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RAPID COMMUNICATION

Simultaneous separation of three isoflavones on oligo-β-cyclodextrin substituted polystyrene-based medium and evaluation adsorption characteristics using AutoDock

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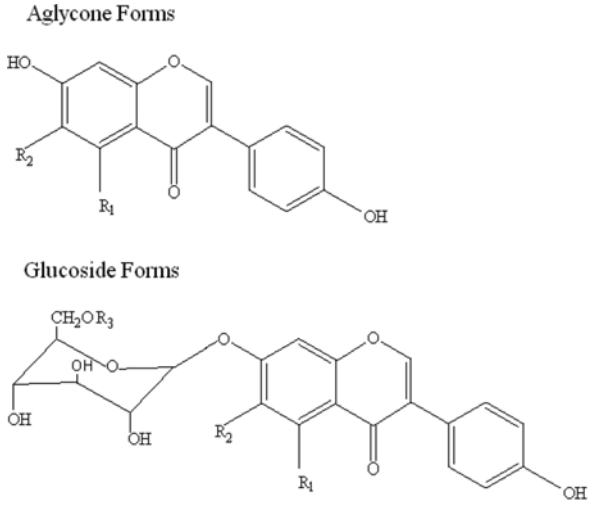
Abstract–The adsorption characteristics between three isoflavones in crude soybean sample and styrene-β-cyclodextrin (S-CD) were studied by molecular mechanics calculations with AutoDock. The discriminatory ability exhibited by S-CD against glycitin, daidzin, and genistin through the differences in the interaction energies and complex geometries could potentially serve for the chromatographic separation. The chromatographic elution order of the three analytes on oligo-β-cyclodextrin substituted polystyrene-based medium (PS-CDP) was predicted depending on the binding free energy values obtained from molecular docking simulations. The experimental results of chromatographic evaluation on PS-CDP were consistent with the simulation prediction. The three isoflavones in sample can be simultaneously separated in one-step under the optimized mobile phase, which consisted of methanol/0.1 mM NH_4AC=65.0/35.0 (v/v) by PS-CDP column chromatography. A glycitin purity of 95.1% with a recovery of approximate 86.3% was achieved by proper peak cutting, and that of daidzin and genistin was 95.8%, 95.4% and 96.2%, 95.7%, respectively.

Keywords: Isoflavone, Separation, Chromatography, Molecular Docking, Medium

INTRODUCTION

In Asian countries, soybeans, which contain large amounts of active components, have long been an essential part of the diet. The health benefits of soybean products have been well documented. Isoflavones are a group of plant-derived phenolic compounds often known as phytoestrogens because of their estrogenic activity. These compounds include three aglycones (daidzein, genistein and glycitein) and nine glucosides (daidzin, genistin, glycitin, acetyldaidzin, acetylgenistin, acetylglycitin, malonydaidzin, malonygenistin and malonyglycitin), whose chemical structures are shown in Fig. 1. These isoflavones may have some important health-enhancing



Isoflavone	R_1	R_2
Daidzin	H	H
Genistin	OH	H
Glycitin	H	OCH_3

Isoflavone	R_1	R_2	R_3
Daidzin	H	H	H
Genistin	OH	H	H
Glycitin	H	OCH_3	H
Acetyldaidzin	H	H	$COCH_3$
Acetylgenistin	OH	H	$COCH_3$
Acetylglycitin	H	OCH_3	$COCH_3$
Malonydaidzin	H	H	$COCH_2COOH$
Malonygenistin	OH	H	$COCH_2COOH$
Malonyglycitin	H	OCH_3	$COCH_2COOH$

Fig. 1. The chemical structure of soybean isoflavones.

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properties. Genistein and daidzein, together with their own glycoside conjugates of genistin and daidzin, are the main components of dietary soy isoflavones. They are mainly responsible for the observed benefits. They have been reported to inhibit tumor cell growth in vivo and in vitro for breast cancer and prostate cancers [1-4]. The biological properties of genistein and genistin have been extensively investigated because of their activities as tyrosine kinase inhibitor, possible anti-oxidant and potential anti-cancer compound [5,6].

