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# Selective Solid-Phase Extraction of Glabridin from Licorice Root using Molecularly Imprinted Polymer

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**Abstract:** A selective molecularly imprinted solid-phase extraction procedure was developed for the selective separation of glabridin from licorice roots samples. The molecularly imprinted polymers (MIPs) synthesized in ethanol-dodecanol solution show high affinity to glabridin in aqueous environment and the affinity can be controlled by adjusting the intensity of the eluents. By using water-methanol (60:40, *v*/*v*) and acetonitrile-trifluoroacetic acid (99.5:0.5, *v*/*v*) as washing and eluting solvents, most interferences originating from licorice matrix were eliminated. The extracts were sufficiently clean to be directly injected into HPLC for further chromatographic analysis. Good linearity was obtained from 0.4 to 500 mg/L ($r = 0.999$) with the relative standard deviations less than 3.6%. The mean recoveries of glabridin from licorice were more than 81.8% at three different concentrations and the limit of detection was 0.07 mg/L. This method is a viable alternative tool to the existing HPLC methods for analyzing the content of glabridin in licorice samples.

**Keywords:** Glabridin, licorice root, molecularly imprinted polymer, selective solid-phase extraction

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## INTRODUCTION

Licorice, the root of the *glycyrrhiza* plant species, is one of the most frequently employed botanicals in foods and traditional medicines to treat diseases such as phthisis, contagious hepatitis, bronchitis, and ague (1,2). Glabridin is a major flavonoid included specifically in *licorice*, and has various physiological activities including antioxidant and anti-inflammatory effects (3–6). In addition, it exhibits inhibition of serotonin re-uptake, and inhibits melanogenesis and inflammation (7,8). Its most common side effect is hypokalemic hypertension, which is secondary to a block of 11β-hydroxysteroid dehydrogenase type 2 at the level of the kidney, leading to an enhanced mineralocorticoid effect of cortisol (9). Although some reports (10–13) have presented analytical methods for determination of glabridin in licorice and its extracts using liquid-liquid extraction (LLE) and solid-phase extraction (SPE) coupled with chromatographic separation, the sample preparing process was considered to be very complicated and time-consuming due to the fact that plant matrix are very complex and many components originating from the licorice may cause interference to the determination.



Molecular imprinting is a rapidly developing technique for the preparation of polymers having specific molecular recognition properties for a given compound, its analogues, or for a single enantiomer (14–16). Molecularly imprinted polymers (MIPs) possess several advantages over their biological counterparts including low cost, ease of preparation, storage stability, high mechanical strength, and applicability in harsh chemical media (17,18). As a technique for the creation of artificial receptor-like binding sites with a ''memory'' for the shape and functional group positions of the template molecule, molecular imprinting has become increasingly attractive in many fields of chemistry and biology, particularly as selective adsorbents for SPE (19–22). The potential value of molecularly imprinted solid-phase extraction (MISPE) lies in the ability to selectively isolate specific compounds or their structural analogs from a complex matrix. However, in the majority of MIPs applications, optimum MIPs synthesis and molecular recognitions occurs in non-polar or low polar systems. The presence of polar solvents, especially water, can disturb the formation of the prepolymerization complex during the imprinting procedure, which obviously limits their further application in environmental and biological matrices.

The purpose of this study is to establish a simple and convenient method for the extraction and separation of glabridin from licorice root using MISPE followed with HPLC determination. The MIPs were synthesized in ethanol-dodecanol system and they show high retention to glabridin in aqueous environment. Compared with conventional

LLE and SPE process, MISPE could get a more clear extract and no interferences originating from the licorice matrix were observed.

## EXPERIMENTAL

### Materials

Licorice root samples are obtained from a local market. Glabridin was purchased from Wako Pure Chemical Industries, Ltd. (Japan) and its molecular structure is shown in Fig. 1. 2-hydroxyethyl methacrylate was purchased from Aldrich (Steinheim, Germany) and purified by distillation. Ethylene glycol dimethacrylate (EDMA) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and was extracted with 2.0 mol/L sodium hydroxide solution and dried over anhydroxide magnesium sulfate. α,α′-Azobis (isobutyronitrile) (AIBN) was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan) and recrystallized prior to use. Trifluoroacetic acid (TFA) was from Acros Organics (Belgium, NJ, USA). All the other reagents used in the experiment were of the highest grade commercially available. Double deionized water was filtered with a 0.45 μm filter membrane before use.



