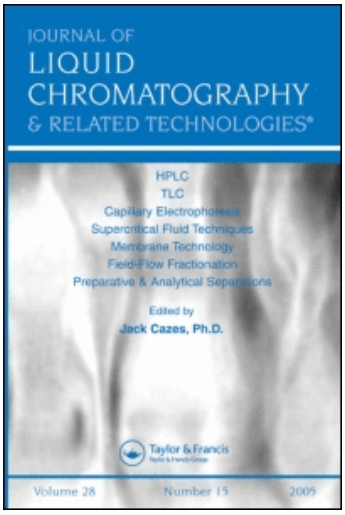

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Study of Separation Conditions of Active Components in Licorice with Two-Dimensional Liquid Chromatography

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Abstract: Licorice, an important natural plant, mainly contains triterpenoids and flavonoids. Glycyrrhizic acid (glycyrrhizin), 18β-glycyrrhetinic acid, liquiritin, isoliquiritigenin, and licochalcone B are typical active components in the two groups. The combination of the RP-RP system was constructed for studying the separation conditions of the above five active components. The different retention characteristics of the five components were exhibited in different two dimensional phase systems. The influences of flow rates, injection concentrations, and temperatures on the separation of these components were studied. The results show that a proper decrease of flow rate is good for gaining high purity of the product. The influences of injection concentration on the separation of active components in a two-dimensional system are not obvious; in the chosen concentration range the nonlinearity of adsorption isotherm for liquiritin is weaker. The study on the influence of temperature and the corresponding thermodynamic analysis show the influence of temperature on the second column is not obvious, but is obvious on the first column; adjusting temperature properly will improve the efficiency of two-dimensional separation and decrease the consumption of organic solvent.

Keywords: Two-dimensional liquid chromatography, Active components, Licorice, Separation conditions

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INTRODUCTION

Licorice is an important natural plant, which contains lots of active components. Triterpenoids and flavonoids are two big groups of active compounds in licorice. The triterpenoids mainly include the glycyrrhizic acid (glycyrrhizin), glycyrrhetinic acid, and their derivatives. The flavonoids include liquiritin, isoliquiritigenin, licochalcone B, etc. It is reported that the glycyrrhizic acid and its hydrolisis glucoside, 18β-glycyrrhetinic acid, exhibit antitumor,[1–3] anticancer,[4] anti-HIV,[5–7] antiviral,[8,9] and other[10–12] activities. In 2003, German scientists had reported that glycyrrhizic acid possessed the activity of suppression against SARS.[13,14] With the development of researching for licorice, the pharmacological activities of licorice flavonoids[15–17] such as the antioxidative[18,19] and antiviral[20] activities from liquiritin; anticancer activities[21,22] and other activity[23] from isoliquiritigenin; and the inhibition to cell proliferation from licochalcone[24,25] were recently noticed. Now, more attention is being paid to the overall extraction and separation of multiple components from the two big groups of licorice.

Two-dimensional liquid chromatography (2D LC) is a powerful tool for separation of complex mixtures. Compared with traditional one-dimensional liquid chromatography, the resolution and separation power of 2D LC have improved greatly. Since 1984, Giddings[26] introduced the concept of 2D separation, the 2D LC techniques are developing rapidly, the change from off-line to on-line is achieved by using a switching technique, and more information of the sample can be obtained by the comprehensive mode than the heart cutting technique.[27–31] Two-dimensional liquid chromatography is often used for analysis, the improved peak capacity can be obtained, and thus more chromatographic peaks can be recognized.[32–34] While two-dimensional liquid chromatography is used for separation and isolation, it can not only improve the peak capacity, but also improve the resolutions between components, especially the closely eluting components.

For many years, two-dimensional liquid chromatography has been widely applied in the field of proteomics research.[35–38] According to our knowledge, there are only a few, numerable, papers published on the separation of active components in natural plants using two-dimensional liquid chromatography. In 2004, Victor Wong et al. separated active components from natural plant *floribundum* using heart cutting RP-2D-LC[39] and analytical multidimensional HPLC.[40] Hanfa Zou's group[41,42] had studied the separation and identification of active compounds of traditional Chinese medicine *chuanxiong* and *gingko* using two-dimensional liquid chromatography.

However, so far there is no report on the separation of active components from licorice by two-dimensional liquid chromatography. In this study, a 2D chromatographic system was constructed to separate the multiple active components from the two big groups of licorice. The five typical active components, glycyrrhizic acid, 18β-glycyrrhetinic acid, liquiritin, isoliquiritigenin,

licochalcone B, which are from two big groups in licorice and the mixtures of them, were chosen as the samples. Based on the high resolution of RP LC, it was frequently used in the separation of natural plant components,[43–46] so in this article two dimensional RP-RP systems were combined. Isocratic elution was adopted in each dimension; each dimension can be operated independently, which permits the use of incompatible mobile phase and can easily be enlarged to a certain scale. Also separation conditions of the active components were studied with the two-dimensional high performance liquid chromatography. The factors effecting the preparative separation, including flow rate, injection concentration, and temperature, were investigated, which are very important in the preparative scale chromatography. At last, thermodynamic analysis was done for the effect of temperature on the separating of active components, and the retention mechanisms were explained from the view of their molecular structures.

EXPERIMENTAL

Apparatus, Columns, and Chemicals

All the experiments were performed with two LC-10ATVP HPLC pumps (Shimazdu, Japan), each fitted with a SPD-10AVP ultraviolet detector (Shimazdu, Japan). Two T-830 column heater/cooler devices (Tian Jin automatic Science Instrument Co. Ltd., China) were used to control the column temperature. Two HH-5 water bath boilers with digital display (Jintan City Medical Instrument Plant, Jiangsu, China) were used to keep the temperature of the mobile phase. A PHS-25A digital acidimeter (Shanghai Precision Instrument Co., Ltd, China) was used to measure the pH of the mobile phase. All determination wavelengths were set at 254 nm.

All the columns used were purchased from Agilent Branch Company (Bejing Caosheng Company, China). The column sizes and the parameters of stationary phase are listed in Table 1.

All the reagents were of Analytical-grade and were purchased from Tianjin Kermel Chemical Reagent Development Center (Tianjin, China), they were filtrated through 0.45 μm filtration membranes before HPLC. All the mobile phases were merged by volume, and were degassed before use.

Liquiritin of 87% purity and 82% purity of licochalcone B were prepared from the extract of *Glycyrrhiza uralensis Fisch*. in our laboratory. They were checked by MS, NMR, UV, and HPLC. Isoliquiritigenin of 96% purity was purchased from Inner Mongolia Yitai Pharmaceutical Co. Ltd. (Inner Mongolia Autonomous Region, China); 95% purity of glycyrrhizic acid and 97% purity of 18β-glycyrrhetinic acid from Xian Ruihong Biological Technology Co. Ltd. (Shanxi, China). The molecular structures of the five components are shown in Figure 1.