Over the past decades, the industrial separation procedures of soy isoflavones depend heavily on solvent extraction and macroporous resin adsorption chromatography [7-12]. Limited by efficiency and cost of separation, the products manufactured using these methods are mostly mixtures of several isoflavones. Along with the increasing demand for soy isoflavones in the clinical medicine and functional food, the exploitation of advanced purification method with higher recovery rates of soy isoflavones, but lower cost, has become an important and challenging task in the pharmaceutical and food processing fields. According to the previous work [13], oligo-β-cyclodextrin substituted polystyrene-based medium (PS-CDP) has been developed for the one-step isolation of the puerarin, the most popular traditional Chinese herbal medicine. Considering the good result of purifying puerarin, we attempted to purify the monomeric isoflavones from crude soybean extracts by using this novel coupled medium.

Recently, improvements in computational simulation technology have provided new non-reagents consuming experimental way and introduced a potentially invaluable tool for the investigation of chromatographic behaviors. There have been many molecular modeling studies, such as molecular dynamics on the chromatographic separations aimed at both the rationalization and prediction of experimental results [14-17]. Modeling is useful to give a theoretical account of the elution order. Compared with the traditional experimental process, which would be complicated, costly and time-consuming, simulation is efficient and inexpensive for chromatography study. We performed molecular docking simulations, using a molecular mechanics method with AutoDock, to evaluate adsorption characteristics of three isoflavones in a crude soybean sample by the novel β-cyclodextrin coupled medium PS-CDP, and to investigate chromatographic behavior on the medium as well as predict the elution order.

The oligo-β-cyclodextrin substituted polystyrene-based medium (PS-CDP) was tentatively utilized for isolating isoflavones by adsorption chromatography. As expected, the data revealed that several individual components of isoflavones in the samples achieved baseline separation on PS-CDP. Meanwhile, the chromatographic elution order predicted by calculation was consistent with the experimental results. The optimum mobile phase composition was selected for the separation of the three isoflavones simultaneously on PS-CDP in one-step, and the isolated fraction of each isoflavone was also identified. This work provides a more efficient and easier method for the purification of soy-isoflavones.

EXPERIMENT

1. Materials

The crude soybean extract powder was donated by Professor Qipeng Yuan, Beijing University of Chemical Technology. The isoflavones standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetonitrile (Merck, Darmstadt, Germany) used were HPLC grade. Acetic acid (HAc), ammonium acetate (NH_4AC), and other reagents were of analytical grade and obtained from Beijing Chemicals Factory (Beijing, China). Water supplied by a Milli-Q water purifier system from Millipore (Bedford, MA, USA) was used in all chromatographic separations and analyses.

2. Computational Molecular Simulation

In this work, the computational simulation method adopted automated molecular docking, which was carried out with docking program AutoDock 4.0 [18]. A Lamarckian genetic algorithm (LGA) in combination with a grid-based energy evaluation method was used for pre-calculating grid maps according to the interatomic potentials of all atom types present in the studied docking molecules, including the Lennard-Jones potentials for van der Waals interactions and Coulomb potentials for electrostatic interactions. A grid map of dimensions 60 Å×60 Å×60 Å, with a grid spacing of 0.375 Å, was placed to cover the styrene-β-cyclodextrin (S-CD) molecule. With the help of AutoDockTools, the atomic partial charges were calculated by the Gasteiger-Marsili method [19,20] and other docking parameters were set as default. For analysis, the representative was selected from the configuration with lowest binding energy.

