1 **Purification of high-purity glycyrrhizin from licorice using**
2 **hydrophilic interaction solid phase extraction coupled with**
3 **preparative reversed-phase liquid chromatography**

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15
16 **Abstract**

17 Glycyrrhizin (GA), a major bioactive compound in licorice, has been
18 extensively used throughout the world as a medicine to treat chronic viral hepatitis and
19 allergic dermatitis. In this study, a new method based on hydrophilic interaction solid
20 phase extraction (HILIC-SPE) and preparative reversed-phase liquid chromatography
21 (prep-RPLC) was developed to purify GA with high purity from the complex licorice
22 extract. Via evaluation of retention behavior of GA and flavonoids in different
23 commercially available columns, a hydrophilic column——Click XIon was finally
24 chosen for the purification due to its excellent resolution toward GA and flavonoids
25 under HILIC mode. To optimize the SPE elution conditions, relative factors including
26 water content, pH and ionic strength had been investigated in chromatographic
27 condition. The result indicated that the most appropriate water content was 30% and

pH at 4.00, as well as salt concentration should be controlled at 5mM. In addition, the optimization revealed that GA experiences both hydrophilic interaction and ion-exchange interaction on the Click XIon material. According to the chromatographic evaluation, the optimized conditions were applied to HILIC-SPE to enrich GA from licorice, which leads to an increased content of GA from 13.67% to 64.22%. Finally, prep-RPLC was performed to obtain GA with purity higher than 99.00%, which demonstrating great prospect in large-scale preparation of GA.

Keywords: Glycyrrhizin / Hydrophilic interaction solid phase extraction / Licorice / preparative reversed-phase liquid chromatography

1 Introduction

Licorice, also named as GanCao, one of the most famous traditional Chinese Medicines (TCMs) which had been used to treat cough, bronchitis, arthritis, adrenal insufficiency, and allergies for more than 4000 years[1]. The major active component of licorice is Glycyrrhizin (GA), a weak acid possess high intense sweetness thus has been used extensively throughout the world as a functional additive in food, beverages, cosmetics and tobacco industries[2]. Modern pharmacological studies have proved that GA possesses many pharmacological bioactivities, such as anticancer, anti-inflammatory, antihepatotoxic, antiviral and antiulcer properties [3-5]. Recent studies also demonstrated its functions regarding attenuating the neuroinflammation and oxidative stress in a rotenone model of Parkinson's disease[6]. Moreover, in many countries, GA has also been used as a therapeutic agent to treat chronic viral hepatitis and allergic dermatitis [7-9]. Purification of GA with high-purity from licorice is of great importance for its pharmacological research and drug industry. Hence, the purification of GA from the natural extract *Licorice*, especially for that of high-purity (>98.00%), had drawn tremendous interest of the scientists. Up to now, it is still a challenge to purify GA with high purity from licorice due to the complicated composition of licorice and ubiquitous interferences with other compounds especially with licorice flavonoids. There are lots of earlier works being done in GA purification.

1 Zhenget al. used polyamide resins and macroporous resins to purify GA, and increased
2 the purity of GA from **11.40%** to **88.95%**[10]. High-speed counter-current
3 chromatography were adopted by Jiang et al to yield GA with purity of **96.8%** within
4 a **350 min** separation procedure[11]. Although these techniques can isolate GA from
5 licorice, their procedures are tedious and time consuming, and the purity of final
6 product remains to be improved.