Table 1. Column sizes and parameters of stationary phase used

Column size I.D. × L. (mm)	Stationary phase	Pore size (Å)	Surface area (m^2/g)	Temperature upper limit (°C)	PH range	Particle size (μm)	Carbon content (%)
4.6 × 150	Zorbax CN	60	300	60	2.0–8.0	5	7
4.6 × 150	Zorbax NH2	70	300	60	2.0–7.5	5	4
4.6 × 150	Zorbax SB-C18	80	180	90	0.8–8.0	5	10
4.6 × 150	Zorbax Extend-C18	80	180	60	2.0–11.5	5	12.5
4.6 × 150	Zorbax Eclipse XDB-C18	80	180	60	2.0–9.0	5	10

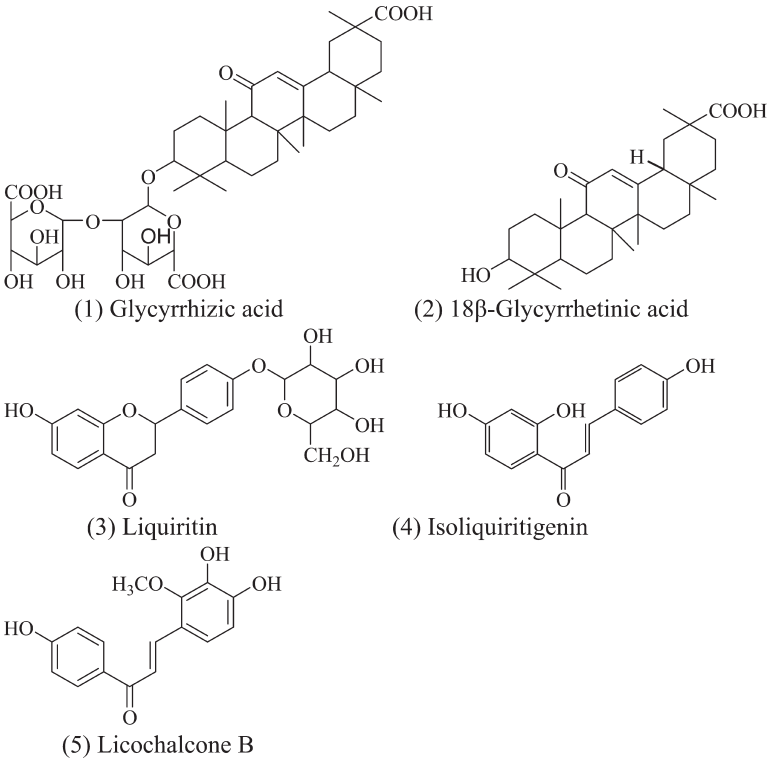

Figure 1. The molecular structures of the five active components in licorice. (1) Glycyrrhizic acid (2) 18β-glycyrrhetinic acid (3) Liquiritin (4) Isoliquiritigenin (5) Licochalcone B.

Sample Preparation

The mixed sample was dissolved in the respective mobile phase, which mainly contains the above five components and their concentrations are as follows: the concentration of glycyrrhizic acid 3.0 mg/mL; 18β-glycyrrhetinic acid 1.5 mg/mL; liquiritin 3.0 mg/mL; isoliquiritigenin 1.0 mg/ml; licochalcone B 5.4 mg/mL, respectively.

Construction of Two-dimensional Phase System–Selection of Phase Systems

Primary Choice of Phase Systems

The primary choice of two dimensional phase system experiments were performed on the columns listed in Table 1, respectively. The two mobile

phases of methanol-water (1:1, v/v) and acetonitrile-water (2:3, v/v) were employed, respectively. A sample of 2 μL was injected into each column every time, and the retention times of the five components were recorded, respectively; flow rate was 1 mL/min. The results are seen in the Results Section.

Selection of Composition of Mobile Phase

Based on the results above, the SB C_{18} and the CN columns were used as the first and the second dimensional columns, respectively. The acetonitrile and water system was used as mobile phase (see Results Section). The composition of the mobile phase was selected. The ratios of 35%, 40%, and 45% acetonitrile were used in the mobile phase. The experiments were performed on the two dimensions, respectively. The other operating conditions are the same as in the section above. (The results are seen in Results Section).

Adjustment of pH Values in Mobile Phase

Based on the results in sections above, the pH values in the mobile phase were adjusted. The pH values of 6, 4, and 2 were adjusted with formic acid, respectively, then repeated as in the above experiment in the 2D system selected. (The results are seen in Results and Discussion).

Effect of Flow Rates on the Two Dimensional Separations

For the cutting fraction of the first dimension, which mainly contains components 1 and 3, the separation conditions were studied in the second dimensional column (CN column). The flow rates were set at 0.5, 0.8, and 1.0 mL/min, respectively, the fraction was injected into the second dimensional column with the mobile phase of acetonitrile-water (2:3, v/v) with pH 6.

Effect of Injection Concentrations on the Two Dimensional Separations

For the fractions mainly containing components 1 and 3, the effects of injection concentration on the 2D separation were studied. The operating conditions are seen in the section of Results and Discussion.

Determination of Isotherms of Liquiritin

The liquiritin sample of 2.299 g 87% purity was dissolved in 150 mL acetonitrile-water mixture (2:3, v/v, pH 6) solution. The sample solution of 2.5, 5, 7.5, 10, 15, 20, 25, and 50 mL was then transferred into 8 volumetric

flasks, respectively, and then added to the acetonitrile and water solution up to 50 mL in all flasks, i.e., the concentrations of liquiritin obtained were 0.67, 1.33, 2.01, 2.66, 3.99, 5.32, 6.67, 13.33 mg/mL respectively. After the SB C_{18} and CN columns were equilibrated by the mobile phase of acetonitrile-water (2:3, v/v) with pH 6, respectively, the Frontal Analysis method was used to determine the isotherms of liquiritin.

The two columns were first permeated by pure water and then permeated by pure acetonitrile; the weight difference of each column in the two situations above was recorded, and the dead volume (V_0) of each column was calculated from the ratio of the weight difference to the density difference. The phase ratios of the two columns were calculated through the formula $F = (1 - V_0)/V_0$, which was 1.25 for the SB C_{18} column, 0.3 for the CN column. The phase ratio of the SB C_{18} column was larger than the usual ones, because it has been used for a long time and some of the pores may be plugged.

Effect of Temperatures on the Two Dimensional Separations

In the mobile phase of acetonitrile-water (2:3, v/v) with pH 6, temperature was adjusted in the range of 25–40°C and then the elution experiments were performed in the second column. In the first dimension, for the components glycyrrhizic acid, 18β-glycyrrhetinic acid, liquiritin, and isoliquiritigenin, the elution experiments were also performed in the same temperature range.

RESULTS AND DISCUSSION

Construction of Two-Dimensional Phase System–Selection of Phase Systems

To represent the results of the separation of five components in the 2D system, the figures, which coordinate the axes of retention times in different columns, are used. For convenience, the five components, namely glycyrrhizic acid, 18β-glycyrrhetinic acid, liquiritin, isoliquiritigenin, and licochalcone B, were numbered from 1 to 5, respectively, in all the figures.