3. Chromatography Separation Procedure

The synthetic media of PS-CDP were slurry packed in 250 mm× 4.6 mm I.D. stainless steel chromatographic column, then the column was connected to an HPLC system. The HPLC system used to separate isoflavones from the crude soybean extract sample was a Shimadzu (Kyoto, Japan) HPLC chromatography system equipped with a Shimadzu LC-20AT pump and a Shimadzu SPD-20A UV detector. Several solvent solutions were used as mobile phases. All the mobile phases were pre-filtrated to remove possible dust before running through the PS-CDP column. The injected samples were prepared by 20 mg crude soybean extract sample dissolved in 10.0 mL of various mobile phases for the optimization of the separation work. All samples were pre-filtrated using a 0.45 μm syringe filter supplied by Xinya Equipment Company (Shanghai, China) to remove dust. The sample solutions were stored in refrigerator at 4 °C before being loaded onto the columns. The injected sample volume was 20 μL at a mobile phase flow velocity of 1.0 mL/min. The column effluent was monitored with a UV detector at 254 nm. The collected fractions of target products were evaporated with a rotary vacuum evaporator at 45-55 °C then for HPLC assay. We performed column chromatography isolation experiments in triplicate. After use, the PS-CDP column was regenerated by flushing with a sufficient amount of methanol.

4. High Performance Liquid Chromatography Analyses

The crude soybean samples, standard samples and each purified fraction were analyzed by high performance liquid chromatography (HPLC). The HPLC system was an Alltech binary gradient HPLC system equipped with a reversed phase C18 column (250× 4.6 mm I.D., 5 μm) from Beijing Analytical Instrument Apparatus Factory, Beijing, China. The mobile phase consisted of methanol/acetic acid/water=38.0/5.0/57.0 (v/v/v). A 20 μL sample was injected

each time and the UV detection wavelength was set at 254 nm used to monitor the eluate.

5. Nuclear Magnetic Resonance Identification

^{1}H nuclear magnetic resonance (NMR) identification of purified fractions was carried out on a BRUKER AV600 spectrometer, operating at a 600.13 MHz ^{1}H frequency. The samples were dissolved with dimethyl sulfoxide-d_6 (DMSO), and the solution was measured with tetramethylsilane (TMS) as the internal reference. Samples were placed in a 5 mm I.D. sample tube. The chemical shifts were given in parts per million (ppm) at 298 K. The sweep width for ^{1}H NMR was 6,009 Hz, 32 FID's of 16 K data points, giving a digital resolution of 0.30 Hz. The acquisition time was 2 s. The structure of a purified fraction was determined by comparing the chemical shift with the reference standard signal.

RESULTS AND DISCUSSION

1. Evaluation of Adsorption Characteristics through Structures and Binding Energies from AutoDock

In this work, the sample of crude soybean extract was the product of solvent extraction. There are three isoflavones, daidzin, genistin and glycitin, in the provided sample, determined through comparing with the sample solution of standard using HPLC analysis. The peak containing daidzin, glycitin and genistin is shown in Fig.

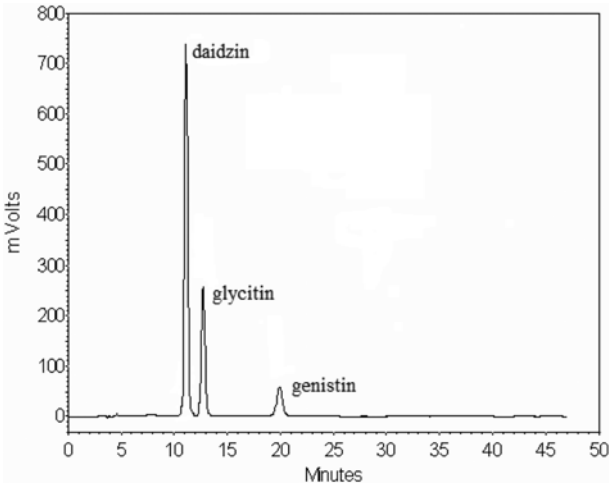

Fig. 2. HPLC chromatograms of the crude soybean extract sample on C18 column. Flow rate: 1.0 mL/min; mobile phase: methanol/acetic acid/water=38.0/5.0/57.0 (v/v/v).

