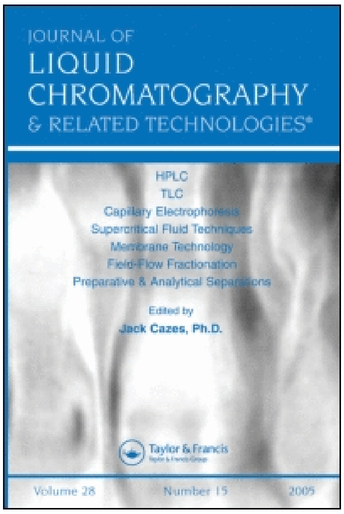

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Countercurrent Chromatographic Separation of Soybean Isoflavones by Two Different Types of Coil Planet Centrifuges with Various Two-Phase Solvent Systems

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ABSTRACT

The countercurrent chromatographic separation of isoflavones from a crude soybean extract was performed with various two-phase solvent systems, using two different types of the coil planet centrifuge (CPC) of the cross-axis CPC and the type-J multilayer CPC. The overall results indicated that the type-J multilayer CPC separates daidzin and genistin

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with an ethyl acetate/ethanol/water (4 : 2 : 7) system at the purity of over 97 and near 100%, respectively, while the cross-axis CPC resolves these compounds in a much longer elution time with an ethyl acetate/1-butanol/water (2 : 1 : 3) system. The type-J multilayer CPC further resolves daidzein and genistein with an *n*-hexane/ethyl acetate/1-butanol/methanol/water (5 : 5 : 0 : 5 : 5) system, and also, simultaneously, all four isoflavones under a linear gradient elution, using the solvent system composed of *n*-hexane/ethyl acetate/1-butanol/methanol/acetic acid/water by shifting the volume ratio from 1 : 2 : 1 : 1 : 1 : 5 to 1 : 2 : 1 : 3 : 0.5 : 5. The present methods may be useful for the separation of isoflavones from other plant extracts.

Key Words: Countercurrent chromatography; Isoflavones; Coil plant centrifuge; Two-phase solvent system.

INTRODUCTION

Countercurrent chromatography (CCC) has been widely used for separation and purification of various natural and synthetic products.[1–4] The method provides advantages over the conventional chromatographic methods by eliminating the solid support, which may cause irreversible adsorption and/or degradation of samples. Among many CCC instruments developed in the past, the type-J multilayer coil planet centrifuge (type-J multilayer CPC) and the cross-axis coil planet centrifuge (cross-axis CPC) have proven to be the most useful models. The type-J multilayer CPC produces a synchronous planetary motion of the separation column, which revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction. The cross-axis CPC, on the other hand, produces a synchronous planetary motion of the column in such a way that it revolves around the vertical axis of the centrifuge, while rotating about its horizontal axis at the same angular velocity. The difference in the planetary motion between these two instruments provides distinctive use of the two-phase solvent systems, where the type-J multilayer CPC performs excellent separation with organic-aqueous two-phase solvent systems, whereas the cross-axis CPC is used for highly polar two-phase solvent systems, such as aqueous-aqueous polymer phase systems.

The present study describes the CCC separation of isoflavones using these two different types of CPC instruments with various two-phase solvent systems. Although, CCC separations of isoflavones from the soybean extract have been reported in the past,[5,6] the present studies will add several new two-phase solvent systems to facilitate the preparative separation of these compounds used for the standards.

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EXPERIMENTAL

Apparatus

The type-J multilayer CPC was obtained from Hitachi Tokyo Electronics (Tokyo, Japan) as a commercial unit.

The cross-axis CPC employed in the present studies, was constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design of the apparatus was previously described in detail.[7–9]

Preparation of Coiled Columns

The multilayer coil for the type-J multilayer CPC was prepared by tightly winding a piece of 2.0 mm ID and 3.0 mm OD Teflon tubing (Flon Kogyo Co., Tokyo, Japan) around the holder hub of 9 cm in diameter, forming tight coiled layers between a pair of flanges spaced 5.6 cm apart. The total column capacity was 106 mL.

The eccentric coil for the cross-axis CPC was prepared by winding a 1 mm ID and 2 mm OD Teflon tubing onto 7.6 cm long, 5 mm OD nylon pipes, forming 20 units of serially connected left-handed coils. A set of these coil units was arranged symmetrically around the holder hub of 7.6 cm diameter in such a way that the axis of each coil unit is parallel to the axis of the holder. Two sets of coil assemblies were mounted on the rotary frame, one on each side, and serially connected with the flow tube. The total column capacity was 26.5 mL.

