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Separation and purification of isoflavones from a crude soybean extract by high-speed counter-current chromatography

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Abstract

A set of isoflavones with a broad range of polarity including daidzin, glycitin, genistin, acetyldaidzin, glycitein, acetylgenistin and daidzein was separated from a crude soybean extract by high-speed counter-current chromatography using a two-step operation. Three solvent systems were used: chloroform–methanol–water (4:3:2, v/v); chloroform–methanol–*n*-butanol–water (4:3:0.5:2, v/v); and methyl *tert*.-butyl ether–tetrahydrofuran–0.5% aqueous trifluoroacetic acid (2:2:0.15:4, v/v). The first solvent system was used for separating less polar isoflavones and the second for more polar isoflavones by eluting the lower organic phase. Genistin and glycitin, which were only partially resolved in the chloroform system, were separated by the third solvent system. Each isolated component showed 98–99% purity as determined by high-performance liquid chromatography analysis. Their structures were identified by LC–MS. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Soybean; Counter-current chromatography; Isoflavones; Daidzin; Glycitin; Genistin; Acetyldaidzin; Glycitein; Acetylgenistin; Daidzein

1. Introduction

Soybeans, which have long been an essential part of the diet in Asian countries, contain large amounts of isoflavones [1]. These include three aglycones (daidzein, genistein and glycitein) and nine glucosides (daidzin, genistin, glycitin, acetyldaidzin, acetylgenistin, acetylglycitin, malony-daidzin, malonygenistin and malonyglycitin), whose chemical structures are shown in Fig. 1. Interest in soy ingredients has increased recently all over the world because epidemiologic studies have shown that their consumption may be associated with low incidence rates of hormone-dependent and independent cancers [2], and with reduction in the risk of various diseases including cardiovascular problem [3], breast cancer [4,5], prostate cancer [6], colon cancer [7], osteoporosis [8], menopausal symptoms [9] and coronary heart disease [10]. In this connection, soy-isoflavones have been reported to have a variety of biological activities including estrogenic [11], anti-oxidative [12,13], anti-osteoporosis [14,15] and anticarcinogenic [16]. Isoflavones may also directly

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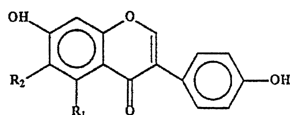
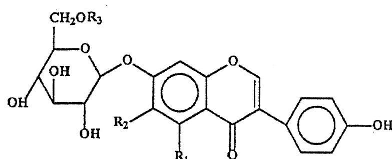
Aglycones:



Compounds	R_1	R_2
Daidzein	H	H
Genistein	OH	H
Glycitein	H	OCH_3

Glucosides:



Compounds	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	$COCH_3$
Malonyldaidzin	H	H	$COCH_2COOH$
Malonylgenistin	OH	H	$COCH_2COOH$
Malonylglycitin	H	OCH_3	$COCH_2COOH$

Fig. 1. Chemical structures of isoflavones from soybeans.

inhibit bone resorption [17]. In animal studies, isoflavones showed anticancer effects inhibiting the growth of human breast and prostatic cancer cells in culture [18]. A recent study proposed that isoflavones may be the main active ingredients responsible for the cholesterol-lowering properties of some soyfoods [19]. Commercial preparations of isoflavone extracts from soybean are sold as nutritional supplements, and in the United States, health claims for foods containing soy protein are now permitted. To evaluate the potential of isoflavones as health-enhancing dietary compounds, pure isoflavones are needed as standards for the quantitative analyses of isoflavones available in soybeans and in typical soyfoods [1].

High-speed counter-current chromatography (HSCCC) is a powerful tool being used worldwide [20,21] for the preparative separation and purification of natural products. The present paper describes the preparative separation of these important compounds from a crude extract of soybeans by normal and reverse phase HSCCC.