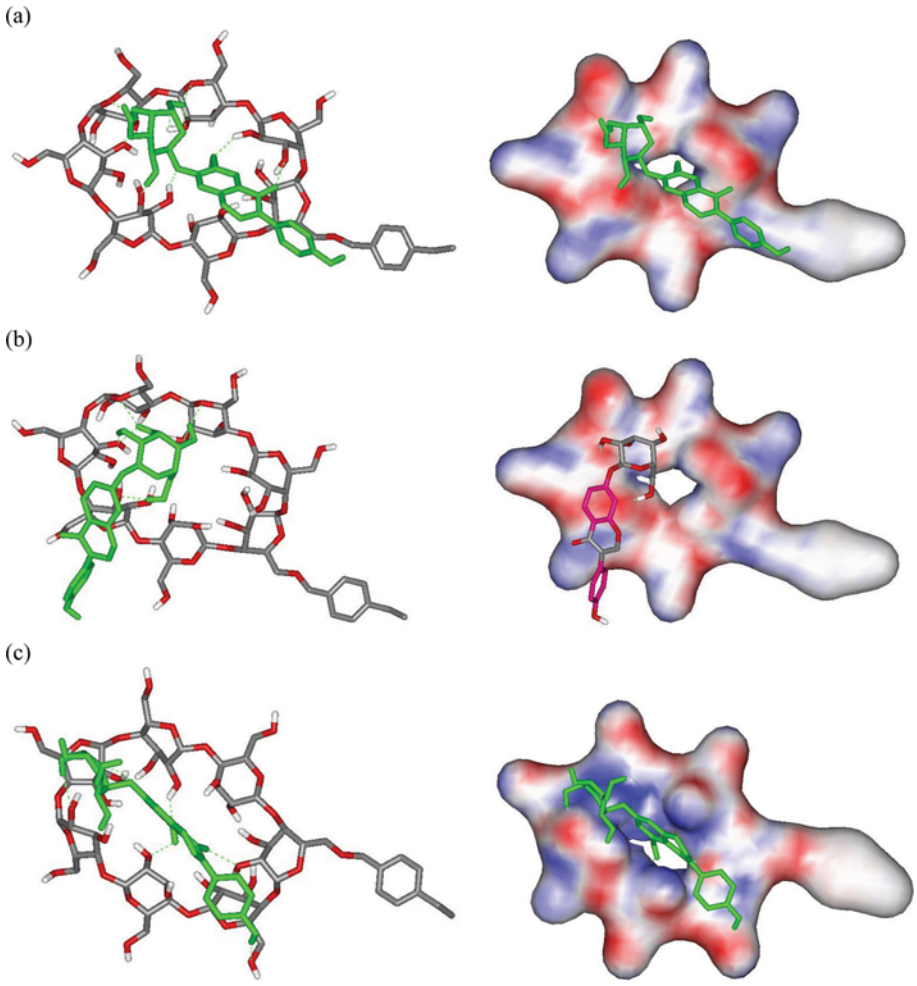

Fig. 3. The dominating configurations of S-CD interacting with the three isoflavones (a) glycitin, (b) daidzin and (c) genistin.

2. A computational molecular modeling approach of molecular docking simulations was adopted to study the chromatographic behaviors of the three target isoflavonoids on PS-CDP.

The polymer of the coupled medium PS-CDP is primarily composed of monomers and functional ligands. To simplify the AutoDock calculations, we assumed that the adsorption on PS-CDP is mainly attributed to the interaction between the constitutional repeating unit of the polymer, i.e. styrene-β-cyclodextrin (S-CD), and the three isoflavones. Chromatographic separation of isoflavones in crude soybean extract sample may occur when molecular recognition by interaction with S-CD is available. The distinction in structures and interaction energies of the S-CD-isoflavone complexes dominates the chromatographic behavior.