7 As one of the most efficient purification techniques, preparative high
8 performance liquid chromatography (prep-HPLC) provides numerous advantages
9 such as high efficiency and good reproducibility in the separation of naturally
10 occurring compounds[12, 13]. Currently, the prep-RPLC has received increasing
11 interest in the separation and determination of GA [14, 15]. However, in large-scale
12 preparation of GA, directly loading the crude extract into prep-RPLC seems
13 impossible due to its complicated chemical composition and widely polarity scope.
14 Herein, sample pretreatment before prep-RPLC is necessary to simplify the crude
15 extract. Of many sample pretreatment techniques, solid-phase extraction (SPE) has the
16 advantages of low cost, simple to operate and rapid phase separation[16-19]. Sorbent
17 plays a significant role in the applications of SPE for different them can provide
18 different selectivity to the targets[18]. Several kinds of sorbents had been early
19 developed by Kyung et al. to extract GA from licorice, but purification of GA with
20 these sorbents in an industrial scale had not been tried yet[20, 21]. Zheng et al. used
21 polyamide resins to enrich GA, but the effect is not so satisfactory that merely
22 increases the content of GA from **11.40%** to **14.96%**[10]. SPE sorbents will influence
23 not only the enrichment but also the loading amount prior to prep-RPLC. Therefore a
24 suitable adsorbent that can selectively enrich GA from licorice was expected to greatly
25 improve the purification efficiency.

26 Hydrophilic interaction liquid chromatography (HILIC) has been perceived as a
27 promising technique in separation of highly polar analytes or those with polar groups
28 such as saccharides and saponins[22-24]. Saponins and flavonoids are two kinds of
29 major chemical compositions in licorice. Flavonoids exhibited remarkably high
30 structural diversities and a wide range of polarity, and seriously interference the

1 separation of GA. GA is an olean-type triterpenesaponin that contains two
2 glycones and three carboxyls (**Fig.1**). Its structural characteristics make GA sufficiently
3 hydrophilic and well retained on the HILIC stationary phase [25]. Moreover, most of
4 flavonoids in licorice can hardly be retained in the HILIC mode, for example
5 prenylated flavonoids and flavonoid aglycones [25]. Therefore, we suspected that SPE
6 in the HILIC mode could enrich GA as well as some saponins that had good
7 hydrophilicity, and remove flavonoids and other compounds of weak hydrophilicity, thus
8 simplify the extract and make it easy to load onto prep-RPLC.

9 In this paper, we developed a HILIC-SPE method to efficiently enrich GA through
10 evaluating the retention behavior of GA and flavonoids on HILIC columns. Excellent
11 results had been achieved by us that the purity of GA was improved from 13.67% to
12 64.22% in one step pretreatment. After enriched by SPE, prep-RPLC was performed
13 to purify and obtain GA with more than 99.0% purity.

14 **2 Material and methods**

15 **2.1 Apparatus and reagents**

16 Alliance HPLC apparatus was employed for sample analysis consisting of a Waters
17 2489 UV detector and a Waters 2695 HPLC pump. Data acquisition and processing
18 were conducted by Waters Empower software (Milford, MA, USA). Column
19 temperature was maintained at 30 °C during experiments.

20 Preparative separation of GA was performed on the Purification Factory (Waters,
21 Milford, MA, USA), which consist of two Waters 2525 binary gradient modules and a
22 2498 ultraviolet (UV) detector (Waters). The equipped autosampler was from
23 Leap Technologies (Carrboro, NC, USA). Data were acquired by a Masslynx
24 workstation (Waters, version 4.1).

25 LC-MS analysis was performed on the same HPLC mentioned above that coupled
26 with a ZQ single quadrupole mass spectrometry (Waters Corporation). The mass
27 spectrometry was equipped with an ESI ion source. Data were acquired and processed
28 by MassLynx version 4.0 (Holliston, MA, USA). Capillary voltage, cone voltage, and
29 source temperature as well as desolvation gas flow rate were set at 3.0 kV, 25.0 kV,

120 °C and 650 L/h, respectively. Desolvation temperature was 350 °C and cone gas flow rate was 50 L/h; mass range was 100 to 1200.