2. Experimental

2.1. Apparatus

Two HSCCC instruments were used in the present studies. The analytical HSCCC instrument employed is a triplet type-J coil planet centrifuge fabricated in the NIH machine shop [22]. It holds three columns symmetrically around the rotary frame at a distance of 7.5 cm from the central axis of the centrifuge. Each column consists of 0.85 mm I.D. Tefzel tubing (Zeus Industrial Products, Raritan, NJ, USA) which is coaxially wound around the holder hub forming 11 coiled layers. These three multilayer coils are connected in series with 0.4 mm I.D. PTFE tubing (Zeus Industrial Products) to make up a total capacity of 175 ml. The β-value varied from 0.5 at the internal terminal to 0.75 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The revolution speed of the apparatus

could be regulated with a controller in the range of 0–1500 rpm. An optimum speed of 1000 rpm was used in the present studies.

The actual preparative separation was performed using a type-J multilayer coil planet centrifuge (P.C. Inc., Potomac, MD, USA). It holds a column holder and a counterweight in the symmetrical positions at a distance of 10 cm from the central axis of the centrifuge. The separation column was prepared by winding a single piece of Tefzel tubing (Zeus Industrial Products) of 1.6 mm I.D. (SW 14), 160 m in length around the column holder hub making 12 layers between a pair of flanges spaced 2 inches apart. The total capacity of the column is about 320 ml. The β-values varied from 0.5 at the internal terminal to 0.75 at the external terminal. The revolution speed of the apparatus was regulated with a controller (Bodine Electric, North Chicago, IL, USA) where an optimum 800 rpm was used in the present studies.

The high-performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-6A system including an LC-6A solvent delivery unit, an SPD-6AV UV–Vis spectrophotometric detector, a Model 7726 injection valve with a 20-μl loop and a CR501 Chromatopac integrator (Shimadzu Corporation, Kyoto, Japan).

2.2. Reagents

Chloroform, methanol, *n*-butanol, methyl *tert*.-butyl ether (MtBE), tetrahydrofuran (THF) and water were of HPLC-grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA) was of HPLC-grade and purchased from Pierce (Rockford, IL, USA).

The crude extract from soybeans was kindly provided by Dr. Qipeng Yuan from the Beijing University of Chemical Technology (Beijing, People's Republic of China).

2.3. Preparation of two-phase solvent system and preparation of sample and sample solutions

The composition of three two-phase solvent systems utilized in the present study were as follows:

Solvent system 1: $CHCl_3$–MeOH–H_2O (4:3:2, v/v).

Solvent system 2: $CHCl_3$–MeOH–*n*-BuOH–H_2O (4:3:0.5:2, v/v).

Solvent system 3: MtBE–THF–*n*-BuOH–0.5% aqueous TFA (2:2:0.15:4, v/v).

After thoroughly equilibrating each solvent mixture in a separating funnel at room temperature, two phases were separated shortly before use. In the present studies the upper phase was used as the stationary phase and the lower phase as the mobile phase.

The sample solutions were prepared by dissolving 60–500 mg of the crude soybean extract in 10–50 ml of a mixture of the upper and lower phases.

2.4. Separation procedure

The column was first entirely filled with the upper stationary phase. This was followed by sample injection through the sample port. The mobile phase was pumped into the inlet of the column at a flow-rate of 1.0 ml/min (analytical) and 2.0 ml/min (preparative) in the head-to-tail elution mode, while the apparatus was rotated at 1000 rpm (analytical) and 800 rpm (preparative). The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S, LKB Instruments, Bromma/Stockholm, Sweden) at 275 nm and collected into test tubes at 2-min intervals using a fraction collector (Ultrorac, LKB Instruments). After the desired peaks were eluted, the apparatus was stopped and the column contents were collected into a graduated cylinder by connecting the inlet of the column to a nitrogen line at 100 p.s.i. The percent retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

2.5. High performance liquid chromatography analyses of CCC peak fractions

The crude soybean extract and each purified fraction were analyzed by HPLC with a Phenomenex Luna C_{18} (2) column (150×4.6 mm I.D., 5 μm) at

262 nm with a mobile phase composed of methanol–5% acetic acid. The elution was programmed at 25:75 (0.01 min)–25:75 (15.00 min)–30:70 (15.01 min)–30:70 (100 min).