Reagents

Standard samples including daidzin, genistin, daidzein, and genistein were purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were of reagent grade.

Preparation of Two-Phase Solvent Systems and Sample Solution

Several two-phase solvent systems were prepared for the CCC separation: methyl *t*-butyl ether (MBE)/acetonitrile/water (2 : 2 : 3), and ethyl acetate/1-butanol/water (2 : 1 : 3) for the cross-axis CPC, while ethyl acetate/ethanol/water (4 : 2 : 7), *n*-hexane/ethyl acetate/1-butanol/methanol/water (5 : 5 : 0 : 5 : 5), *n*-hexane/ethyl acetate/1-butanol/methanol/acetic acid/water (1 : 2 : 1 : 1 : 1 : 5) and (1 : 2 : 1 : 3 : 0.5 : 5) for the type-J multilayer CPC.

Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated after two clear layers formed. Sample solutions were prepared by dissolving the desired amounts of samples in 0.5 mL of equal volume, of each phase of the two-phase solvent system used for separation.

Measurements of Partition Coefficients of Isoflavones

Successful CCC separation highly depends on the choice of the two-phase solvent system, which provides suitable partition coefficient values for a set of analytes. In the present study, partition coefficient (K) of each standard sample was determined spectrophotometrically, using a simple test tube experiment described by Oka et al.[10] as follows: Two milliliters of each phase of an equilibrated solvent system were delivered into a test tube to which about 1 mg of the sample was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the two clear layers were formed, a 1 mL aliquot of each phase was diluted with 2 mL of methanol, and the absorbance was measured at 260 nm using a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan). The K value was obtained by dividing the absorbance value of the upper organic phase by that of the lower aqueous phase.

Extraction of Crude Isoflavones from Soybean Embryo Bud

Crude isoflavone extract was prepared from soybean embryo bud according to the procedure by Ohta et al.[11] as follows: Soybean embryo bud flour (128 g) was thoroughly extracted with 1 liter of hot ethanol two times for 1 hour. The combined ethanol solution was concentrated under vacuum. The extract was stirred with acetone (700 mL) and filtered, and the filtrate was again concentrated under vacuum. The residue was suspended in water (350 mL), and ethyl acetate (1000 mL) was added to precipitate the target compounds. After filtrating and removing the solvent of each layer, white precipitate (12 g), the ethyl acetate extract (9.4 g) and the aqueous residue (10.9 g) were obtained. This white precipitate was submitted to the CCC separation in the present studies.

CCC Separation of Isoflavones

Each separation was initiated by completely filling the column with the stationary phase, followed by injection of the sample solution into the column inlet. Then, the mobile phase was pumped into the column using a reciprocating pump (Model KHU-W-52H, Kyowa Seimitsu Co., Tokyo, Japan), while the column was rotated at 800 rpm. The effluent from the outlet of the column was collected in test tubes using a fraction collector (Model SF-200, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each collected fraction was diluted with 2 mL of methanol and the absorbance was measured at 260 nm using a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan).

An aliquot of each fraction was also analyzed by high-performance liquid chromatography (HPLC). The HPLC equipment included a reciprocating pump (Model LC-10ATVP), an UV detector (Model SPD-10AVP), and a variable input recorder (Model C-R6A).

Analytical conditions for HPLC were as follows: column, ODS-silica (Wakopak® Handy-ODS, 4.6 mm ID × 150 mm, Wako Pure Chemicals, Osaka, Japan); eluent, aqueous 30% methanol; flow rate, 1.0 mL/min; column temperature, room temperature; detection, 260 nm and injection volume, 10 μL.

