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ISOLIQUIRITIGENIN EXTRACTED FROM LICORICE *Glycyrrhiza uralensis* ROOTS BY A FACILE CONVERSION TECHNIQUE

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*Isoliquiritigenin (**1**), one of the major constituents of licorice, is a natural pigment with a simple chalcone structure 4,2′,4′-trihydroxychalcone. This study was performed to determine whether liquiritin, isoliquiritin, and liquiritigenin can be converted into isoliquiritigenin using alkaline conversion after acid hydrolysis. An orthogonal L_9 $(3)^4$ test design was used in the extraction mode and used for optimization of the extract conditions, and the yield from the conventional procedure and from the optimum procedure to extract **1** was compared. The results demonstrated that the yield of the novel procedure was increased about 27 times compared with the conventional procedure, which indicated that the novel procedure is powerful in separating and purifying isoliquiritigenin.*

Keywords: isoliquiritigenin, extraction, hydrolysis.

The root of *Glycyrrhiza uralensis* Fisch. (Leguminosae) is widely used as a harmonizing ingredient in many traditional herbal formulations. It is used in more formulations than any other herb in oriental medicine. Although it is considered to be the quintessential "servant" herb, it is often referred to as the "King of Herbs." It can be used for various purposes such as cough, pharyngitis, bronchitis, bronchial asthma, ague, hepatitis, and gastric ulcer [1, 2]. Flavonoids in it are the main bioactive compounds. Pharmacological investigations concluded that they have antioxidant properties [3], prevent atherosclerosis, and have antibacterial activities [4]. Among them, the most remarkable one is isoliquiritigenin (**1**), a simple chalcone-type flavonoid that has been evaluated in terms of its antioxidative effects [5, 6], antiplatelet aggregation effects [7], anti-ischemia effects [8], anti-inflammatory properties [9, 10], antispasmodic effects [11], and estrogenic properties [12]. Moreover, **1** is already used as an antiarrhythmia and anticancer medicine in a few countries. It has been suggested that **1**, a specific constituent in licorice, merits investigation as a potential chemopreventive agent in humans.

Methods of preparation of **1** from licorice roots were investigated by different researchers. Column chromatography, high-performance liquid chromatography (HPLC) [13], and high-speed countercurrent chromatography have been utilized [14]. However, the concentration of isoliquiritigenin (**1**) in licorice is very low, approximately accounting for 0.045% in the raw material [15]. Until recently, there was lack of knowledge on the conversion of liquiritin (**2**), isoliquiritin (**3**), and liquiritigenin (**4**) into **1** by alkaline conversion after acid hydrolysis.

Compounds **2** and **3** are flavonoid glycosides; in an acid medium, **2** and **3** can be hydrolyzed into **4** and **1**, respectively. Compound **1** is a chalcone, which is formed by a Michael-type nucleophilic attack of a phenol group on the unsaturated ketone, giving a flavanone such as **4**. This isomerization can occur in an alkaline medium [16]. It is hoped that **1** can be obtained from **2**, **3**, and **4** using our proposed procedure. The present report deals with the isolation of **1** from *Glycyrrhiza uralensis* Fisch. licorice roots by a facile conversion technique for the first time, and demonstrates that the process of **2**, **3**, and **4** conversion into **1** can be carried out using crude extract of roots.

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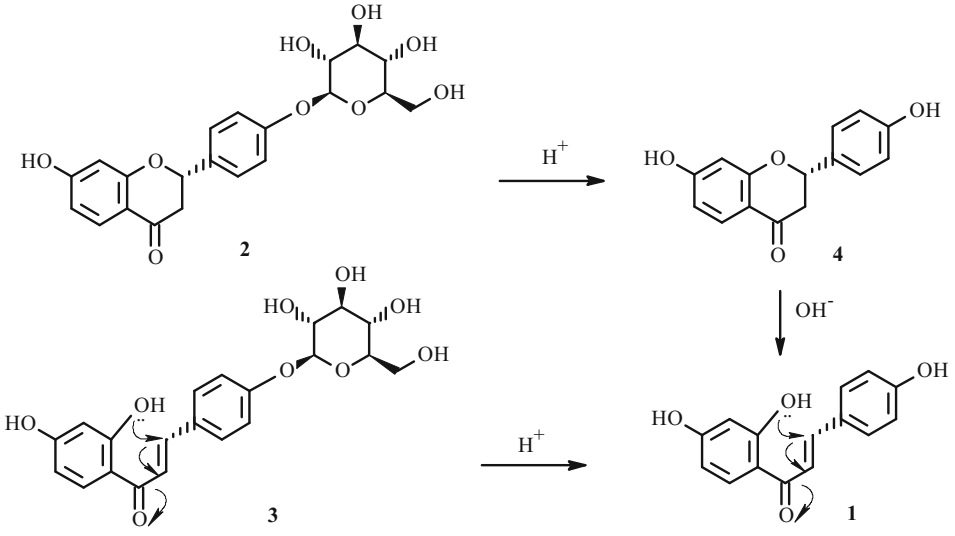
TABLE 1. Linear Regression Results