2.6. LC–MS structure identification

All of the compounds studied have different molecular weights so that identification by their $(M+H)^+$ ions suffices to identify them. All analyses were performed with a Finnigan LCQ MS (Finnigan TSQ-700 mass spectrometer; Finnigan, San Jose, CA, USA) coupled with a HP series 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). HPLC utilized a YMC ProC-18 (3 μm) reversed-phase column (YMC, USA) eluting with 0.25% acetic acid solution and 0.25% acetic acid in acetonitrile at a flow-rate of 1.0 ml/min with a gradient of 0–30% acetonitrile over 40 min followed by 30–50% for an additional 10 min. The entire column eluate was introduced into the MS source with positive ESI using a standard high flow tube method.

3. Results and discussion

An HPLC method was first used for the analysis of the soybean extract according to a previously reported method [23] with a minor modification as indicated above. Fig. 2 shows the resulting chromatogram of the crude soybean extract.

HSCCC separation of the crude soybean extract was initiated using a simple ternary solvent system composed of $CHCl_3$–MeOH–H_2O at a volume ratio of 4:3:2 (v/v, solvent system 1) which has been commonly used for the separation of flavonoids. The result of this separation of 150 mg of crude soybean extract is shown in Fig. 3, and the separation yielded 5–10 mg of four of the less polar isoflavones of daidzein, glycitein, acetyldaidzin and acetylgenistin with purity ranging from 98 to 99%, as determined by HPLC analysis. Polar isoflavones, such as genistin, glycitin and daidzin, were still retained on the column due to their large partition coefficient (K) values in this solvent system. In order to separate them, the above solvent system was made slightly more hydrophilic by adding n-butanol. Fig. 4 shows the separation of 60 mg of the crude soybean extract

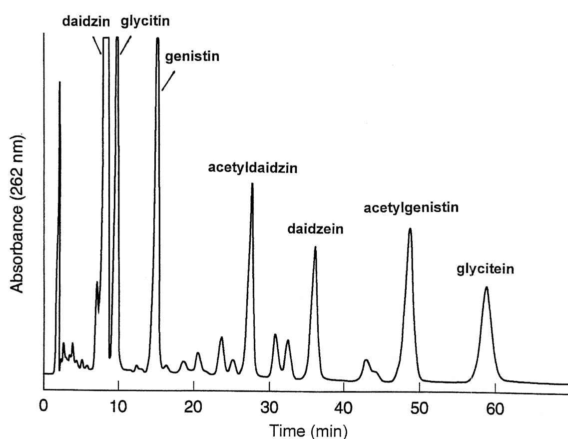


Fig. 2. HPLC analyses of the crude soybean extract. Column: Shim-pack VP ODS column (150×4.6 mm I.D.); mobile phase: methanol–5% acetic acid: 25:75 (0.01 min)–25:75 (15.00 min)–30:70 (15.01 min)–30:70 (100 min); flow-rate: 1.0 ml/min; UV wavelength: 262 nm.