The search of the most stable complex between S-CD and the three isoflavones in sample was performed using the method as described in Section 2.2 with AutoDock 4.0 software. The results of molecular docking between S-CD and the isoflavones are presented for the typical equilibrated conformations with minimum binding free energy (ΔG) in Fig. 3. On the left sides of the structures in Fig. 3, the green stick configurations represent the isoflavones and tubular bonds of red stood for oxygen atoms, the gray for carbon atoms. The hydrogen bonding can be determined by AutoDock 4.0. The bonds of green dashed lines represent hydrogen bonds. The hydrogen bonding mainly emerged from the interaction between hydroxyl groups of the β-CD moieties, the ether bonds formed as a consequence of the cross-linking reaction between these, and the phenol groups of the isoflavones. From the structures in Fig. 3, the visible differences in the geometry of complexes between S-CD and the three analytes of glycitin, daidzin and genistin can be found. Variations in the geometries to attain the energetically most favorable complexes strongly depend on the size and shape of the isoflavone molecules. These suggest the potential differences in adsorption characteristics and the chromatographic behaviors of the three isoflavones on the β-CD coupled medium. In addition, for the three isoflavones, the hydrogen bonding could play an important role in the retardation on PS-CDP.

The binding free energies between S-CD and the three isoflavones obtained from molecular docking with AutoDock are expressed in Fig. 4. It indicates that the three isoflavones in the sample possessed different binding free energies with S-CD from each other. Genistin has the highest interaction energy with S-CD among the three isoflavones, and glycitin has the lowest. As the value of binding free energy is high, the interaction between analyte and S-CD is strong. The glycitin complex is less stable than the daidzin complex, as shown by the ΔG of −3.37 and −3.60 kcal/mol, respectively. Similar result was also observed for the daidzin and genistin by the ΔG of −3.60 and −4.05 kcal/mol. This implies different chromatographic behavior on the β-CD coupled medium, namely, glycitin had the shortest retention time on PS-CDP, while genistin had the longest one. The binding free energy differences ($\Delta\Delta G$) range from 0.23 to 0.68 kcal/mol after calculation. In the chromatographic context, these $\Delta\Delta G$ values, if valid, would be sufficient for separation of the liquid mixtures [21]. Consequently, the actual binding free energy values predicted the chromatographic elution orders of glycitin>daidzin>genistin on PS-CDP.

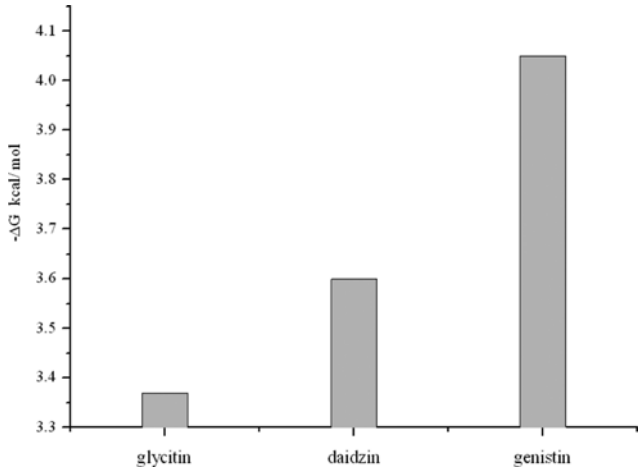

Fig. 4. Binding free energies at 298 K of the complexes between S-CD and the isoflavones obtained from AutoDock 4.0.

2. One-step Simultaneous Separation of Three Isoflavones on PS-CDP

According to the aforementioned, the PS-CDP column has a discriminatory ability against the three isoflavones in the sample depending on the distinctions in complex geometries (Fig. 3) and binding energies (Fig. 4) by molecular docking simulations. Hence, the optimum conditions of chromatography operation on PS-CDP such as optimum mobile phase and operation mode should be ascertained in order to achieve thorough separation of isoflavones from the crude soybean extracts. The composition of the mobile phase is of paramount importance in the chromatographic purification of the target product. Thus, the subsequent study is to select solvent solutions and adjust the polarity of the mobile phase. The optimum mobile phase composition is experimentally determined on the basis of the retention factor, the purity and recovery of the individual target product. With respect to operation mode, isocratic elution mode was designed aiming at facilitating the operation and shortening the separation time.