Compound	Regression analysis equation	Correlation coefficient
1	y = 188418x + 25143	0.9997
2	y = 39069x + 58540	0.9998
3	y = 84390x + 27098	0.9998
4	y = 93582x – 57420	0.9993

TABLE 2. Analysis of $L_9(3)^4$ Test Results

No.	Hydrolysis			Blank	Yield of **1**, %
	time, h	[HCl] mol/L	temperature, °C		
1	1	0.5	60	1	0.053
2	1	1.5	75	2	0.537
3	1	2.5	90	3	0.524
4	2	0.5	75	3	0.117
5	2	1.5	90	1	0.834
6	2	2.5	60	2	0.254
7	3	0.5	90	2	0.119
8	3	1.5	60	3	0.335
9	3	2.5	75	1	0.625
K1	0.371	0.096	0.214	0.504	
K2	0.402	0.569	0.426	0.303	
K3	0.360	0.468	0.492	0.325	
R	0.042	0.473	0.278	0.201	

R: refers to the result of extreme analysis.



The chromatogram of licorice extract is shown in Fig. 1 (liquiritin 14.6 min, isoliquiritin 28.6 min, liquiritigenin 32.3 min, and isoliquiritigenin 50.7 min). The regression equations are given in Table 1, which shows good linear relationships between the peak areas and concentrations.

To test the stability of licorice extract and the precision of the procedure, detection of the four compounds in the sample using the same reagents and instrument was performed in five consecutive days (Table 1). We used SN > 3 to calculate the lower limit of detection, which was 83 ng/mL for isoliquiritigenin, 118 ng/mL for isoliquiritin, 75 ng/mL for liquiritigenin, and 199 ng/mL for liquiritin. The recoveries of the flavonoids were 97.6–99.7% with relative standard deviations of 2.12–3.41%. Analysis of licorice extract showed acceptable precision and accuracy.