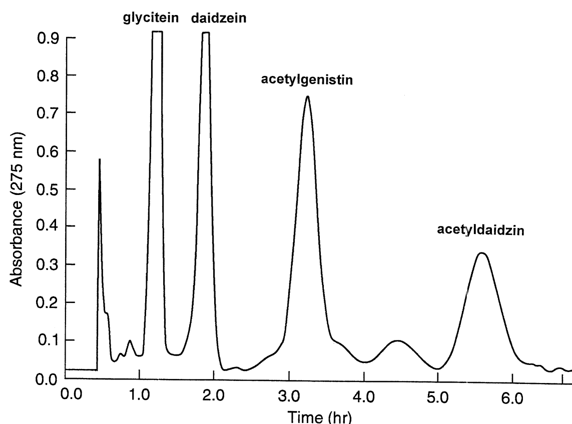


Fig. 3. Chromatogram of the separation of the crude soybean extract by preparative HSCCC. Solvent system 2: $CHCl_3$–MeOH–H_2O (4:3:2, v/v); stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 150 mg; retention of stationary phase: 85%; UV wavelength: 275 nm. (1) Glycitein; (2) daidzein; (3) acetylgenistin; (4) acetyldaidzin.

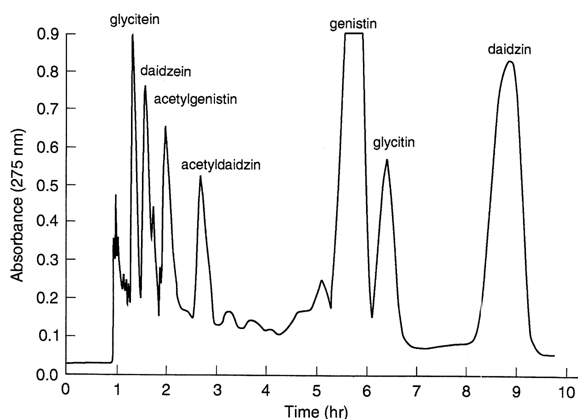


Fig. 4. Chromatogram of the separation of the crude soybean extract by analytical HSCCC. Solvent system 1: $CHCl_3$–MeOH–n-BuOH–H_2O (4:3:0.5:2, v/v); stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow-rate: 1.0 ml/min; revolution speed: 1000 rpm; sample size: 60 mg; retention of stationary phase: 79%; UV wavelength: 275 nm.

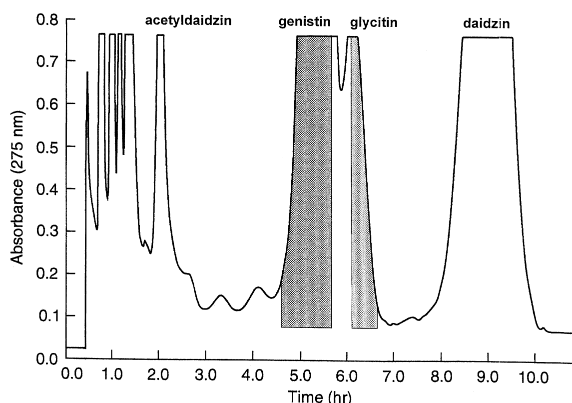


Fig. 5. Chromatogram of the separation of the crude soybean extract by preparative HSCCC. Solvent system 1: $CHCl_3$–MeOH–n-BuOH–H_2O (4:3:0.5:2, v/v); stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 150 mg; retention of stationary phase: 85%; UV wavelength: 275 nm.

by analytical HSCCC with the solvent system composed of $CHCl_3$–MeOH–n-BuOH–H_2O at 4:3:0.5:2 (v/v, solvent system 2). Elution with the lower organic phase produced a good separation of polar components, such as genistin, glycitin, daidzin and acetyldaidzin, while the resolution between the less polar components was reduced due to their lower K-values.