Unitary C18 column (150 mm × 4.6 mm, I.D., 5 μm), Unitary C18 column (250 mm × 20 mm, I.D., 10 μm), Unitary Diol column (150 mm × 4.6 mm, I.D., 5 μm), XAmide column (150 mm × 4.6 mm, I.D., 5 μm), Click XIon column (150 mm × 4.6 mm, I.D., 5 μm), and Click XIon SPE cartridge (30 g / 60 mL) were all purchased from Acchrom Technologies Co., Ltd. (Beijing, China). Reference compounds liquiritin, liquiritigenin, isoliquiritigenin and licochalcone A (purity > 98%) were acquired from Sichuan Victory Biotechnology Co., Ltd. (Sichuan, China), and GA was purchased from Acchrom Technologies Co., Ltd. (Beijing, China) with purity greater than 70%. Industrial grade ethanol and analytical grade acetonitrile (ACN) were purchased from Yuwang Chemical Reagent Factory (Shandong, China). HPLC-grade ethanol was purchased from Kermel (Tianjin, China). HPLC-grade formic acid (FA) and ammonium formate (NH₄FA) were purchased from J&K Chemical (Hebei, China). Water for HPLC mobile phase was prepared by a Milli-Q system (Billerica, MA, USA).

2.2 Sample preparation

Licorice (*Glycyrrhiza inflata bat.*) was obtained from Qinghai Lake Pharmaceutical Co., Ltd. and its rhizome (1 Kg) was smashed into powders and extracted by an ultrasonic-assisted method for three times with 1 L, 0.8 L and 0.6 L of ethanol/water (70:30, v/v) solutions for 3h respectively. Subsequently, three filtrates were combined and rotated to dryness. Finally, 347.9 g solids extract was obtained and 3.4 g of them re-dissolved in 20 mL ethanol/water (70:30, v/v) solution and filtered through a 0.45 μm pore size membrane. The solid concentration was determined as 171.4 mg/mL.

2.3 Chromatographic conditions

For HILIC evaluation, the separation was performed on a XAmide column (150 mm × 4.6 mm, I.D., 5 μm). The mobile phase A was acetonitrile, B was 100 mM ammonium formate in water (pH 6.33), and C was water. Gradient elution steps were as follows:

0-30min, 80-50% A, 10-40% C, the proportion of mobile phase B was maintained at 10%, so the salt concentration in the whole mobile phase system was kept at 10 mM. The flowrate was 1.0 mL/min. Detection was carried out at 265 nm. The column temperature was maintained at 30 °C.

For the selection of HILIC material, three columns were used including XAmide column (150 mm × 4.6 mm, I.D., 5 µm), Unitary Diol column (150 mm × 4.6 mm, I.D., 5 µm), and Click XIon column (150 mm × 4.6 mm, I.D., 5 µm). The mobile phase conditions were the same as the HILIC evaluation.

The mobile phase optimization was carried out on Click XIon column (150 mm × 4.6 mm, I.D., 5 µm). On account of connectivity between the extract solvent and SPE loading solvent, we chose ethanol as an organic phase, which was also more economic and of less pollution. 100 mM NH₄FA was prepared as stock solutions and adjusted to the required pH (pH 6.35, pH 5.00, pH 4.26, pH 3.37 and pH 2.77) by adding different amount of FA. Binary mobile phases were prepared by mixing ethanol with NH₄FA stock solutions. The NH₄FA concentrations in this work represent final concentration in the mobile phase. The flow rate was maintained at 1.0 mL/min; column temperature 30 °C and UV detection 265 nm.

The purity determination was performed on an analytical Unitary C18 column (150 mm × 4.6 mm, I.D., 5 µm). The mobile phase A was acetonitrile, the mobile phase B was 0.1% FA in water. Gradient elution condition was 0-5 min, 5% A; 5-25 min, 5-95% A; and 25-30 min, 95% A. The flow rate was 1.0 mL/min. Detection was carried out at 265 nm and the column temperature was maintained at 30 °C. The purity of GA was estimated by calculating the area under the curve (AUC) in 265 nm.