### HPLC Analysis

HPLC analysis was performed using a liquid chromatography system containing a Waters 600s Multisolvent Delivery System and a Waters 616 pump (Milford, MA, USA), a Waters 486 Tunable Absorbance UV detector (Milford, MA, USA), and a Rheodyne injection valve (20 μL sample loop). Autochro 2000 data software (Younglin, Anyang, Korea) was used as the data acquisition system. The analytical column

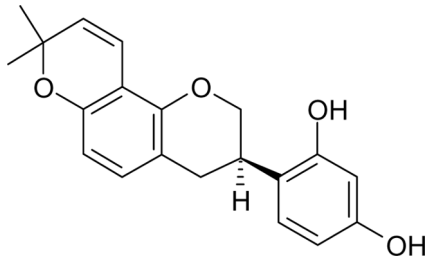


*Figure 1.* Molecular structure of glabridin.

(Optimapak, $C_{18}$, 5 μm, 150 mm × 4.6 mm I.D.) was purchased from RStech Co. (Daejeon, Korea). The mobile phase was water-acetonitrile (50:50, *v*/*v*) and the flow rate was set at 1.0 mL/min. Chromatographic assay was carried out at ambient temperature and the UV wavelength was set at 230 nm.

## Preparation of Molecularly Imprinted Polymers

Glabridin imprinted polymers were prepared by thermal-initiated polymerization within a 25 mL thick-walled glass tube. The polymerization mixture was composed of 3.0 mg glabridin, 2.0 mL 2-hydroxyethyl methacrylate, 5.0 mL EDMA, and 0.059 g AIBN, dissolved in appropriate porogenic solvents (ethanol:dodecanol = 9:2, *v*/*v*). The solution was sonicated for 10 min and purged with helium gas for 10 min before being sealed inside the tube under a helium environment. Polymerization was performed at a temperature of 56°C in a water bath for 24 h. After the polymerization, the polymers were grinded and sieved through a 32 μm sieve, and then suspended in acetone until the upper solution became clear. Finally, the particles were dried and put into a HPLC column and washed with tetrahydrofuran, methanol-acetic acid-trifluoracetic acid (80:19:1, *v*/*v*) to remove the templates. After washing with methanol and being dried in a drying oven (45°C), the particles were stored for further use. Non-imprinted blank polymers (NIP, in the absence of a template) were prepared and treated in an identical manner.

## Procedure of Molecularly Imprinted Solid-Phase Extraction

150 mg of the imprinted particles was respectively packed in an empty polypropylene cartridge and preconditioned with 6.0 mL methanol and 4.0 mL methanol-water (40:60, *v*/*v*). The licorice samples obtained from the local market were oven-dried, sliced, crushed into powder, and then extracted according to our previously reported method (23). Briefly, 1.0 g licorice powders were extracted with 50 mL ethanol/water (30:70, *v*/*v*) for 60 min at 50°C. The obtained extraction solution 0.5 mL was loaded into the MISPE cartridge and cleanup using 4.0 mL of water-methanol (60:40, *v*/*v*) and then eluted using 4.0 mL of acetonitrile-TFA (99.5:0.5, *v*/*v*). The filtrate were evaporated to dryness and reconstituted in 100 μL of mobile phase for further HPLC analysis.