Primary Choice of Phase System

Based on the experimental results, the five columns with different stationary phases Zorbax CN, NH2, SB-C_{18}, Extend-C_{18}, Eclipse XDB-C_{18}, and two mobile phases were selected.

In the mobile phase of methanol-water (1:1, v/v), the total elution times of the five components are long in the five columns. The component 5 (licochalcone B) had not yet eluted in 120 minutes in the three stationary phases of

C_{18}. The peak shapes are also poor for the five components in this mobile phase. Actually, in the study of the separation conditions of licorice components, ethanol-water was also employed, but the results are similar to that of methanol-water and the operating pressures are high, so there are no results given here.

In the mobile phase of acetonitrile-water (2:3, v/v), the total elution time of the five components is very short in the NH2 column, which is less than 3 minutes, and the five components can not be separated in this stationary phase.

But in the mobile phase of acetonitrile-water (2:3, v/v), in the CN and the three C_{18} columns, the elution times of the five components are moderate and the peak shapes of the five components are better than of that in methanol-water.

Figure 2 shows the retention times of the five components in the 2D systems combined by the SB C_{18}, Extend C_{18} and XDB C_{18} vs. CN columns with acetonitrile-water (2:3, v/v) as mobile phase, respectively. The corresponding retention times of the five components in the three C_{18} columns are

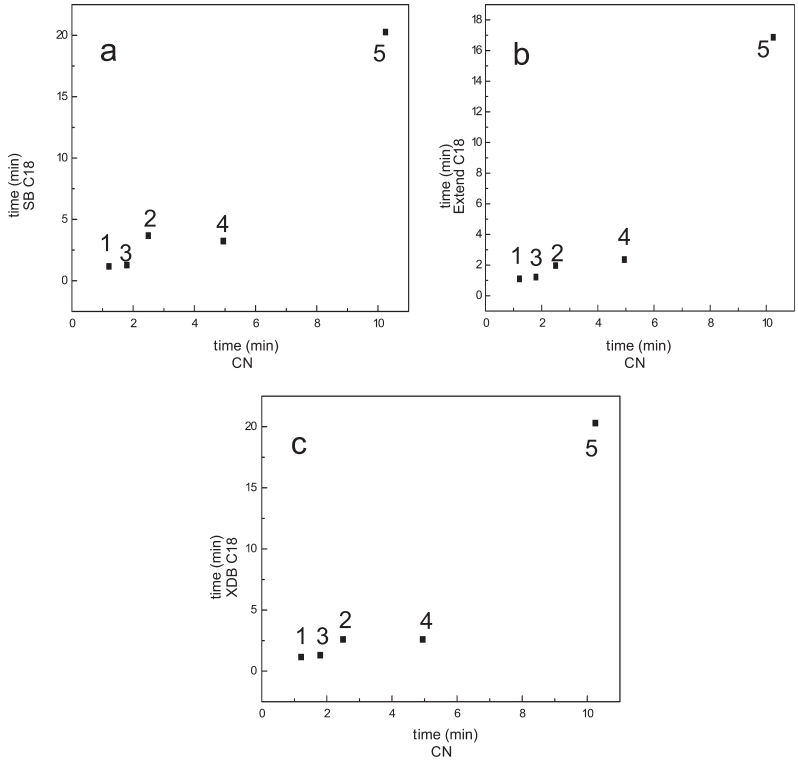

Figure 2. Retention time plot for the five components in the selected 2D systems. The first column was (a) SB C18, (b) Extend C18, and (c) XDB C18 respectively, the second column was CN column. The mobile phase was acetonitrile-water (2:3, v/v) in both columns. Room temperature 16–17°C. Flow rate 1 mL/min.

similar, but in actual operation, their peak shapes are very different; the peak shapes in the SB C_{18} column are better than those in the other two C_{18} columns in general.

Based on the above results, acetonitrile-water and the combination of stationary phases of CN and SB C_{18} were primarily chosen to construct 2D phase systems.

Selection of Composition of Mobile Phase

In the chosen 2D phase systems, the mobile phase composition and pH value can be selected and adjusted to improve the resolution between the licorice components.

Figure 3 shows the effect of the ratio of acetonitrile in mobile phase on the retention times of the five components in the selected 2D system. With the increasing of the ratio of acetonitrile in mobile phase, the total elution time in both dimensions reduces the first dimension, which is determined directly by the retention time of the more retained component licochalcone B, which decreases rapidly, and the retention time in the mobile phase A (40% acetonitrile) is about one third of that in the mobile phase B (35% acetonitrile). However, with the increasing of acetonitrile concentration, the resolution between different components will decrease accordingly. In the mobile phase B (35% acetonitrile), the peak of 18β-glycyrrhetinic acid broadens. Therefore, the mobile phase A (40% acetonitrile) is proper for the separation of these components.

Adjustment of pH Values in Mobile Phase

In the chosen phase system, the effect of pH values on the retention times of licorice components in the 2D system is shown in Figure 4. From Figure 4, in the CN column, with the decreasing of pH, the retention times of 18β-glycyrrhetinic acid and glycyrrhizic acid increase first, then reduce gradually. In the SB C_{18} column, with the increasing of pH, the retention time of licochalcone B increases first, and then decreases, while the retention time of glycyrrhizic acid reduces all along, and the retention time of 18β-glycyrrhetinic acid increases. Along with the changes of pH, the retention times of the five components vary, even the elution order changes.

The effect of pH on the separation selectivity is important. D. V. McCalley always noticed that the elution order would change with pH in the analysis of the Cinchona alkaloids[47] and tobacco alkaloids.[48] In the study of Martin Gilar et al.,[34] they constructed a 2D RP-RP system using the same kind of C_{18} as stationary phase and the same solution with different pH as mobile phase, which had the highest practical peak capacity for the analytes. The effect of pH on the separation of licorice components