Fig. 5 shows separation of 150 mg of the crude soybean extract by preparative HSCCC with the same solvent system ($CHCl_3$–MeOH–n-BuOH–H_2O, 4:3:0.5:2, v/v). Although a 2.5-fold increase in sample size still produced a good separation of daidzin and acetyldaidzin, genistin and glycitin were only partially resolved. In order to improve this separation of genistin and glycitin, a new solvent system composed of MtBE–THF–n-BuOH–0.5% aqueous TFA, 2:2:0.15:4 (v/v, solvent system 3) was tested. Fig. 6 shows the separation of 100 mg of the crude soybean extract by preparative HSCCC with solvent system 3. Using the aqueous phase as the mobile phase, isoflavones were eluted in a decreasing order of polarity, as observed in reversed-phase HPLC. The result shows that genistin was well resolved from glycitin and the other isoflavones,

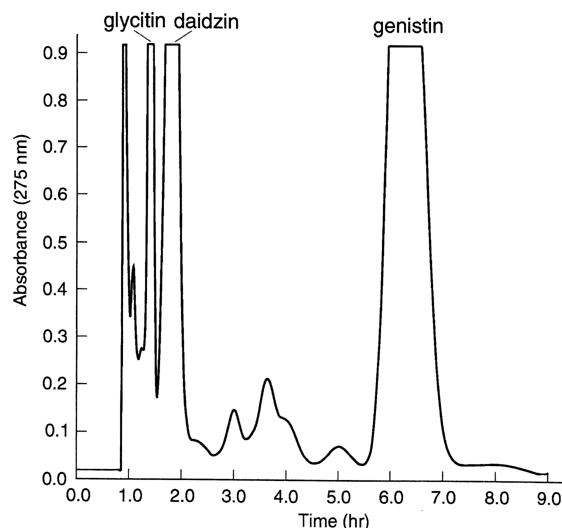


Fig. 6. Chromatogram of the separation of the crude soybean extract by preparative HSCCC. Solvent system: MtBE–THF–n-BuOH–0.5% aqueous TFA (2:2:0.15:4, v/v); stationary phase: upper phase; mobile phase: lower phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 100 mg; retention of stationary phase: 67.5%; UV wavelength: 275 nm.

whereas glycitin was only partially resolved from the polar isoflavones.

The above result suggests that the one-step separation of all components with a single solvent system is not practical. However, their combined use resolves all isoflavones compounds as described below:

Fig. 7 shows the chromatogram obtained from 55 mg of a combined peak fraction containing genistin and glycitin (Fig. 5) by preparative HSCCC with solvent system 3. Complete separation of these two compounds was realized. This suggested that the separation of genistin and glycitin from the crude soybean extract can be remarkably improved and scaled up by a combined two-step separation using solvent systems 2 and 3. Fig. 8 shows the separation of 500 mg of the crude soybean extract by preparative HSCCC with solvent system 2 ($CHCl_3$–MeOH–n-BuOH–H_2O at 4:3:0.5:2, v/v). This separation yielded about 100 mg of daidzin with a purity over 98.5%, as determined by HPLC. The combined fractions, which contain genistin and glycitin, were separated by the second run using solvent system 3 (MtBE–THF–n-BuOH–0.5% aqueous TFA at 2:2:0.15:4, v/v). The result is shown in Fig. 9, and this separation yielded genistin (106 mg) and glycitin (30 mg) each with over 99% purity, as determined by HPLC (Fig. 10). The chemical struc-

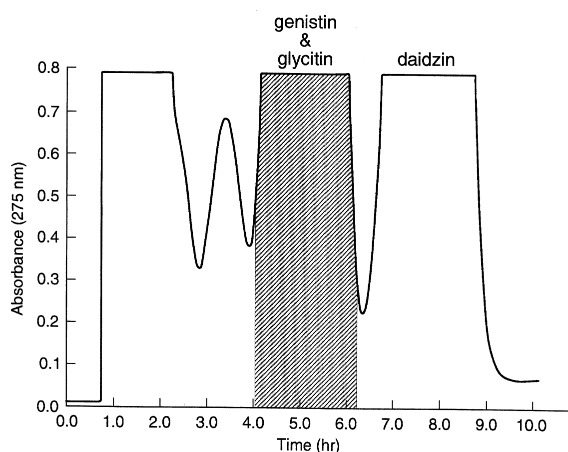


Fig. 8. Chromatogram of the separation of the crude soybean extract by preparative HSCCC. Solvent system 1: $CHCl_3$–MeOH–n-BuOH–H_2O (4:3:0.5:2, v/v); stationary phase: upper phase; mobile phase: lower phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 500 mg; retention of stationary phase: 72%; UV wavelength: 275 nm.