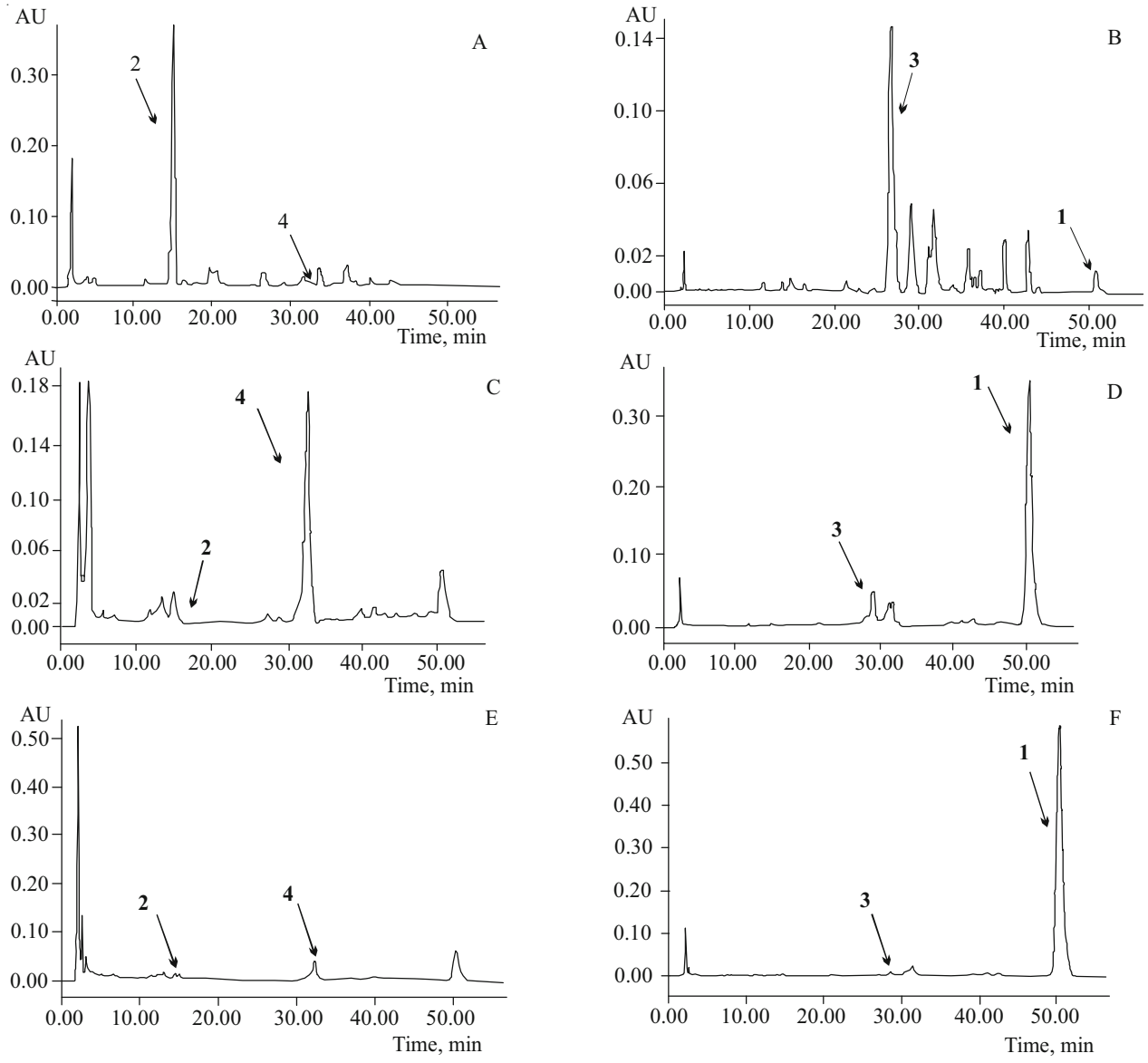


Fig. 1. **4** and **2** (A, C, and E) were detected at 275 nm; **1** and **3** were detected at 370 nm (B, D, and F) successively by HPLC. The concentration of alcohol extract (A and B), alcohol extract after acid hydrolysis (C and D), and alcohol extract after acid hydrolysis and alkaline conversion (E and F) was compared.

The extraction parameters of **1** from licorice are optimized for efficient extraction and to avoid co-extraction of undesired compounds and degradation of **1**. Since various parameters potentially affect the extraction process, the optimization of the experimental conditions is a critical step in the development of a solvent extraction process. In fact, hydrolysis time, concentrations of HCl, and hydrolysis temperature are generally considered to be the most important factors based on the single-factor test. In the present study, all selected factors were examined using an orthogonal $L_9 (3)^4$ test design. The yield of **1** was subject to analysis by statistical methods. The results of orthogonal test and extreme difference analysis are presented in Table 2. The analysis of variance was performed by statistical software SPSS 12.0.

The results of experiments presented in Table 2 indicated that the maximum extraction yield of the crude extract was 0.834%. However, we cannot select the best extraction conditions based only on these outcomes in Table 2, and a further orthogonal analysis was warranted. Thus, K and R values were calculated and are listed in Table 2. As shown in Table 2, we have found that the influence on the mean extraction yields of the compounds decreases in the order: B > C >A with regarding to the R values. The hydrolysis temperature was found to be the most important determinant of the yield of **1**. In other words, the maximum yield of **1** was obtained when the concentration of HCl, hydrolysis time, and hydrolysis temperature were 1.5 mol/L, 2 h, and 90°C, respectively.

Firstly, the concentration of **1**, **2**, and **3** is 0.030%, 1.292%, and 0.331%, respectively. Compound **4** could not be detected in the extract obtained from the conventional procedure. HPLC chromatograms are shown in Fig. 1 (A and B). Secondly, 1.5 mol/L HCl was utilized to hydrolyze **2** and **3** into **4** and **1** at 90°C for 2 h; Fig. 1 (C and D) shows that the concentration of **2** and **3** decreased rapidly, while the concentration of **4** and **1** increased rapidly, proving that **2** and **3** were hydrolyzed into **4** and **1**, respectively. Finally, the hydrolyzed solution was adjusted to pH 14 using NaOH for 2 h; Fig. 1 (E and F) shows that the concentration of **4** decreased sharply while the concentration of **1** increased dramatically, showing that **4** is converted to isoliquiritigenin in an alkaline medium. The concentration of **1** is 0.834%. Thus, the yield of **1** increased about 27 times. In other words, the conversion rate of **2**, **3**, and **4** into **1** reached 80.92%.

