. Chromatogram of the extracts of licorice by different injection volume (Column: C18, 40/63 μm, 4.6 × 250 mm, 10, 100, and 200 μL; FR: 0.5 mL/min, 252 nm).

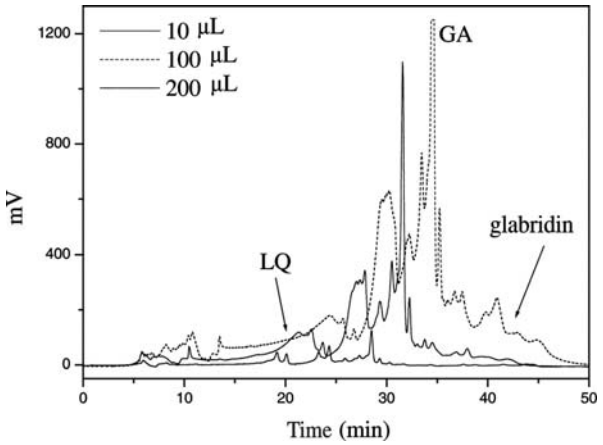

Fig. 7. Chromatogram of the extracts of licorice by different injection volume (Column: C18, 12 μm, 4.6 × 250 mm, 10, 100, and 200 μL; FR: 0.5 mL/min, 252 nm).

compounds could still be detected in the 12 μm column except for glabridin. As the particle size became larger, the column efficiency and resolution deteriorated, because of the smaller contact area of the sample with the surface of the solid packings and due to larger diffusivity and longer flow paths. Hence, from the results, it was determine that a particle size of 12 μm can be used in the preparative column.

CONCLUSION

In this study, a simple and convenient method for the simultaneous extraction and separation of liquiritin, glycyrrhizic acid, and glabridin is described. Preparative columns with different packing sizes were investigated to isolate the three compounds from the extracts of licorice. Under the optimum conditions, the 12 μm column showed better separation for the three compounds from licorice. The extracted amounts of LQ, GA, and glabridin were 0.29, 1.43, and 0.07 mg/g and the recoveries were 80.8, 89.7, and 72.5%, respectively.

Table 4. Peak width with different injection volumes and particle sizes

Injection volume (μL)	Peak width					
	40/63 μm			12 μm		
	LQ	GA	Glabridin	LQ	GA	Glabridin
10	1.38	1.54	1.02	0.87	0.78	0.50
30	1.78	1.61	1.12	1.23	0.88	0.63
50	1.84	1.66	1.52	1.38	1.14	0.77
100	*	1.68	*	1.78	1.61	*
150	*	1.70	*	1.96	1.79	*
200	*	1.82	*	*	1.84	*

*Not detected.

Acknowledgements The authors gratefully acknowledge financial support by the Center for Advanced Bioseparation Technology and Inha University, Korea.

Received February 4, 2008; accepted July 24, 2008

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