## RESULTS AND DISCUSSION

### Chromatographic Evaluation of the MIPs

In order to synthesize MIPs that demonstrate specific recognition for glabridin in aqueous environment, the MIPs were prepared in ethanol-dodecanol systems using 2-hydroxyethyl methacrylate as a hydrophilic monomer to improve the special affinity and selectivity of the obtained MIPs in the polar environment. The schematic illustrations of the imprinting formation and molecular recognition processes are shown in Fig. 2. The obtained MIPs particles were packing into a HPLC column (100 mm × 3.2 mm I.D.) for further chromatographic evaluation of its recognition ability in aqueous environment. Glabridin cannot be washed out from the column within 60 min when water was used as a mobile phase. At the same condition, unrelated molecules with template such as ethyl resorcinol, ethyl resorcinol, caffeine, tryptophan, and chromene were eluted out in less than 15 min. Moreover, glabridin was washed out from the blank (NIP) column in less than 10 min, which indicates that the retention ability of the MIPs to glabridin was attributed to special imprinted recognition. Furthermore, with increasing of the acidity (acetic



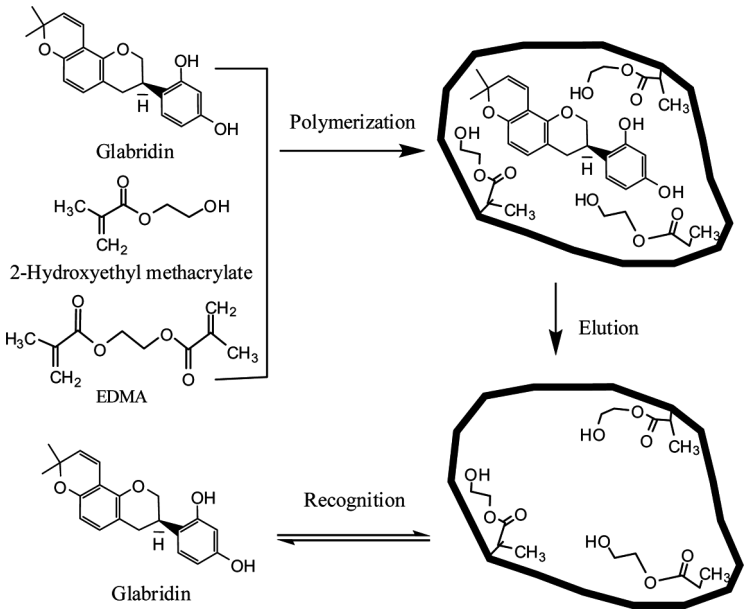


*Figure 2.* Schematic illustrations of the imprint formation and molecular recognition.

acid and TFA) of the mobile phase, the special affinity was destroyed and glabridin could be washed out. When the portion of trifluoracetic acid in the mobile phase is above 0.5%, glabridin could be eluted within 5 minutes. These values, taken together with the elution profiles, demonstrate that the MIPs have high affinity for glabridin and the retention ability can be easily controlled by adjusting the properties of the mobile phase. These characteristics lend the MIPs potential applicability as selective adsorbents for use in the enrichment and separation of glabridin from biological samples.

## The Selectivity of Molecularly Imprinted Solid-Phase Extraction (MISPE)



One of the main difficulties in the development of an analytical method for a complex natural matrix is the sample pretreatment, which becomes more complicated when the analyte was at low concentration and the interferences may disturb the determination. The existing methods typically LLE and SPE involve low selectivity and many components originating from the licorice matrix may cause interferences to the determination. Due to their special selectivity for conducting target analyte isolation, MIPs were proved to be a better selection, giving higher recovery values and a clearer extract (Fig. 3). The chromatography of licorice samples after LLE, SPE using the $C_{18}$ sorbent, and MISPE were shown in Fig. 4. $C_{18}$, the usual solid support for the SPE procedure, showed the recoveries of glabridin were between 76% and 82% with high relative standard deviations, which was attributed to nonspecial interactions such as hydrophobic and hydrophilic interactions between the various components of the sample matrix with $C_{18}$ sorbents. The chromatogram of licorice roots after MISPE processes indicate that there were no interferences from the biological matrix after the MISPE process, which demonstrates the high selectivity and affinity of the synthesized MIPs in the water environment.