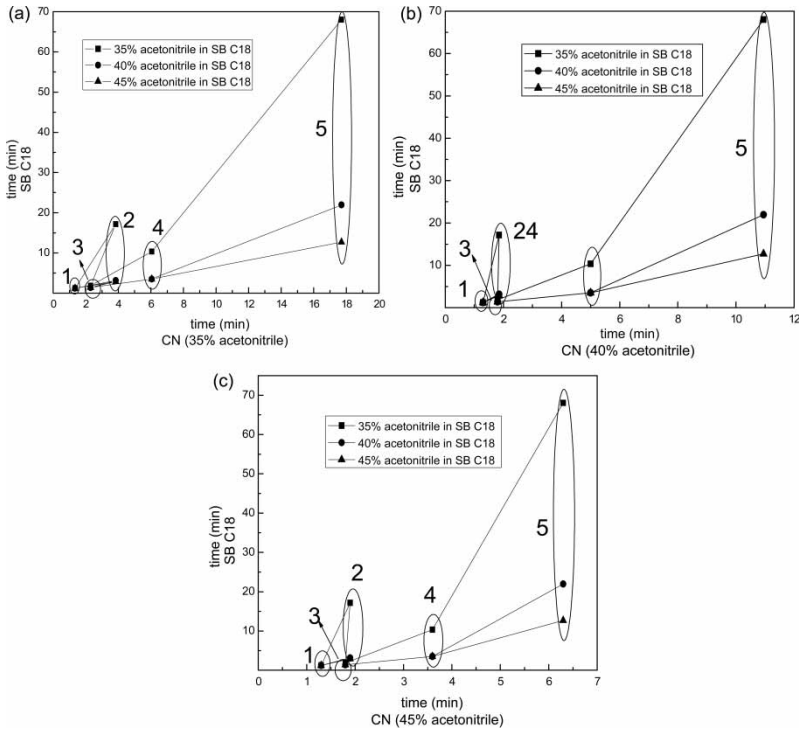

Figure 3. The effect of ratio of acetonitrile on the retention times of the five components in the selected 2D system. In the first column (SB C_{18}), three ratios of acetonitrile in mobile phases were used, i.e., 35% (square, ■), 40% (circle, ●) and 45% (triangle, ▲) acetonitrile, respectively. The irregular shapes in all the figures are caused by the overlap in the different ratios. In the second column (CN), one ratio in mobile phase was kept to different ratios in the first column. In Figure 3a in the second column, the kept ratio is 35% acetonitrile in mobile phase, in Figure 3b in the second column, the kept ratio is 40% acetonitrile in mobile phase and in Figure 3c in the second column, the kept ratio is 45% acetonitrile in mobile phase. Room temperature 16–19°C. Flow rate 1 mL/min.

perhaps is related to the properties of the components, because there are carboxyl groups in the structures of glycyrrhizic acid and 18β-glycyrrhetinic acid; also, flavonoids contain phenolic hydroxyl groups which are weakly acidic groups. The changes of pH would affect the balance of the sub-ionization of these components in the mobile phase, thus there are more complicated separation mechanisms due to this changes.

Based on the above results, the SB C_{18} and CN columns were selected as the first and the second dimensions, respectively, and acetonitrile-water (2:3, v/v) with pH 6 was selected as the mobile phase for both dimensions.

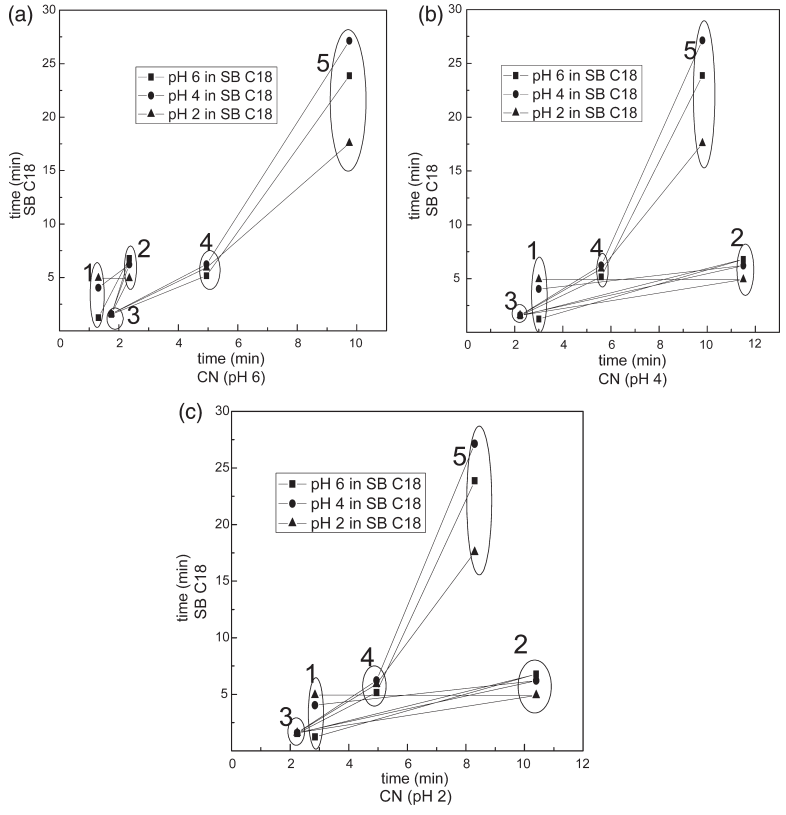

Figure 4. The effect of pH values on the retention times of the five components in the selected 2D system. In the first column (SB C_{18}), three pH values were used, i.e., 6 (square, ■), 4 (circle, ●), and 2 (triangle, ▲) in the mobile phase of acetonitrile-water (2:3, v/v), respectively. The irregular shapes in all the figures are caused by the overlap in the different pH values. In the second column (CN), one pH value was kept to different pH values in the first column. In Figure 4a in the second column, the kept pH value is 6 in the mobile phase, in Figure 4b in the second column, the kept pH value is 4 in the mobile phase and in Figure 4c in the second column, the kept pH value is 2 in the mobile phase. Room temperature 16–17°C. Flow rate 1 mL/min.

Effect of Flow Rates on the Two Dimensional Separations

The separation of closely eluting components is still one of the key problems in 2D separation. The fraction eluted from the first dimension, which mainly contained glycyrrhizic acid and liquiritin, was separated again in the second dimension.

The Van Deemter curve was determined for the component of liquiritin (Figure 5), with the increasing of flow velocity, the HETP for liquiritin increases. So, in the separation of closely eluting components in licorice,

the decreasing of flow rate is good for gaining the high purity products, the increasing of flow rate is also good to enhance productivity. In practice, the flow rates should be selected according to the demands of final products. In this study, the flow rate of 0.5 mL/min was chosen for separating components in licorice in the second dimension.

Effect of Injection Concentrations on the Two Dimensional Separations

In the first dimension, the separation of component 3 (liquiritin) and component 1 (glycyrrhizic acid) is difficult, but they can be separated well in the second dimension with the phase system and flow rate determined in earlier sections (Figure 6). With the increasing of resolution in second column, the impurities before and after liquiritin can be removed from the sample and, thus, higher purities of glycyrrhizic acid and liquiritin products can be obtained. Figure 6 shows the effect of injection concentration of liquiritin on the elution curves in the second dimensional separation. With the increasing of injection concentration of liquiritin, its retention time decreases gradually, but it is not obvious. The resolution between liquiritin and the impurity before it decreases, will affect the 2D separation.