RESULTS AND DISCUSSION

Cross-Axis CPC Separation

Figure 1 illustrates the K values of four standard test samples including daidzin, genistin, daidzein, and genistein in the two-phase solvent system composed of *n*-hexane/ethyl acetate/1-butanol/methanol/water, at various volume ratios, as indicated in the diagram. This solvent system provides a broad range of hydrophobicity simply by adjusting the volume ratios and, therefore, it is useful for estimating the polarity of the analytes. As expected, the K values of both glycosides (daidzin and genistin) and their aglycones (daidzein and genistein) were increased as the hydrophobicity of the solvent system was decreased. Among those, a volume ratio of 0 : 5 : 0 : 0 : 5 was first selected for CCC separation, since the K values of daidzin and gensitin are relatively well separated between each other with an ideal range of K value at around 1. However, this solvent system did not provide satisfactory separation between daidzin and genistin because of broad spreading of the genistin peak due to its large K value. Therefore, we have selected two additional solvent systems composed of ethyl acetate/1-butanol/water (2 : 1 : 3)[12] and MBE/acetonitrile/water (2 : 2 : 3).

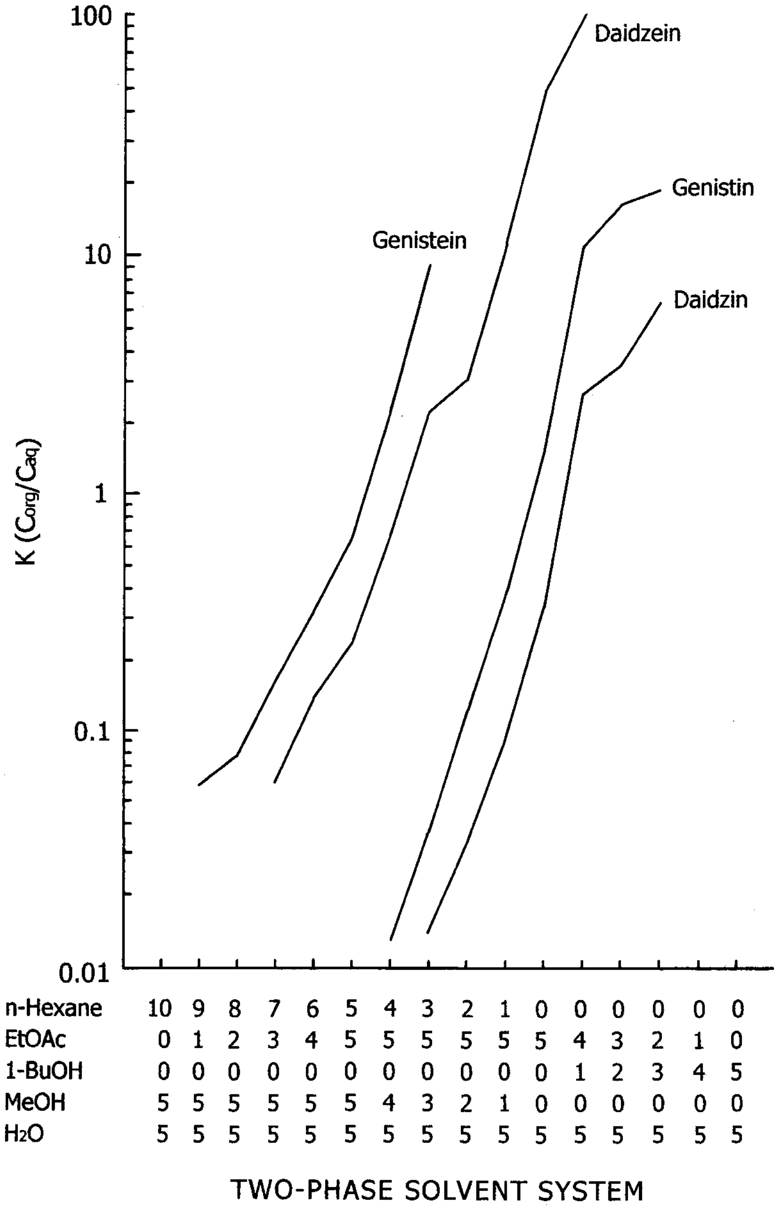

Figure 1. Partition coefficients of ($K = C_{org}/C_{aq}$) of standard isoflavones in *n*-hexane/ethyl acetate/1-butanol/methanol/water system determined by the test tube experiments (see text). EtOAc = ethyl acetate; 1-BuOH = 1-butanol; MeOH = methanol.