Moreover, two blank samples (Negative reference substance) were also treated under the same MISPE process and no peaks were observed at the same retention time of glabridin in the chromatogram, which indicated that there was no ''template leakage'' happening during the MISPE procedure. Compared with MIPs which were showing high affinity towards the target and it was necessary to present TFA in the eluting solvent to effectively desorb the analyte, the blank polymer (NIP) showed similar recovery of glabridin in each step of the extraction, irrespective of the solvent applied. Due to the fact that some part of the analyte was eluted out in washing step, so the recoveries of glabridin in elution

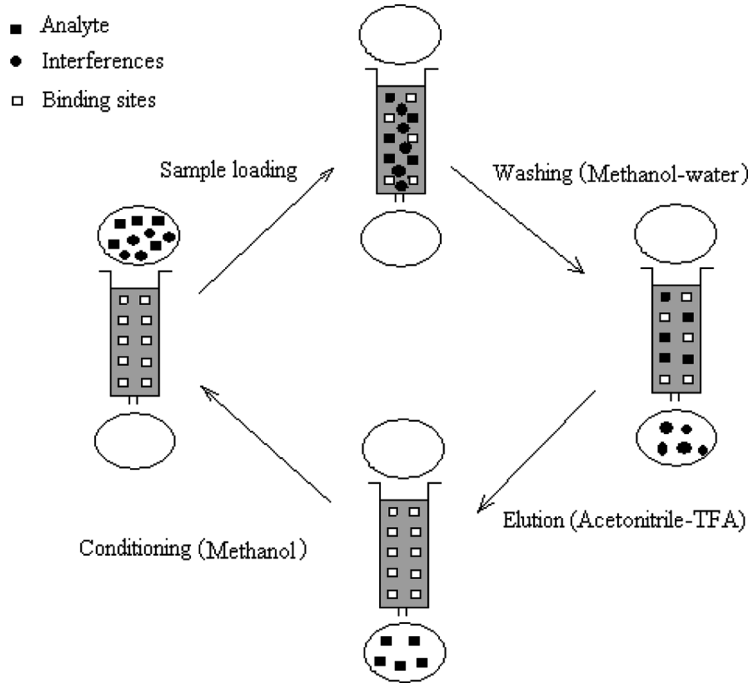


*Figure 3.* Schematic illustrations of molecularly imprinted solid-phase extraction.



step were low (42∼56%), which also indicated that the affinity of MIPs was due to special molecular recognition.

## Optimization of Washing and Eluting Conditions

To optimize the process of selective MISPE, the parameters of the washing and the eluting step were investigated. It is important to apply a wash step immediately after the extraction in dealing with the natural samples, as this ensures reduction of the matrix interference in the separation of analyte. Based on the solvent system should resemble the natural system and the earlier chromatographic evaluation, water-methanol (60:40, $v/v$) was selected as the washing solvent. For the purpose of determining the minimum volume of washing solvent able to efficiently rinse the endogenetic components, different volumes ranged from 1.0 to 8.0 mL were investigated and 4.0 mL was found to be the optimum volume of the washing solvent.