2.4 SPE procedure

After activation and equilibration in 240 mL of ethanol/water solution (70:30, v/v), the 20 mL (171.4 mg/mL) crude extract sample was loaded into a Click XIon solid phase extraction cartridge (30 g/60 mL), then 240 mL of ethanol/water solution (70:30, v/v) was used as the first elution step solvent to obtain a non-GA fraction. All solvents used in the above procedure were adjusted to pH 4.0 and 5 mM salt environment with

FA and NH₄FA, respectively. Eventually, a GA fraction was collected with 320 mL of ethanol/water solution (40:60, v/v) with 5% FA. The two fractions were concentrated by rotary evaporation in vacuum at 50 °C and lyophilized in parallel.

2.5 Prep-RPLC conditions

The isolation of GA was performed on prep-RPLC system using a Unitary C18 column (250 mm × 20 mm, I.D., 10 μm). The mobile phase was 0.1% FA in ethanol/water (50:50, v/v). Isocratic elution was performed for 30 min. The detection wavelength was 265 nm, and flow rate was 20 mL/min. 193.72 mg GA-fraction powders were totally purified after injections of four times.

3 Result and discussion

3.1 Evaluation of HILIC separation

To estimate the feasibility of HILIC-SPE, we evaluate the retention behaviors of GA and flavonoids in HILIC mode with reference GA and four predominant flavonoid compounds from licorice, including liquiritin, liquiritigenin, isoliquiritigenin and licochalcone A. As shown in **Fig. 1 (A)**, good chromatographic retention is observed for GA on the HILIC column. In contrast, flavonoids were completely eluted near the dead time. This result demonstrates that glycyrrhizin and flavonoid compounds have distinct retention behaviors in HILIC mode. This is probably due to the difference in the numbers and diversities of hydrophilic glycosyls in GA and flavonoids. As further proof, licorice crude extracts was analyzed on XAmide column with the same analysis conditions of reference compounds. As displayed in **Fig.1 (B)**, the peak was clearly separated into two parts which is in accordance with the chromatogram of reference compounds. After validating by LC-MS, GA was demonstrated exist in the well-retained part (**Fig.S1, Table. S1 and S2**). Therefore, HILIC displayed considerable potential for the enrichment of GA in licorice.

3.2 Selection of HILIC stationary phases

Choosing proper stationary phases is a key part in chromatographic separation.

Based on above results, we tended to adopt a HILIC stationary phase with better retention for GA in order to enhance separation selectivity. The retention of GA on three commercially available HILIC columns with different materials was compared under the same mobile phase conditions. The chromatograms were shown in **Fig. 2**. Unitary Diol column contains diol groups bonding with the silica sphere. XAmide column contains amide group. Click XIon contains zwitterionic groups grafted to silica via polar groups. The weak retention for GA was observed on the Unitary Diol column (**Fig. 2A**). On XAmide column, the retention was improved (**Fig. 2B**). Whereas, the Click XIon column shows strongest retention of GA (**Fig. 2C**), which is of beneficial to SPE separation. As a result, we choose Click XIon material for the enrichment of GA in licorice.

3.3 Optimization of mobile phase conditions

In order to further improve the separation selectivity between GA and flavonoids, the mobile phase conditions were optimized on the Click XIon column. Various mobile phase conditions, including water content, pH value, and salt concentration were investigated. It is worth mentioned that, most mobile phase in hydrophilic mode were based on acetonitrile, a toxic solvent as we all knows [26-28]. In this paper, ethanol was tried to displace acetonitrile as it is environmental friendly and of low pollution. The challenge of the displacement was that the eluting power of water/ethanol is much stronger than that of water/acetonitrile in the HILIC mode, thus it required GA could well retained on stationary phase. Fortunately, GA has showed sufficient retention on the Click XIon material when ethanol was tested. Thus ethanol was employed in the mobile phase optimization steps instead of acetonitrile.

Since mobile phase strength plays a vital role in HILIC separation, the influence of water content to the retention behavior of GA on the Click XIon column was firstly studied. When the content of water increased from 20% to 35%. The retention factor (k) of GA is decreased from 16.8 to 2.3 (**Fig. 3A**), which is in accordance with typical retention characteristic of HILIC. Thus, the retention of GA was significantly susceptible to the change of the water content in the Click XIon column.