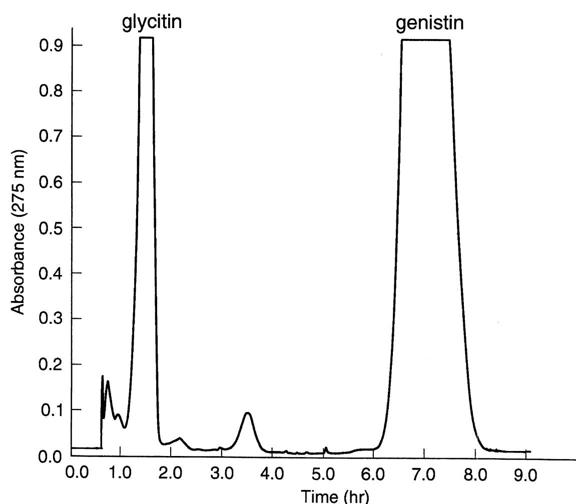


Fig. 7. Chromatogram of the separation of the combined fractions which contain genistin and glycitin from Fig. 5 by preparative HSCCC. Solvent system: MtBE–THF–0.5% aqueous TFA (2:2:4, v/v); stationary phase: upper phase; mobile phase: lower phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 55 mg; retention of stationary phase: 82%; UV wavelength: 275 nm.

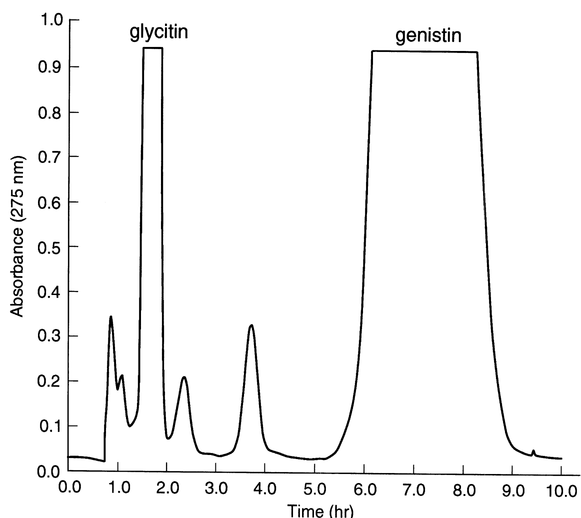


Fig. 9. Chromatogram of the separation of the combined sample from Fig. 8 by preparative HSCCC. Solvent system 3: MtBE–THF–n-BuOH–0.5% aqueous TFA (2:2:0.15:4, v/v); stationary phase: upper phase; mobile phase: lower phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 150 mg; retention of stationary phase: 82%; UV wavelength: 275 nm.