The isotherms of liquiritin in the two stationary phases are determined with Frontal Analysis method. The sample is approximated as a single component, and then the relationship between the concentrations of q and C was fitted

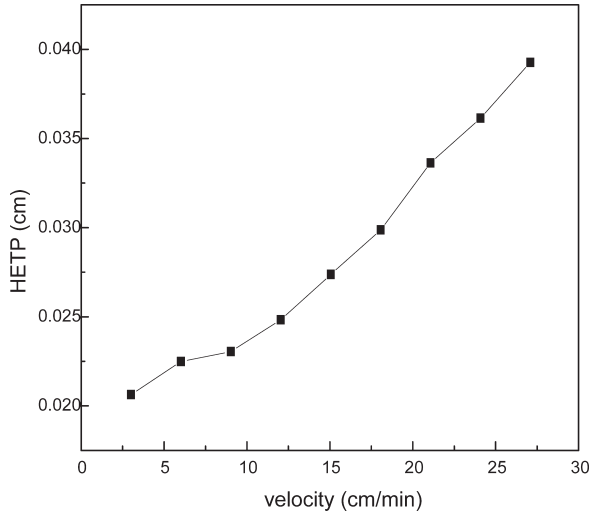

Figure 5. The relationship of between HETP and velocity for liquiritin in CN column with the mobile phase of acetonitrile-water (2:3, v/v) with pH 6.

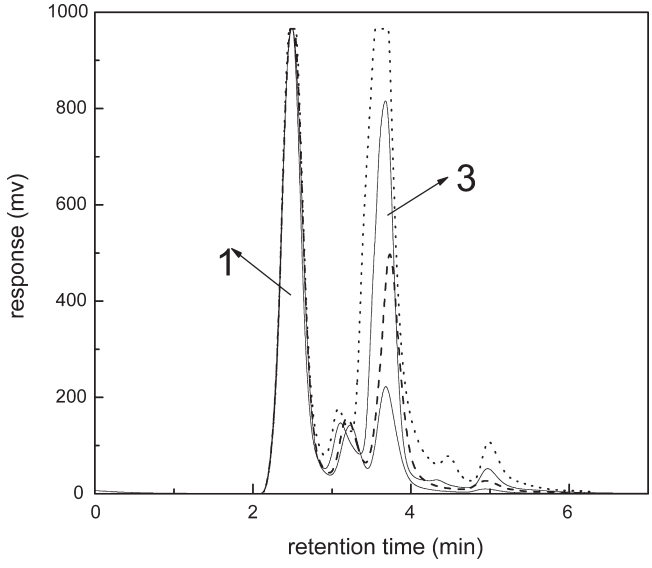

Figure 6. The effect of injection concentration of liquiritin on the elution curves in the second dimension. Mobile phase acetonitrile-water (2:3, v/v) with pH 6, flow rate 0.5 mL/min, injection volume 5 μL. The concentrations of liquiritin were 1, 3, 6, and 15 mg/mL, respectively. Room temperature was 19°C.

through the software Oringin 7.5, where q and C are the concentrations in stationary phase and mobile phase, respectively. Using Levenberg-Marquardt method to iterate 100 times, the Langmuir isotherms results are shown in Figure 7, which indicates that the non-linearity of isotherms in the two dimensional stationary phases is weaker. This is the reason why the influence of injection concentration on the 2D separation is not observed.

Effect of Temperatures on the Two Dimensional Separations

In fact, the changes in temperature would improve the efficiency of separation and resolution of the two dimensional liquid chromatography. In the temperature range of 25–40°C, the effect of temperatures on the separation of liquiritin and glycyrrhizic acid is not obvious in the second dimension.

The effect of temperature on the separation of components 1–4 in the first dimension is also studied (Figure 8). From Figure 8, the effect of temperature on the separation of components 2 and 4 are obvious in the SB C_{18} column. The raising of the temperature is good for the 2D separation of active components, which can not only improve the efficiency of the separation, but also decreases the consumption of organic solvent in the separate process.

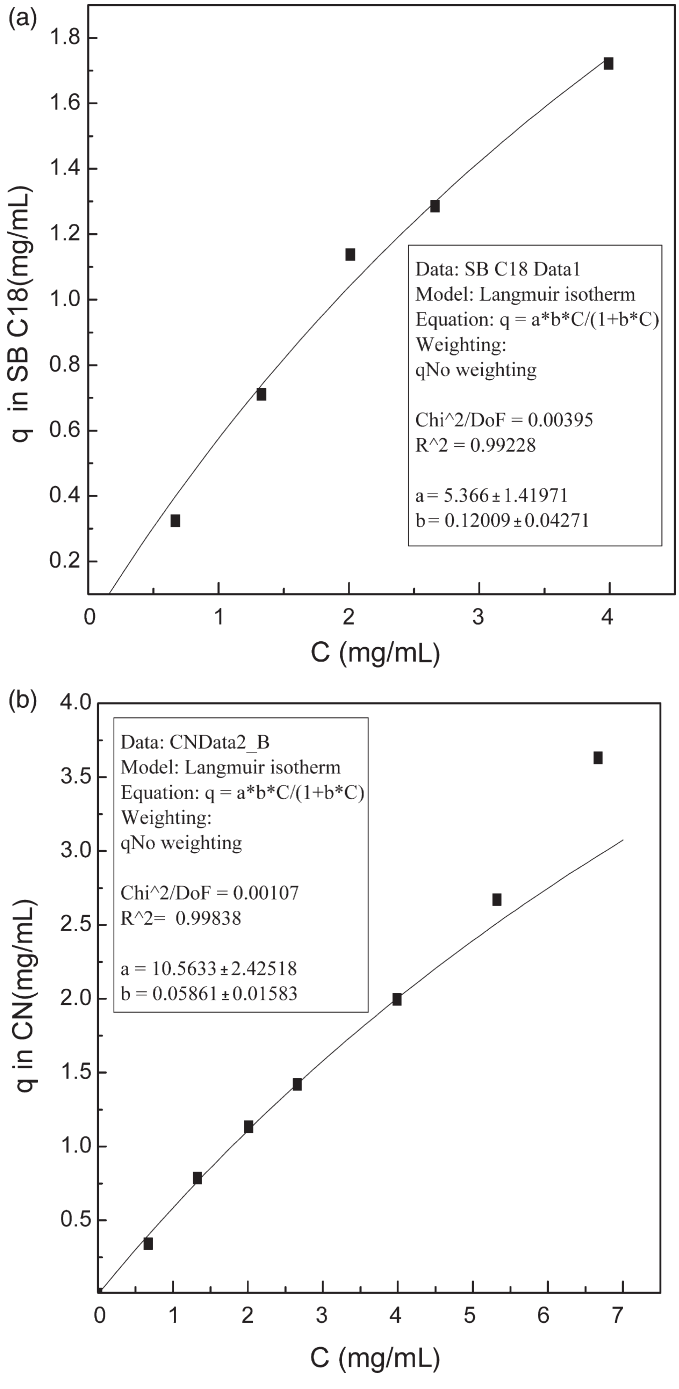

Figure 7. Isotherms of liquiritin in the stationary phases of SB C_{18} and CN.