Yellow needle crystals were isolated by polyamide columns. ^{1}H NMR (600 MHz, $CDCl_3$, δ, ppm, J/Hz): 8.01 (1H, d, J = 8.9, H-6′), 7.64 (2H, d, J = 8.5, H-2, H-6), 7.73 (2H, s, H-α, H-β), 7.87 (2H, d, J = 8.5, H-3, H-5), 6.44 (1H, dd, J = 8.9, 2.1, H-5′), 6.32 (1H, d, J = 2.1, H-3′). The data are consistent with the literature [13] on isoliquiritigenin.

In the orthogonal test for optimization of the extraction conditions, alkaline conversion parameters were not considered. However, in pre-experiment we found that **4** can be easily converted into **1** at room temperature, so that hydrolysis time, concentration of HCl, and hydrolysis temperature are considered to be the most important factors in this study.

Facile conversion was successfully applied to separate and purify **1**, the main bioactive component of the Chinese medicinal herb *Glycyrrhiza uralensis* Fisch., a particular plant species of licorice. With alkaline conversion after acid hydrolysis, the yield of **1** increased about 27 times compared with the conventional procedure. The overall results of the present study indicate that the procedure mentioned in this study is a powerful one in separating and purifying **1**.

EXPERIMENTAL

Reagents and Materials. Licorice was purchased from the First Affiliated Hospital of Shihezi University Medical College. Standard samples of liquiritigenin (> 99%), isoliquiritigenin (> 99%), and isoliquiritin (> 98%) were obtained from the Phytochemistry Department of Shihezi University. Liquiritin (> 98%) was purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products. All standard samples were stored at –20°C until use. HPLC grade acetonitrile (TEDIA, Fairfield, OH, USA) and Milli-Q water purified on a Milli-Q® Ultrapure water purification system was used for extraction and isolation. Analytical grade alcohol (Huada, Guangdong, China) and analytical grade methanoic acid (Qinshengda, Beijing, China) were utilized in this investigation.

HPLC System and Conditions. Analytical experiments were performed on a Waters Delta 600 system with a Waters 2996 Diode Array Detector. Compounds **2**, **3**, **4**, and **1** in licorice root were studied. The separation system consists of a Dikma Kromasil C_{18} (4.6 mm × 150 mm, 5 μm) column and a gradient elution system containing acetonitrile (A) and 3% methanoic acid (v/v, B) in water, using a gradient program of 13–20% A in 0–8 min, 20% A in 8–20 min, 20–30% A in 20–33 min, 30–32% A in 33–40 min, 32–30% A in 40–45 min, and 30% A in 45–57 min. The flow rate was 0.8 mL·min^{-1} and the column temperature was maintained at 30°C. Compounds **1** and **3** were detected at 370 nm; **4** and **2** were detected at 275 nm.

Optimization of Extraction Procedure. An orthogonal L_9 $(3)^4$ test design was used in the extraction mode, which was used for optimization of the extraction conditions. In this study, 12 g licorice powder was suspended with a 100 mL volume of HCl solution. Acid hydrolysis was carried out at temperatures of 60°C, 75°C, and 90°C; 0.5 mol/L, 1.5 mol/L, and 2.5 mol/L HCl solutions were utilized in this hydrolysis process; hydrolysis time was 1, 2, and 3 h on the basis of the single-factor test. The extract was adjusted to pH 14 by NaOH, then adjusted to pH 7 using HCl. The extract was evaporated to dryness at 60°C under reduced pressure. The dry residue was extracted twice by ethanol at 90°C, 100 mL each time. HPLC was used to determine the concentration of **2**, **3**, **4**, and **1** in the ethanol extract.

In order to compare the difference between the conventional procedure and the optimum procedure screened through the orthogonal L_9 $(3)^4$ test, the conventional procedure to extract **1** was performed on 12 g licorice powder extracted twice by ethanol at 90°C, 100 mL each time.

Isolation and Identification of Isoliquiritigenin. Polyamide columns (50 g) were prepared and primed with 80 mL 100% ethanol followed by 100 mL 50% ethanol; 5 g of the sample was loaded, the alkaline conversion having ended after acid hydrolysis. Polyamide column chromatography eluting with 400 mL 50% ethanol yielded isoliquiritigenin solvent. The solvent was removed from the samples at 20°C with N_2 gas and the residual aqueous phases were shell-frozen on dry ice prior to freeze-drying. The freeze-dried samples were stored at –20°C until NMR analyses were performed.

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