Solvent solutions such as methanol-water, acetonitrile-water, acetic acid-water, ammonium acetate-water and their mixtures were employed as mobile phases, based on the previous data of puerarin in reference [13] and the hydrogen bond between the adsorbent and adsorbate. The separation efficiency of solvent systems was assessed as chromatograms on PS-CDP column. From the profiles of the column using different mobile phases, methanol-ammonium acetate-water and methanol-acetic acid-water were better than other single or binary solvents. Thus, further studies about these two solvent systems proceeded in order to screen out the best one. In Fig. 5, the results demonstrate that mixtures composed of methanol-ammonium acetate-water are superior to methanol-acetic acid-water. The total separation of isoflavones in one-step was achieved while the proportions to volume ratio of methanol and ammonium acetate were adjusted to the appropriate value. The optimum mobile phase composition was confirmed as methanol/0.1 mM NH_4AC=65.0/35.0 (v/v), and the corresponding chromatogram is illustrated in Fig. 6, which corresponds to their prediction elution order glycitin, daidzin, and genistin by molecular docking.

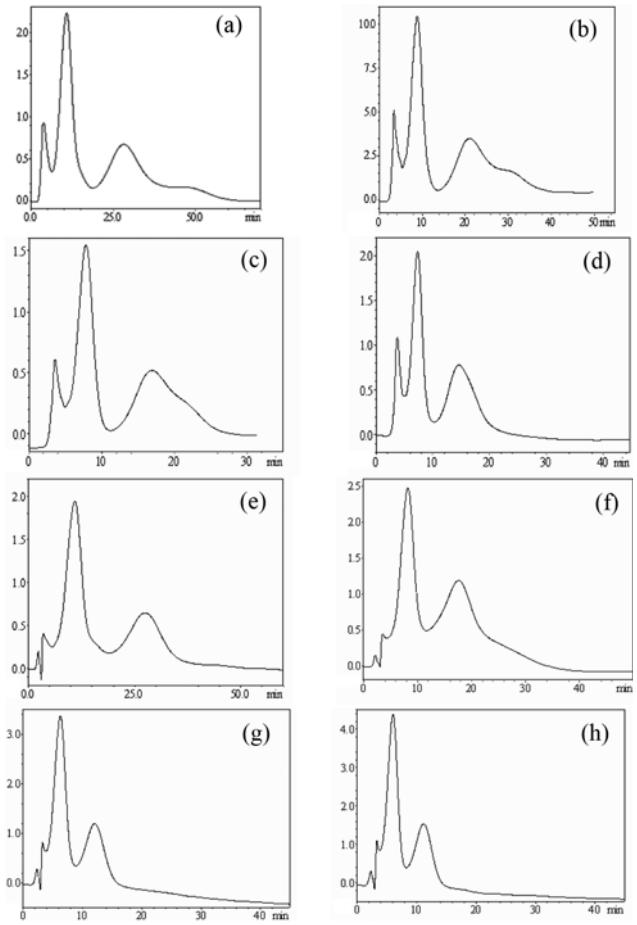

Fig. 5. Chromatograms of purifying isoflavones on PS-CDP column by different mobile phases. Flow rate: 1.0 mL/min; mobile phase: (a) Methanol/0.1 mM NH_4AC=60.0/40.0 (v/v), (b) methanol/0.1 mM NH_4AC=70.0/30.0 (v/v), (c) methanol/0.1 mM NH_4AC=75.0/25.0 (v/v), (d) methanol/0.1 mM NH_4AC=80.0/20.0 (v/v), (e) methanol/7.0%HAC=60.0/40.0, (f) methanol/7.0%HAC=70.0/30.0, (g) methanol/30.0%HAC=50.0/50.0, (h) methanol/30.0%HAC=60.0/40.0.