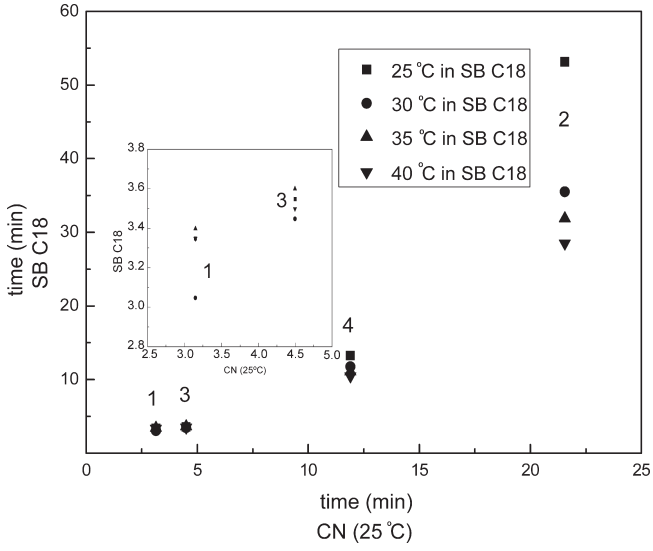

Figure 8. The relationships between the retention times of the four components and temperatures in the 2D system. In the SB C_{18} column, operating temperatures were 25°C (square, ■), 30°C (circle, •), 35°C (regular triangle, ▲), and 40°C (inverse triangle, ▼), respectively. In the CN column operating temperature was kept at 25°C. Mobile phase was acetonitrile-water (2:3, v/v) with pH 6 in both dimensions. Flow rate was 0.5 mL/min.

Thermodynamic Analysis of Temperature Effect on Separation of Licorice Components—Enthalpy and Entropy

The relationship between the logarithm of retention factors, k, and the reciprocal value of thermodynamic absolute temperature is:

$$\ln k = \frac{\Delta H}{RT} + \frac{\Delta S}{R} - \ln F$$

in RP-HPLC.[49] In the above equation, R is the universal gas constant 8.314 J mol^{-1} K^{-1}, F is the phase ratio of the column, i.e., the ratio of the volumes of stationary to mobile phase. ΔH and ΔS are the change in enthalpy and change in entropy, respectively, connected with the solute transfer from the mobile phase to the stationary phase. In this paper, the k value was obtained by the formula $k = t_R Q/V_0 - 1$, Q is the flow rate. In a certain temperature range, ln k and 1/T are linear, the ΔH and ΔS can be calculated by the slope and intercept, respectively. In the temperature range of 25–40°C, the relationships of ln k and the 1/T for the four components in the SB C_{18} column are shown in Figure 9. The regression equations and the calculated changes in entropy and changes in enthalpy for the four components in licorice are listed in Table 2.

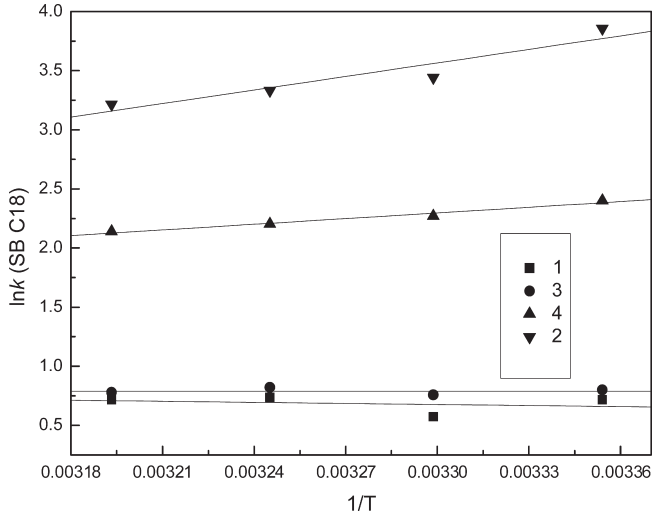

Figure 9. Relationship between ln k and $1/\mathrm{T}$ for the four components in the SB C_{18} column.

The typical changes in enthalpy of small molecules are about 10–15 kJ mol^{-1},[50] for the four components in licorice their enthalpy changes are about from 0.012 to 32 kJ mol^{-1}. In the experiment, the 18β-glycyrrhetinic acid and isoliquiritigenin in the SB C_{18} column are far away from each other, and their changes in enthalpy are negative, so the raising of temperature is good for the desorption of the two components. It is a better way through adjusting temperature to accelerate the separation of these components, and when the temperature is lower than 40°C, the activities of the components will also not be affected.

Molecular Structures and Retention

From the molecular structures of the five active components, the retention times of glycyrrhizic acid and liquiritin are small in the two dimensions.

Table 2. Regression equations for relationship between ln k and $1/\mathrm{T}$ and the calculated changes in entropy and changes in enthalpy for the four licorice components

Components	Regression equations	ΔH	ΔS
1	$\ln k_1 = 1.67 - 300.95/\mathrm{T}$	2502.04	15.87
2	$\ln k_2 = -9.04 + 3818.81/\mathrm{T}$	−31749.60	−73.27
3	$\ln k_3 = 0.79 - 1.46/\mathrm{T}$	12.15	8.59
4	$\ln k_4 = -2.98 + 1598.71/\mathrm{T}$	−13291.68	−22.78

The glycyrrhizic acid and liquiritin contain a glycosyl unit in their molecular structures and are probably easy to be dissolved in the mobile phase, and thus they were eluted faster. Glycyrrhitic acid and 18β-glycyrrhetinic acid belong to triterpenoids, they are similar in structure, but there is no glycosyl unit in 18β-glycyrrhetinic acid, which makes it easy to be adsorbed in stationary phase; therefore, its retention time is longer than glycyrrhitic acid. Though both isoliquiritigenin and licochalcone B belong to the chalcones, their retentions are quite different under the same conditions. Licochalcone B is not easy to be eluted from the stationary phase of C_{18}, probably because the functional group CH3 is easy to be adsorbed on the surface of stationary phase of C_{18} and this makes it difficult to be eluted.

CONCLUSIONS

The results show that the two dimensional liquid chromatography is a good tool to separate the multiple components from licorice. A suitable two-dimensional RP-RP system was found for the separation of these active components in this study, which was combined by SB C_{18} and CN as the first and the second dimensional stationary phases, respectively, and acetonitrile-water (2:3, v/v) with pH 6 as the mobile phase in each dimension. When the separation conditions, including the two dimensional phase system and factors which affected the separation, are considered carefully and arranged properly, the more active components with high purities in licorice can be separated effectively through two-dimensional liquid chromatography. The results indicated that to decrease the flow rate properly is good for gaining high purified components; to adjust the temperature properly will improve the efficiency of two-dimensional separation and decrease the consumption of organic solvent. To a certain extent, the influence of injection concentration on the separation of active components in two-dimensional system is not obvious.