Figure 2 illustrates the CCC separations of the soybean precipitates using the cross-axis CPC with these two solvent systems. As shown in Figure 2A, MBE/acetonitrile/water (2 : 2 : 3) separated the two peaks, daidzin as the first peak and genistin as the second peak, where the purities of these fractions were 78.6% for daidzin and 99.1% for genistin by HPLC analysis. The solvent system composed of ethyl acetate/1-butanol/water (2 : 1 : 3) improved the separation between these two peaks, as shown in Figure 2B, in which the purities of these compounds were 93.8% for daidzin and 100% for genistin. However, the method required a long separation time of over 10 hours.

Type-J Multilayer CPC Separation

In order to improve the separation of the soybean isoflavones, the type-J multilayer CPC was used with three more different types of two-phase

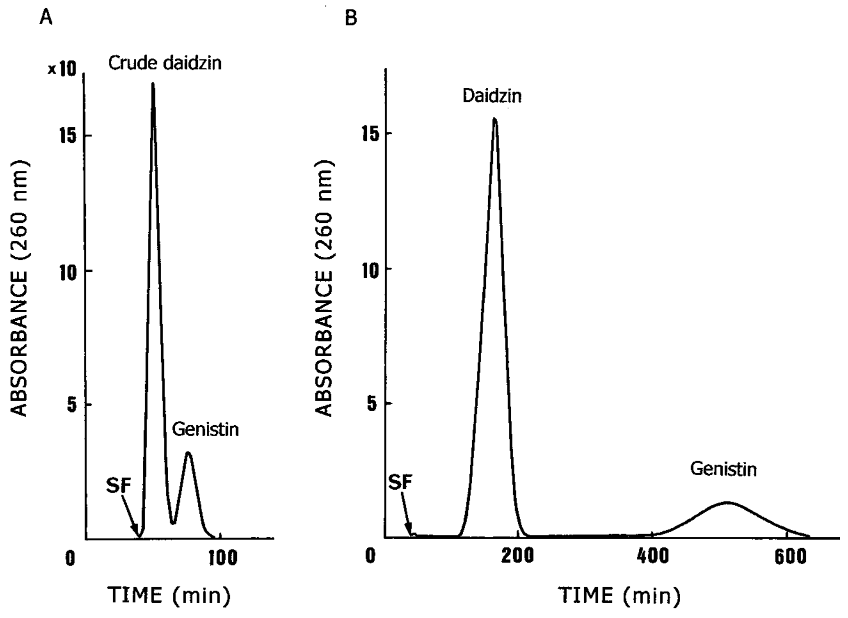

Figure 2. CCC chromatograms of soybean white precipitates using the cross-axis CPC. Experimental conditions: apparatus: cross-axis CPC equipped with a pair of eccentric coil assemblies, 1 mm ID and 26.5 mL total column capacity; sample: soybean white precipitate (A) 13 mg; (B) 5.4 mg; solvent system: (A) methyl *t*-butyl ether/acetonitorile/water (2 : 2 : 3); (B) ethyl acetate/1-butanol/water (2 : 1 : 3); mobile phase: lower phase; flow rate: 0.4 mL/min; revolution: 800 rpm. SF = solvent front.

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Table 1. Partition coefficients of standard isoflavones with three different two-phase solvent systems.

Solvent system	Ethyl acetate/ 1-butanol/water (2 : 1 : 3)	Ethyl acetate/ 1-propanol/water (4 : 2 : 7)	Ethyl acetate/ ethanol/water (4 : 2 : 7)	n-Hexane/ethyl acetate/1-butanol/ methanol/acetic acid/water	
				(12 : 1 : 1 : 1 : 5)	(1 : 2 : 1 : 3 : 0.5 : 5)
Glycoside					
Daidzin	2.57	1.87	0.63	0.17	0.04
Genistin	11.9	5.94	2.72	0.63	0.04
Aglycon					
Daidzein	54.0	19.4	17.2	3.79	0.36
Genistein	37.5	43.0	29.6	9.35	1.00