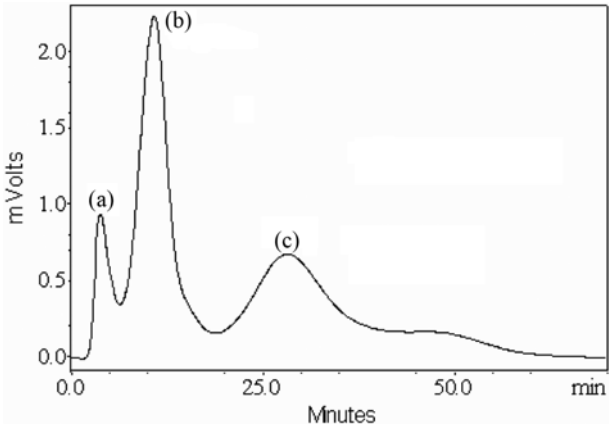

Fig. 6. Chromatogram of simultaneous separation of isoflavones on PS-CDP column under optimal mobile phase. Flow rate: 1.0 mL/min, mobile phase: methanol/0.1 mM NH_4AC=65.0/35.0 (v/v).

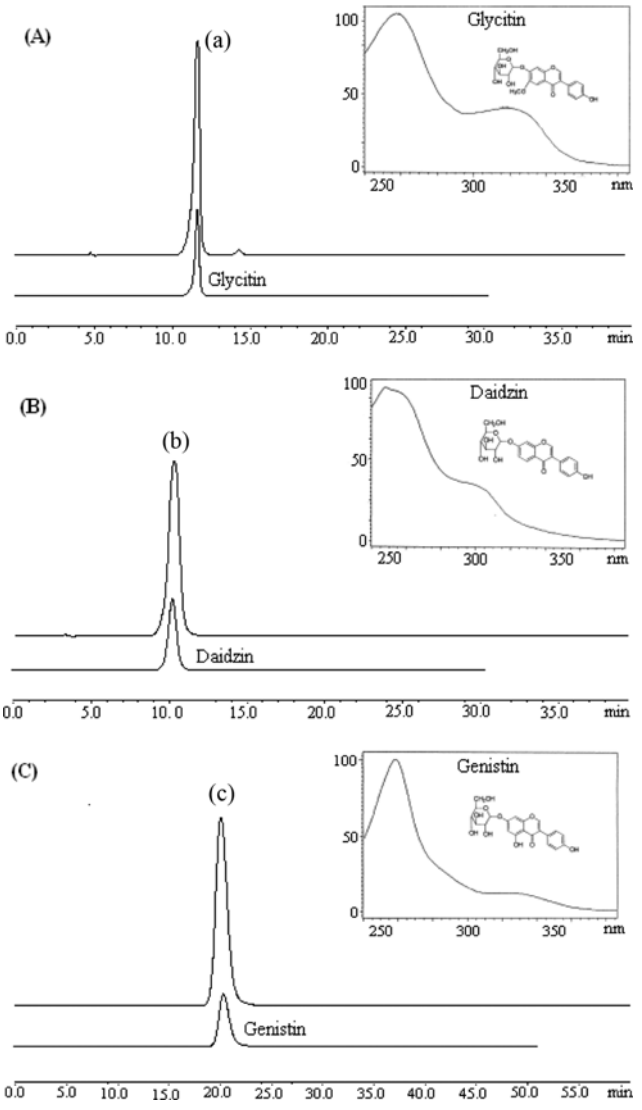

Fig. 7. HPLC analyses and UV spectra of the peaks (a), (b), and (c) in Fig. 6. Column: RPC18; flow rate: 1.0 mL/min; mobile phase: methanol/acetic acid/water=38.0/5.0/57.0 (v/v/v).