REFERENCES

1. Agarwal, M.K.; Iqbal, M.; Athar, M. Inhibitory effect of 18β-glycyrrhetinic acid on 12-O-tetradecanoyl phorbol-13-acetate-induced cutaneous oxidative stress and tumor promotion in mice. Redox Rep. **2005**, *10*, 151–157.
2. Takei, M.; Kobayashi, M.; Herndon, D.N.; Pollard, R.B.; Suzuki, F. Glycyrrhizin inhibits the manifestations of anti-inflammatory responses that appear in association with systemic inflammatory response syndrome (SIRS)-like reactions. Cytokine **2006**, *35* (5–6), 295–301.
3. Matsui, S.; Matsumoto, H.; Sonoda, Y.; Ando, K.; Aizu-Yokota, E.; Sato, T.; Kasahara, T. Glycyrrhizin and related compounds down-regulate production of inflammatory chemokines IL-8 and eotaxin 1 in a human lung fibroblast cell line. Int. J. Immunopharmacol. **2004**, *15*, 1633–1644.

4. Fujisawa, Y.; Sakamoto, M.; Matsushita, M.; Fujita, T.; Nishioka, K. Glycyrrhizin inhibits the lytic pathway of complement—possible mechanism of its anti-inflammatory effect on liver cells in viral hepatitis. Microbiol. Immunol. **2000**, *44* (9), 799–804.
5. Ito, M.; Sato, A.; Hirabayashi, K.; Tanabe, F.; Shigeta, S.; Baba, M. De Clercq, E.; Nakashima, H.; Yamamoto, N. Mechanism of inhibitory effect of glycyrrhizin on replication of human immunodeficiency virus (HIV). Antiviral Res. **1988**, *10*, 289–298.
6. Mori, K.; Sakai, H.; Suzuki, S.; Sugai, K.; Akutsu, Y.; Ishikawa, M.; Seino, Y.; Ishida, N.; Uchida, T.; Kariyone, S.; Endo, Y.; Miura, A. Effect of glycyrrhizin (SNMC: Stronger Neo-Minophagen C) in hemophilia patients with HIV infection. Tohoku J. Exp. Med. **1989**, *158*, 25–35.
7. Sasaki, H.; Takei, M.; Kobayashi, M.; Pollard, R.B.; Suzuki, F. Effect of glycyrrhizin, an active component of licorice roots, on HIV replication in cultures of peripheral blood mononuclear cells from HIV sero(+) patients. Pathobiology **2002**, *70*, 229–236.
8. Pompei, R.; Flore, O.; Marccialis, M.A.; Pani, A.; Loddo, B. Glycyrrhizic acid inhibits virus growth and inactivates virus particles. Nature **1979**, *281*, 689–690.
9. Utsunomiya, T.; Kobayashi, M.; Pollard, R.B.; Suzuki, F. Glycyrrhizin, an active component of licorice roots, reduces morbidity and mortality of mice infected with lethal doses of influenza virus. Antimicrob. Agents Chemother. **1997**, *41*, 551–556.
10. Soo, L.-C.; Jeong, K.-Y.; Sook, H.-E. Glycyrrhizin protection against 3-morpholinosydnonime-induced mitochondrial dysfunction and cell death in lung epithelial cells. Life Sci. **2007**, *80* (19), 1759–1767.
11. Jeong, H.G.; You, H.J.; Park, S.J.; Moon, A.R.; Chung, Y.C.; Kang, S.K.; Chun, H.K. Hepatoprotective effects of 18β-glycyrrhetinic acid on carbon tetrachloride-induced liver injury: inhibition of cytochrome P450 2E1 expression. Pharmacol. Res. **2002**, *46*, 221–227.
12. Ito, A.; Hayashi, N.; Katayama, K.; Hagiwara, H.; Kasahara, A.; Kashiwagi, T.; Yoshihara, H.; Fusamoto, H.; Kamada, T. Effect of glycyrrhizin on viral replication and quasispecies in patients with type C chronic hepatitis. Int. Hepatol. Commun. **1997**, *50* (6), 233–238.
13. Cinatl, J.; Morgenstern, B.; Bauer, G.; Chandra, P.; Rabenau, H.; Doerr, H.W. Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus. Lancet **2003**, *361* (9374), 2045–2046.
14. Hoever, G.; Baltina, L.; Michaelis, M.; Kondratenko, R.; Baltina, L.; Tolstikov, G.A.; Doerr, H.W.; Cinatl, J., Jr. Antiviral activity of glycyrrhizic acid derivatives against SARS-coronavirus. J. Med. Chem. **2005**, *48*, 1256–1259.
15. Rafi, M.M.; Vastano, B.C.; Zhu, N.; Ho, C.-T.; Ghai, G.; Rosen, R.T.; Gallo, M.A.; DiPaola, R.S. Novel polyphenol molecule isolated from licorice root (*Glycrrhiza glabra*) induces apoptosis, G2/M cell cycle arrest, and Bcl-2 phosphorylation in tumor cell lines? J. Agric. Food Chem. **2002**, *50* (4), 677–684.
16. Fukai, T.; Marumo, A.; Kaitou, K.; Kanda, T.; Nomura, T. Anti-Helicobacter pylori flavonoids from licorice extract. Life Sci. **2002**, *71*, 1449–1463.
17. Kumar, S.; Sharma, A.; Madan, B.; Singhal, V.; Ghosh, B. Isoliquiritigenin inhibits IκB kinase activity and ROS generation to block TNF-α induced expression of cell adhesion molecules on human endothelial cells. Biochem. Pharmacol **2007**, *73* (10), 1602–1612.