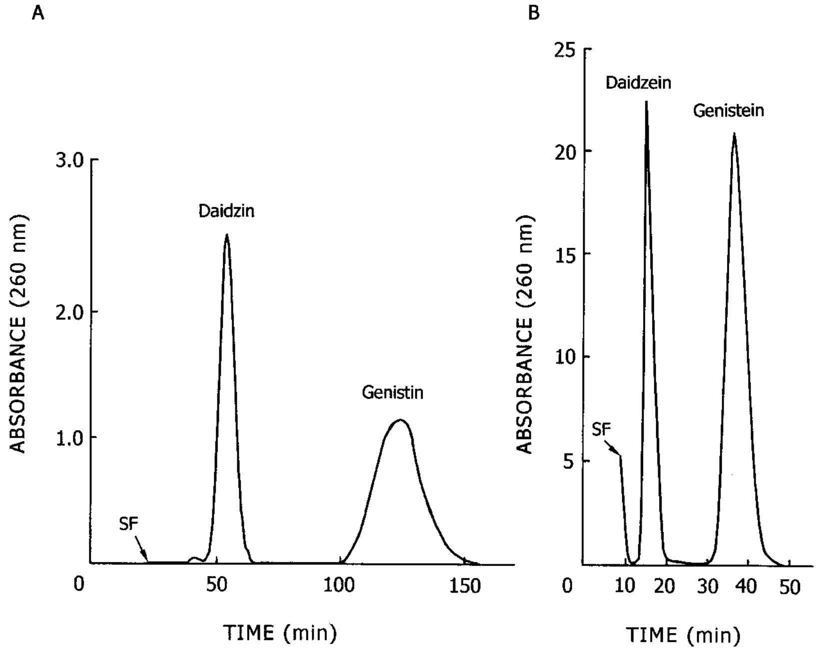

Figure 3. CCC chromatograms of standard isoflavones using the type-J multilayer CPC. Experimental conditions: apparatus: type-J multilayer CPC equipped with a multilayer coil assembly, 2 mm ID and 106 mL column capacity; sample: (A) 1 mg each of daidzin and genistin; (B) 1 mg each of daidzein and genistein; solvent system: (A) ethyl acetate/ethanol/water (4 : 2 : 7); (B) *n*-hexane/ethyl acetate/1-butanol/methanol/water (5 : 5 : 0 : 5 : 5); mobile phase: lower phase; flow rate: 1 mL/min; revolution: 800 rpm. SF = solvent front.

solvent systems, each containing polar alcohol to increase the polarity of the system. Table 1 lists the partition coefficients of the standard isoflavones in these solvent systems. Among these solvent systems, the ethyl acetate/ethanol/water (4 : 2 : 7) system was selected for the separation of daidzin and genistin.

Figure 3 illustrates the CCC chromatograms of the standard isoflavones of daidzin and genistin (A), and daidzein and genistein (B). These separations were successfully performed using the two-phase solvent systems composed of ethyl acetate/ethanol/water (4 : 2 : 7) described above, and of *n*-hexane/ethyl acetate/1-butanol/methanol/water (5 : 5 : 0 : 5 : 5), which was selected from the experimental data shown in Figure 1.

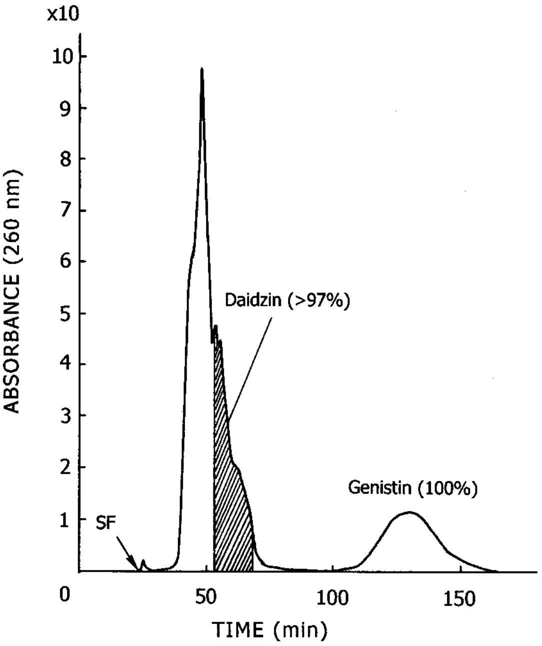

Figure 4. CCC chromatogram of soybean white precipitate using the type-J multilayer CPC. Experimental conditions: sample: soybean white precipitate (50 mg); solvent system: ethyl acetate/ethanol/water (4 : 2 : 7). Other experimental conditions were the same as those described in Figure 3. SF = solvent front.