Given that the ionization of GA and zwitterionic groups on the Click XIon material is pH dependent, pH values in the mobile phase should be taken into consideration in the method development. The separation of GA was conducted on the Click XIon column in the pH range of **2.77-6.35**. Salt concentration was fixed at **10 mM**. As presented in **Fig. 3B**, the retention of GA increases with pH till the value reached **4.26** and became reverse once the value was larger than **4.26**. Thus, the improved retentions of GA were observed in the pH 2.77-4.26. The result could have been caused by electrostatic interaction. The ionization of GA (**pKa 2.6**) increases within the **pH 2.77-4.26**, while the zwitterionic groups on the Click XIon column were positively charged. Thus, the most appropriate pH for retention of GA should be kept around **4.26**. Furthermore, the ionization of GA is barely changed in the pH range of **4.26-6.35**, whereas the negatively charged characteristic of the stationary phase would be enhanced with the increase of pH. As a result, the reduced retentions of GA presented within pH **4.26-6.35**.

As electrostatic attraction might be involved in the retention of GA on the Click XIon column, the influence of salt concentration on the solute retention was further investigated. The proportion of ethanol was maintained at **75%**. NH_4FA in different concentrations (**5, 10, 20, 30, 40, 50 mM**) was added because of its compatibility to organic modifier. The pH of buffer was all controlled at 4.28. As displayed in **Fig. 3C**, the retention factor of GA decreases with the increase of buffer concentration in mobile phases, which is due to the decrease of ion-exchange interactions between GA and zwitterionic groups on the surface of the Click XIon column as a result of the electrostatic screen effect. Indeed, not only hydrophilic interaction, electrostatic interaction also provides significant contribution to the retention of GA on Click XIon stationary phase.

As a conclusion, although the retention of GA in Click XIon increases with the volume percentage of ethanol, the solubility of GA decreases when the proportion of ethanol increases from **65%** to **80%**. Therefore, in consideration of both the retention and solubility of GA, **70%** ethanol/water (v/v) was used to extract GA from licorice powders, and after a membrane concentration treatment, the crude extract of

1 **70%**ethanol/water (v/v) could be directly loaded on the SPE, saving the trouble of
2 solvent exchange and making the protocolsimpler. According to the optimization of
3 pH and buffer concentration, all solvents used in the activation, equilibration, loading
4 and washing procedurein the SPEshould be adjusted to **pH 4.26** in the mixture of **5**
5 **mM** NH₄FA for the sake of best retention of GA. After above procedure, a proper
6 solution was needed to quickly elute GA from the SPE cartridge. Theoretically,
7 ethanol solutions with a high percentage of water (>**35%**), pH lower than 2 and high
8 concentrations of NH₄FA (>**50 mM**) could be used, but plenty of salt will suffer from
9 tedious post-treatment. Thereby**5%** FA in **40%** ethanol/water (v/v) was finally chosen
10 to elute GA from the SPE sorbent because of the retention characteristics of HILIC
11 and GA has a high solubility in **40%** ethanol/water (v/v), and FA was added to inhibit
12 the ion exchange interaction.

13 **3.4 Enrichment of GA from licorice**

14 The SPE procedure wasestablished according to chromatographic evaluation.The
15 Click XIon material was used in the SPE for theenrichment of GA from complex
16 matrices. **3.4 g** ofcrude extract was loaded on a Click XIon cartridge (30 g/60 mL). As
17 a result, **0.98 g**GA fraction was obtained. The content of GA greatly increases from
18 **13.67%** in the extract (**Fig. 4A**) to **64.22%** in the GAfraction (**Fig. 4B**). In addition, it
19 is noticed that theHILIC-SPE is effective in resolving the co-elution problem of GA in
20 C18 column. Many compounds in the non-GA fraction, such as peaks**17, 18** and **19**
21 (**Fig. 4C**), have close retention times to GA in RPLC (**Fig. 4B**).Separation in RP mode
22 will have difficulties in isolating GA and collecting it with high-purity. However,
23 these compounds were separated into different fractions by HILIC-SPE. The
24 complexity of the GA-fraction was largely reduced, which isbeneficial to the
25 preparation and purification of GA with high efficiency.