18. Waya, J.; Belinky, P. Antioxidant Constituents from Licorice Roots: Isolation, structure elucidation and antioxidation, capacity toward LDL oxidation. Free Rad. Biol. Med. **1997**, *23* (2), 302–313.
19. Amer, M.; Metwalli, M. Topical liquiritin improves melasma. Int. J. Dermatol. **2000**, *39* (4), 299–301.
20. Hatano, T.; Yasubara, T.; Miyamoto, K.; Okuda, T. Anti-human immunodeficiency virus phenolics from licorice. Chem. Pharm. Bull. **1988**, *36* (6), 2286–2288.
21. Jung, J.I.; Lim, S.S.; Choi, H.J.; Cho, H.J.; Shin, H.K.; Kim, E.J.; Chung, W.-Y.; Park, K.-K.; Park, J.H.Y. Isoliquiritigenin induces apoptosis by depolarizing mitochondrial membranes in prostate cancer cells. J. Nutrit. Biochem. **2006**, *17* (10), 689–696.
22. Kanazawa, M.; Satomi, Y.; Mizutani, Y.; Ukimura, O.; Kawauchi, A.; Sakai, T.; Baba, M.; Okuyama, T.; Nishino, H.; Miki, T. Isoliquiritigenin inhibits the growth of prostate cancer. Eur. Urol. **2003**, *43* (5), 580–586.
23. Hsu, Y.L.; Kuo, P.L.; Lin, L.T.; Lin, C.C. Isoliquiritigenin inhibits cell proliferation and induces apoptosis in human hepatoma cells. Planta Med. **2005**, *71* (2), 130–134.
24. Barfod, L.; Kemp, K.; Hansen, M.; Kharazmi, A. Chalcones from Chinese liquorice inhibit proliferation of T cells and production of cytokines. Int. J. Immunopharmacol. **2002**, *2* (4), 545–555.
25. Liu, Y.-C.; Hsieh, C.-W.; Wu, C.-C.; Wung, B.-S. Chalcone inhibits the activation of NF-κB and STAT3 in endothelial cells via endogenous electrophile. Life Sci. **2007**, *80* (15), 1420–1430.
26. Giddings, J.C. Two-dimensional separations: Concept and promise. Anal. Chem. **1984**, *56*, 1258–1270A.
27. Ma, S.; Chen, L.X.; Luo, G.A.; Ren, K.N.; Wu, J.F.; Wang, Y.M. Off-line comprehensive two-dimensional high-performance liquid chromatography system with size exclusion column and reverse phase column for separation of complex traditional Chinese medicine Qingkailing injection. J. Chromatogr. A **2006**, *1127* (1–2), 207–213.
28. Stroink, T.; Wiese, G.; Teeuwsen, J.; Lingeman, H.; Waterval, J.C.M.; Bult, A.; Jong, G.J.; de. Underberg, W.J.M. On-line coupling of SEC and RP-LC for the determination of structurally related enkephalins in cerebrospinal fluid. J. Pharm. Biomed. Anal. **2002**, *30*, 1393–1401.
29. Huber, J.F.K.; Ecker, R.; Van Der Linden, E.; Oreans, M. Column switching in high-pressure liquid chromatography. J. Chromatogr. **1973**, *83* (29), 267–277.
30. Erni, F.; Frei, R.W. Two-dimensional column liquid chromatographic technique for resolution of complex mixtures. J. Chromatogr. **1978**, *149*, 561–569.
31. Bushey, M.M.; Jorgenson, J.W. Automated instrumentation for comprehensive two-dimensional high-performance liquid chromatography of proteins. Anal. Chem. **1990**, *62*, 161–167.
32. Lim, K.B.; Kassel, D.B. Phosphopeptides enrichment using on-line two-dimensional strong cation exchange followed by reversed-phase liquid chromatography/mass spectrometry. Anal. Chem. **2006**, *354* (2), 213–219.
33. Wagner, K.; Miliotis, T.; Marko-Varga, G.; Bischoff, R.; Unger, K.K. An automated on-line multidimensional HPLC system for protein and peptide mapping with integrate sample preparation. Anal. Chem. **2002**, *74*, 809–820.
34. Gilar, M.; Olivova, P.; Daly, A.E.; Gebler, J.C. Orthogonality of separation in two-dimensional liquid chromatography. Anal. Chem. **2005**, *77*, 6426–6434.

35. Peng, J.; Elias, J.E.; Thoreen, C.C.; Licklider, L.J.; Gygi, S.P. Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large scale protein analysis: the yeast proteome. J. Proteome Res. **2005**, *4*, 2283–2293.
36. Machtejevas, E.; Andrecht, S.; Lubda, D.; Unger, K.K. Monolithic silica columns of various format in automated sample clean-up/multidimensional liquid chromatography/mass spectrometry for peptidomics. J. Chromatogr. A **2007**, *1144*, 97–101.
37. Karty, J.A.; Running, W.E.; Reilly, J.P. Two dimensional liquid phase separations of proteins using online fractionation and concentration between chromatographic dimensions. J. Chromatogr. B. **2007**, *847* (2), 103–113.
38. Machtejevas, E.; Denoyel, R.; Meneses, J.M.; Kudirkaitė, V.; Grimes, B.A.; Lubda, D.; Unger, K.K. Sulphonic acid strong cation-exchange restricted access columns in sample cleanup for profiling of endogenous peptides in multidimensional liquid chromatography: Structure and function of strong cation-exchange restricted access materials. J. Chromatogr. A **2006**, *1123* (1), 38–46.
39. Wong, V.; Shalliker, R.A. Isolation of the active constituents in natural materials by "heart-cutting" isocratic reversed-phase two-dimensional liquid chromatography. J. Chromatogr. A **2004**, *1036*, 15–24.
40. Wong, V.; Sweeney, A.P.; Shalliker, R.A. Using analytical multidimensional isocratic HPLC methods of separation to isolate active constituents in natural products. J. Sepn. Sci. **2004**, *27* (1–2), 47–52.
41. Hu, L.; Chen, X.; Kong, L.; Su, X.; Ye, M.; Zou, H. Improved performance of comprehensive two-dimensional HPLC separation of traditional Chinese medicines by using a silica monolithic column and normalization of peak heights. J. Chromatogr. A **2005**, *1092* (2), 191–198.
42. Chen, X.; Kong, L.; Su, X.; Fu, H.; Ni, J.; Zhao, R.; Zou, H. Separation and identification of compounds in Rhizoma chuanxiong by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry. J. Chromatogr. A **2004**, *1040* (2), 169–178.
43. Whelan, T.J.; Gray, M.J.; Slonecker, P.J.; Shalliker, R.A.; Wilson, M.A. Study of the selectivity of reversed-phase columns for the separation of polycarboxylic acids and polyphenol compounds. J. Chromatogr. A **2005**, *1097* (1–2), 148–156.
44. Blahová, E.; Jandera, P.; Cacciola, F.; Mondello, L. Two-dimensional and serial column reversed-phase separation of phenolic antioxidants on octadecyl-, polyethyleneglycol-, and pentafluorophenylpropyl-silica columns. J. Sepn. Sci. **2006**, *29*, 555–566.
45. Jandera, P.; Skeifikova, V.; Rehova, L.; Hajek, T.; Baldrianova, L.; Skopova, G.; Kellner, V.; Horna, A. RP-HPLC analysis of phenolic compounds and flavonoids in beverages and plant extracts using a CoulArray detector. J. Sepn. Sci. **2005**, *28* (9–10), 1005–1022.
46. Cong, J.; Lin, B. Separation of Liquiritin by simulated moving bed chromatography. J. Chromatogr. A **2007**, *1145* (1–2), 190–194.
47. McCalley, D.V. Analysis of the Cinchona alkaloids by high-performance liquid chromatography and other separation techniques. J. Chromatogr. A **2002**, *967*, 1–19.
48. McCalley, D.V. Evaluation of reversed-phase columns for the analysis of very basic compounds by high-performance liquid chromatography: Application to the determination of the tobaccs alkaloids. J. Chromatogr. A **1993**, *636*, 213–220.

49. Greibrokk, T.; Andersen, T. High-temperature liquid chromatography. J. Chromatogr. A **2003**, *1000*, 743–755.
50. Xu, J.; Tanaka, H.; Shoyama, Y. One-step immunochromatographic separation and ELISA quantification of glycyrrhizin from traditional Chinese medicines. J. Chromatogr. B **2007**, *850* (1–2), 53–58.

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