Figure 4 illustrates the CCC chromatogram of soybean precipitates (50 mg) using the above ethyl acetate/ethanol/water (4 : 2 : 7) system. The sample solution was prepared by dissolving the sample mixture in a small amount of dimethylsulfoxide before adding equal volumes of each phase of two-phase solvent system used for separation, since the soybean precipitates were not well dissolved in either phase directly. Dimethylsulfoxide, added in the sample solution, eluted at the solvent front without interfering with the separation of the target compounds. This method led us to the successful preparative separation of soybean precipitates, yielding 4.4 mg of daidzin at purity of over 97.0% (recovery 8.8%) from the first peak (fractions between No. 53–69, shadowed in the chromatogram), and 7.9 mg of genistin at almost 100% purity from the entire fractions of the second peak (recovery 15.8%). This method improved the separation time almost 2.5 hours.

In order to separate both glycosides and aglycones of isoflavones, simultaneously, in one run, a gradient elution was performed. Figure 5 illus-

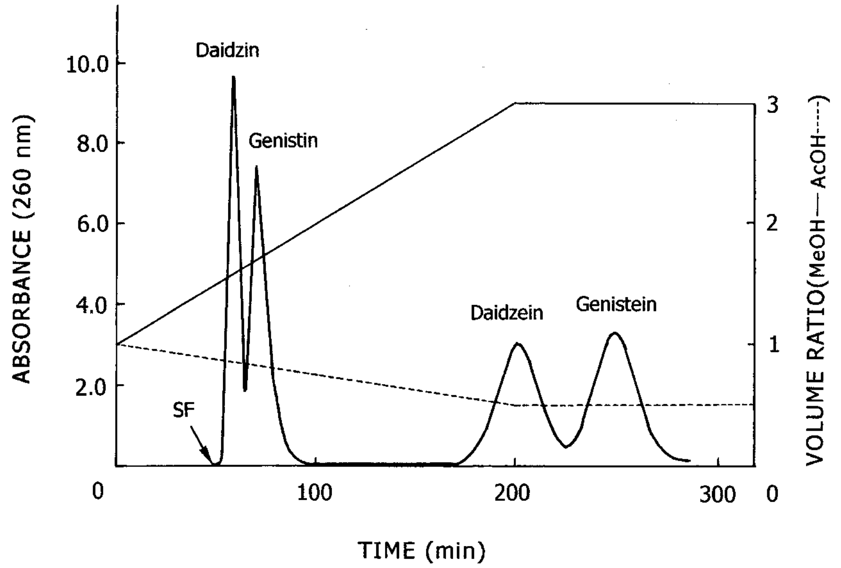

Figure 5. CCC chromatogram of standard isoflavones using the type-J multilayer CPC with linear gradient elution. Experimental conditions: sample: 1 mg each of daidzin, genistin, daidzein, and genistein; solvent system: *n*-hexane/ethyl acetate/1-butanol/methanol/acetic acid/water (1 : 2 : 1 : 1 : 1 : 5) and (1 : 2 : 1 : 3 : 0.5 : 5) with a linear gradient elution; mobile phase: lower phase. Other experimental conditions were the same as those described in Figure 3. SF = solvent front.

trates the CCC chromatogram of the four standard isoflavones using the type-J multilayer CPC. The gradient elution was performed using a two-phase solvent system composed of *n*-hexane/ethyl acetate/1-butanol/methanol/acetic acid/water[5] by linearly shifting the volume ratio from 1 : 2 : 1 : 1 : 1 : 5 to 1 : 2 : 1 : 3 : 0.5 : 5. The separation was completed in less than 5 hours.

CONCLUSIONS

The CCC separation of soybean isoflavones was examined using two different types of CPCs with various two-phase solvent systems. Overall results suggests that the type-J multilayer CPC is useful for the CCC separation of daidzin and genistin with the ethyl acetate/ethanol/water (4 : 2 : 7) system, and of daidzein and genistein with *n*-hexane/ethyl acetate/

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1-butanol/methanol/water (5 : 5 : 0 : 5 : 5) system. The simultaneous separation of these four isoflavones was also accomplished using a linear gradient elution of a two-phase solvent system composed of *n*-hexane/ethyl acetate/1-butanol/methanol/acetic acid/water by shifting the volume ratio from 1 : 2 : 1 : 1 : 1 : 5 to 1 : 2 : 1 : 3 : 0.5 : 5. The present methods may be useful for the preparation of isoflavones from the plant extracts.

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