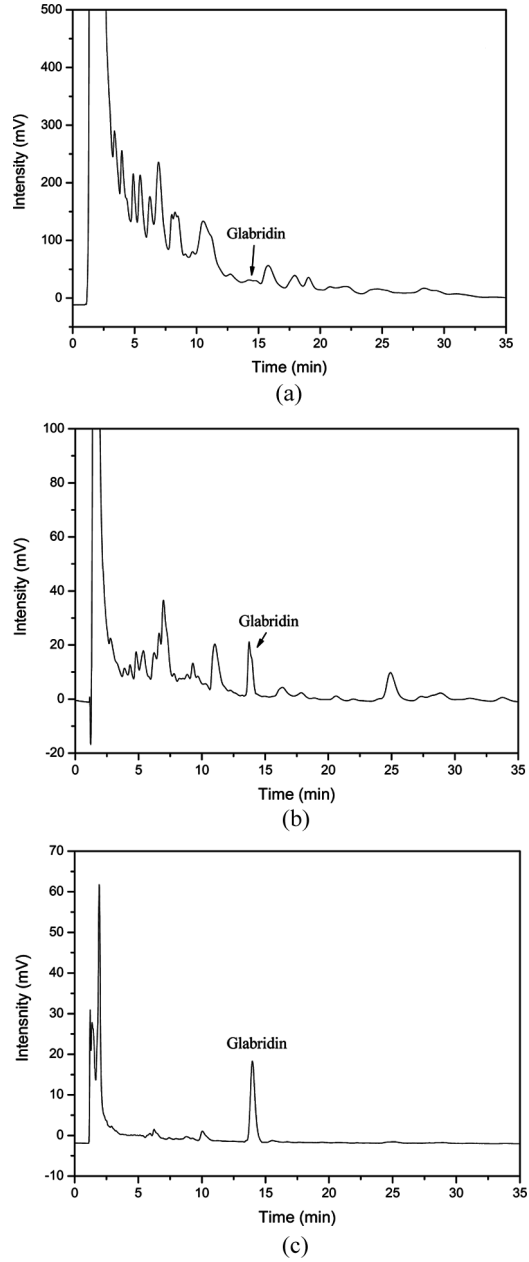


***Figure 4.*** Chromatogrpham of licorice samples after different extraction process. (a) Liquid-liquid extraction; (b) solid-phase extraction using $C_{18}$ sorbent; (c) molecularly imprinted solid-phase extraction; mobile phase: water-acetonitrile (50:50, *v*/*v*); flow rate: 1.0 mL/min; injection volume: 10 μL).

In order to obtain the highest recovery of glabridin from licorice samples, a series of elution solutions, including water, methanol, and acetonitrile with TFA in different proportions were investigated. Considering the recovery and dryness time of the extracts, acetonitrile-TFA (99.5:0.5, *v*/*v*) was selected as the eluting solution. With further increase of the eluting strength of acetonitrile-TFA, the recoveries of glabridin were nearly constant and additional interference from the biological matrix was observed. After optimization, 4.0 mL was found to be the optimum volume of the elution solution.

## Validation of the Proposed Method



Calibration curves were constructed using the areas of the chromatographic peaks measured at nine increasing glabridin concentrations, in a range of 0.4 to 500 mg/L. Good linearity was obtained throughout the concentration range and the linear correlation equations were $Y = 1.62 \times 10^5\ X - 69.42$ with correlation coefficients of $r = 0.999$. The mean recoveries of glabridin in licorice samples were evaluated by spiking three different levels (2.0, 10, 50 mg/L) of standard analyte to samples in replicates of three (Table 1). The precision and accuracy of the method (intra-day) evaluated as RSD at the three different spiked levels were ranged from 2.2 to 4.5% and the inter-day reproducibility was below 6.5% in all cases. Based on a signal-to-noise ratio of 3, the limits of determinations (LOD) of glabridin were 0.07 mg/L. Three licorice samples were randomly collected from local markets and analyzed by the developed MISPE-HPLC method. The concentrations of glabridin in these samples were in a range of 0.90∼0.94 mg/g, which was consistent with our previous results by LLE.

**Table 1.** The recoveries of glabridin in three spiked samples (n = 3)

| | 2.0 mg/L | | 10 mg/L | | 50 mg/L | |
|---|---|---|---|---|---|---|
| Spiked level | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
| #1 | 83.6 | 3.1 | 82.2 | 4.5 | 91.6 | 3.8 |
| #2 | 86.5 | 4.4 | 87.1 | 2.2 | 89.4 | 4.1 |
| #3 | 81.8 | 3.6 | 85.7 | 3.6 | 92.3 | 4.3 |
| #4* | 42.0 | 3.9 | 49.6 | 4.1 | 56.1 | 5.3 |

*#4 was obtained using non-imprinted polymer as sorbents of MISPE.

## CONCLUSION

A simple and sensitive MISPE-HPLC assay procedure is described for the selective extraction and determination of glabridin in licorice samples. Glabridin MIPs prepared in ethanol-dodecanol systems show high affinity to analyte in an aqueous environment and were successfully applied as a special adsorbent of SPE to selectively extract glabridin from licorice root. Good linearity was obtained from 0.4 to 500 mg/L ($r = 0.999$), with relative standard deviations less than 3.6%. This method is a viable alternative tool to the existing HPLC methods for analyzing the content of glabridin in licorice samples.

## ACKNOWLEDGEMENTS

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