26 To further investigate the performance of SPE, LC-MS analysis wasemployed to
27 determine the chemical components in theGA and non-GA fractions.The molecular
28 weights of all the detected peaks in the GAfraction were greater than 800 Da (**Table.**
29 **S3**), which could be ascribed to saponins tentatively [29].The above results reveal

that HILIC-SPE is feasible to achieve class separation of saponins in licorice, which also helps pharmacological research and phytochemical separation of saponin compounds.

3.5 Purification of GA by preparative RPLC

After purifying the crude extract by SPE, prep-RPLC was followed to further separate GA from the GA fraction on a Unitary C18 column. In order to acquire a satisfactory resolution, chromatographic conditions were optimized on analytical Unitary C18 column, and then transferred to a preparative scale. The mobile phase condition was set as 0.1% FA in 50% ethanol/water (v/v). On this condition the GA-fraction obtained from the SPE can be directly loaded to the RP column after a simple concentration pretreatment without the risk of solvent effect. Fraction from 14.00 min to 16.00 min was collected during injections of four times (the preparation chromatogram in Fig. 5A). After drying by lyophilization 49.6 mg white powder was obtained. In addition, it is noteworthy that “heart-cutting” was used as a repeated purification strategy to ensure the purity of GA. Purity analysis indicates that HPLC purity of the collected GA was higher than 99.0% (Fig. 5B).

4 Conclusion

In this study, a new method was successfully developed to efficiently purify GA with high purity through application of HILIC-SPE and prep-RPLC. After SPE enrichment, the content of GA was fast increased from 13.67% to 64.22%. Some substances co-eluted with GA in the RPLC can be removed from the sample by the HILIC-SPE. Meanwhile, this HILIC-SPE procedure may manage a class separation of saponins, which will be significant to the phytochemical separation and bioactivity research of saponin compounds. Prep-RPLC can further separate GA into purity higher than 99.0%. The whole process is environmental friendly, of low toxicity and good solvent compatibility. Thus, this protocol has the prospect of large-scale preparation of high-purity GA, laying the foundation of a wide use of GA in the pharmaceutical industry and other fields.

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Fig.1. (A) The chromatogram of GA and four flavonoids from licorice on a XAmide column in HILIC mode. (B) The chromatogram of licorice crude extract on a XAmide column in HILIC mode. Conditions: mobile phase, ACN (A1) and water (C1), 0-30 min, 80%-50% A1, concentration of NH_4FA buffer was 10 mM, flow rate: 1.0 mL/min; column temperature: 30 °C; UV detection: 265 nm.

Fig.2. The chromatograms of GA on three different HILIC columns. Conditions: mobile phase, ACN (A) and water (C), 0-30min, 80%-50% A. Concentration of NH_4FA buffer was 10mM; flow rate: 1.0 mL/min; column temperature: 30 °C; UV detection: 265 nm.

Fig. 3. (A) The influence of water content on the retention of GA, chromatographic conditions: 5mMNH₄FA in ethanol (concentration as noted), pH 4.21, isocratic elution. (B) The effect of different pH in the mobile phase on the retention of GA, chromatographic conditions: 10 mM NH₄FA in 75% ethanol, pH as noted, isocratic elution. (C) The influence of buffer concentration on the retention of GA, chromatographic conditions: 75% ethanol with NH₄FA buffer (concentration as noted), pH 4.28, isocratic elution.

Fig. 4. HPLC chromatogram of crude extracts (A), GA fraction (B), and non-GA fraction (C) on the Unitary C18 column (150 mm $\times$ 4.6 mm, I.D., 5 μ m), chromatographic conditions: ACN (A1) and 0.1% FA in water (B1), 0–60 min, 5–95% A1. UV detection: 265 nm; flow rate: 1.0 mL/min.

Fig. 5. Preparation chromatogram of GA (A) and purity analysis (B)