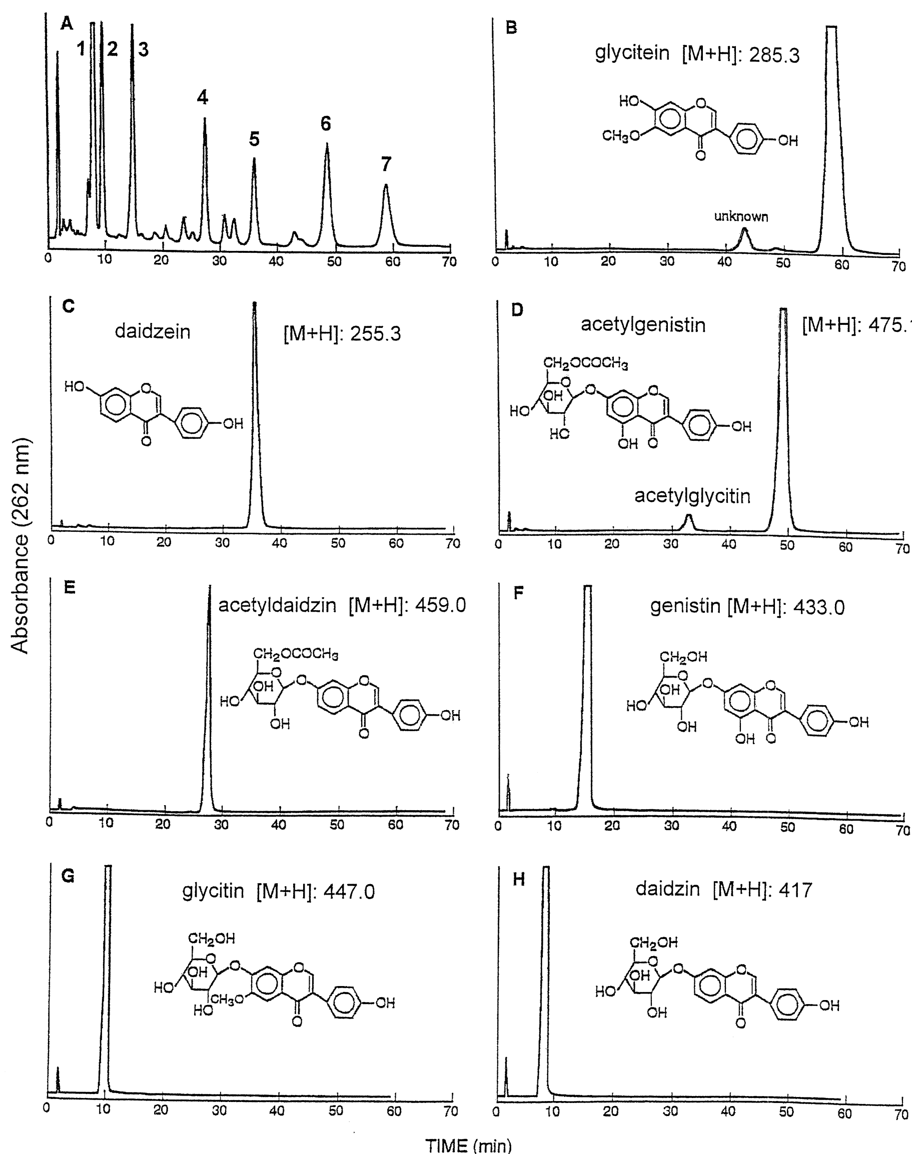


Fig. 10. HPLC analyses of the purified peak fractions from the HSCCC separations. Column: Shim-pack VP ODS column (150×4.6 mm I.D.); mobile phase: methanol–5% acetic acid: 25:75; flow-rate: 1.0 ml/min; UV wavelength: 262 nm. (A) The crude soybean extract: (A-1) daidzin, (A-2) glycitin, (A-3) genistin, (A-4) acetyldaidzin, (A-5) daidzein, (A-6) acetylgenistin, (A-7) glycitein; (B) peak (1) in Fig. 3; (C) peak 2 in Fig. 3; (D) peak 3 in Fig. 3; (E) peak 5 in Fig. 3; (F) peak 1 in Fig. 9; (G) peak 4 in Fig. 9; (H) peak 4 in Fig. 8.

tures of the purified compounds were identified by LC–MS, and the results of their [M+H] values are also given in Fig. 10.

The results of our studies clearly demonstrate that the combined use of a pair of two-phase solvent systems in HSCCC enables excellent purification of a set of isoflavones with a broad spectrum of polarity from a large amount of crude soybean extract. The results also clearly demonstrate that HSCCC provides a great flexibility and selectivity to meet the need of resolving multiple components in a crude extract.

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