3. HPLC Analysis and NMR Identification

The fractions of peaks (a), (b), and (c) in Fig. 6 were collected and analyzed by HPLC. Confirmed through comparing with the sample solutions of standard, they were the target products of glycitin, daidzin, and genistin, respectively. The HPLC and UV spectra analysis is shown in Fig. 7. The UV absorption spectra of the purified fractions shown in Fig. 7 are consistent with the reference standard [22]. The purities and recoveries of glycitin, daidzin, and genistin in peaks a, b, and c of Fig. 6 cuts were 95.1% and 86.3%, 95.8% and 95.4%, 96.2% and 95.7%, respectively, as determined by HPLC analysis. It was proved that the three isoflavones in sample obtained simultaneous separation on PS-CDP with higher separation efficiency under optimal mobile phase compared with previous methods mentioned above.

The structures of the purified fractions were further identified and characterized by NMR. The 600 MHz ^{1}H NMR spectral data of the purified fraction corresponding to daidzin were as follows:

^{1}H NMR (DMSO, 600 MHz) δ: 9.54 (1H, br.s, 4′-OH), 8.39 (1H, s, 2-H), 8.06 (1H, d, J=8.8 Hz, 5-H), 7.40 (2H, d, J=8.4 Hz, 2′, 6′-H), 7.15 (1H, d, J=8.8 Hz, 6-H), 6.80 (2H, d, J=8.4 Hz, 3′, 5′-H), 5.11 (1H, d, J=7.4 Hz, glc 1″-H), 3.70 (1H, d, J=10.6 Hz, glc 6″b-H), 3.47 (1H, m, 5-H), 3.45 (1H, m, glc 6″a-H), 3.31 (1H, m, glc 2″-H), 3.25 (1H, m, glc 3″-H), 3.20 (1H, m, glc 4″-H). The 600 MHz ^{1}H NMR spectral data of the purified fraction corresponding to glycitin were as follows: ^{1}H NMR (DMSO, 600 MHz) δ: 9.48 (1H, br.s, 4′-OH), 8.35 (1H, s, 2-H), 7.45 (1H, d, J=8.8 Hz, 5-H), 7.32 (1H, s, 8-H), 7.10 (2H, d, J=8.6 Hz, 2′, 6′-H), 6.82 (2H, d, J=8.5 Hz, 3′, 5′-H), 5.15 (1H, d, J=7.3 Hz, glc 1″-H), 3.88 (3H, s, OCH3), 3.28 (1H, m, glc 2″), 3.28 (1H, m, glc 3″), 3.16 (1H, m, glc 4″). The 600 MHz ^{1}H NMR spectral data of the purified fraction corresponding to genistin were as follows: ^{1}H NMR (DMSO, 600 MHz) δ: 9.60 (1H, br.s, 4′-OH), 8.43 (1H, s, 2-H), 7.39 (2H, d, J=8.5 Hz, 2′, 6′-H), 6.82 (2H, d, J=8.5 Hz, 3′, 5′-H), 6.72 (1H, s, 8-H), 6.47 (1H, s, 6-H), 5.05 (1H, d, J=7.3 Hz, glc 1″-H), 3.70 (1H, d, J=10.1 Hz, glc 6″b-H), 3.45 (1H, dd, J=10.1, 4.7 Hz, glc 6″a-H), 3.16-3.28 (3H, t, glc 3″, 2″, 4″-H). These data are in agreement with the reported data in the literature [23-25]. Thus, it can be safe to conclude that the simultaneously separated three target components were daidzin, glycitin, and genistin, respectively.

CONCLUSION

Molecular docking simulations were used to investigate the separation mechanism and adsorption behavior about the novel medium PS-CDP. The predicted elution order was validated by experimental data, giving as supporting information that this simulation calculation provides an effective and non-reagent consuming method for the development of chromatography technology. The three isoflavones in crude soybean sample were completely separated in one-step using the optimum mobile phase by PS-CDP column. To the best of our knowledge, no observations of simultaneous isolation of isoflavones have been reported for CDP substituted polystyrene-based media.

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