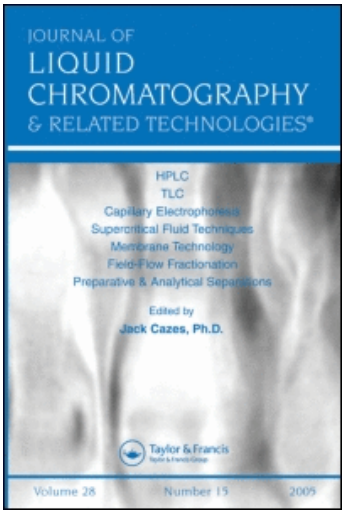

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PREPARATIVE SEPARATION OF ISOFLAVONES FROM SOYBEAN BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

As a potential anticancer agent, the isoflavones contained in soybeans were considered for separation by reversed-phase high performance liquid chromatography (RP-HPLC). The mobile phases were the ternary system of water/acetonitrile/acetic acid. In this work, the four different columns of μ-Bondapak (0.39 × 30 cm, 10 μm), and the other three empty stainless steel columns (0.39 × 30, 1 × 25, 1″ × 25 cm) packed with 15 μm C_{18} packings were used. The experimental variables were the gradient conditions and mobile phase composition. The increased number of gradient steps enabled the separation of isoflavones in the larger columns. The separation condition of 1″ × 25 cm column was determined by experiments of the systematic sequence of the smaller columns. For the largest column, 2 mL of sample was separated with the three gradient steps at 18 mL/min of mobile phase flow rate. The isoflavones of daidzein, glycitin, genistin, daidzin, and glycitein were identified by LC-MS with the purified components.

INTRODUCTION

Soybeans have been a popular Korean diet for centuries. As it contains 40% protein and 20% lipid, with only a small amount of starch, soybeans are

considered to be closer to the meat group rather than grain products. Recently, other good effects of soybeans have been studied.[1,2] Soybeans are unique because they contain compounds called isoflavones and the structures of these molecules are very similar to the human natural estrogens.[3,4] Estrogens are hormones that our bodies make and require for normal growth and development, and to maintain good adult health not only in women, but also in men. It has been reported that these isoflavones compounds play an important role in cancer prevention, inhibit tumor initiation, oxidative damage, moderation of menopausal symptoms, and other health effects.[5]

Some researches have been established for the separation of isoflavones. Some kinds of isoflavone glycosides were isolated from the hypocotyls of soybean seeds[6] and a simple analytical method has been developed for routine quantification of the isoflavones of daidzein and genistein in food.[7] By solid phase extraction and reversed-phase HPLC, the purification as well as qualitative and quantitative determination of isoflavones in plant material were performed.[2] Chromatographic methods have been developed to determine the separation conditions of the mixture from analytical to preparative column.[8] From the analytical column, the separation condition was determined and it was modified to adapt to the preparative column. For the scale-up of column, the effect of changing the column dimensions can be estimated by considering the ratios of the cross-sectional areas of column and column volumes so both the volumetric flow rate and the amount injected should be modified.[9] Usually the volumetric flow rate and injection amounts are proportional to the cross section areas and the total volume of the column, respectively. In this work, the analytical and preparative reversed-phase HPLC systems with four different sizes of column were used to separate preparatively some of isoflavones. The linear gradient modes and mobile phase compositions were changed to obtain the desirable experimental conditions.

EXPERIMENTAL

Chemicals

The standard chemical of daidzein was obtained from Sigma, USA and HPLC-grade methanol and acetonitrile were purchased from J. T. Baker, USA. The hypocotyls of soybean prepared by Shindongbang Co. (Ansan, Korea) were extracted with an 80% aqueous methanol, then filtered through a filter (HA-0.45 μm, FH-0.5 μm).

Instrumentation and Method

The experiments were performed with two HPLC systems. Analytical HPLC (Waters Co.): equipped with a 600S solvent delivery and control system, 2487 dual λ absorbance detector and Millennium 32 data acquisition system. Preparative HPLC (Waters Co.): 600E pump, 486 detector and Chromate 3.0

data acquisition system (Interface Eng.). Sufficient time was allowed for the stabilization of the column and detector signal after each injection, and the solvents in reservoirs were continuously stripped with helium to degas the mobile phase. The dual wavelengths were fixed at 260 nm and 290 nm for the analytical and preparative HPLC systems, respectively.

Four different types of the columns used in this experiments were μ-Bondapak (0.39 × 30 cm, 10 μm, Waters Co.), and the other three empty stainless steel columns (0.39 × 30, 1 × 25, 1″ × 25 cm) in-house packed with 15 μm C_{18} (Lichrospher, Merck). With μ-Bondapak and the analytical column (0.39 × 30 cm), the flow rate was 1mL/min and the injection volume was 10 μL. The two larger preparative columns (1 × 25 cm, 1″ × 25 cm) were installed on the preparative HPLC, where the wavelength was set at 290 nm. For the two columns, the flow rate and amount of sample injected were 4 mL/min, 0.2 mL and 18 mL/min, 2 mL, respectively. The ternary mobile phases were water, acetonitrile, and acetic acid. The adjustable experimental variables were the conditions of gradient modes and mobile phase compositions. The experimental conditions were listed with the four columns in Table 1.

Table 1

Gradient Conditions Used in this Work

		Mobile Phase Profile	
Column	Run Time (min)	% Reservoir A[a]	% Reservoir B[b]
μ-Bondapak	0	100	0
0.39cm x 30cm	60	70	30
	0	100	0
	17	96.5	3.5
1cm x 25 cm	20	91	9.0
	65	85	15
	100	60	40
	0	100	0
1″ x 25cm	30	90	10
	70	88	12
	200	60	40

[a] Reservoir A: Water/acetonitrile/acetic acid (94.9/5.0/0.1, vol. %).
[b] Reservoir B: Water/acetonitrile/acetic acid (5.0/94.9/0.1, vol. %).

Some peaks were collected and freeze-dried by (Labconco, Labconco Co. USA) and then analyzed by LC-MS (SSQ7000, Finnigan Co. USA) for qualification. The daidzein peak was qualitatively reconfirmed by adding the pure component into the extracted sample. Figure 1 shows the chemical structures of some isoflavones identified in this work.

RESULTS AND DISCUSSION

To separate the isoflavones contained in hypocotyl supplied by Shindongbang Co., the commercial C_{18} μ-Bondapak column was utilized. The mobile phases were composed of water with acetic acid and acetonitrile. Gradient mode was applied; the mobile phase composition of a reservoir A was 94.9/5.0/0.1 vol.%, water/acetonitrile/acetic acid, while that of a reservoir B was 5.0/94.9/0.1 vol.%, water/acetonitrile/acetic acid. These mobile phase compositions in the reservoirs A and B were kept constant throughout in this work. With these types of experimental columns, gradient times were adjusted, so the total run times were varied in the range of between 60 and 130 min by varying the flow rates.

As the composition of mobile phase was linearly changed and the content of acetonitrile was increased, the late-eluting components came out of the column fast with relatively good resolutions. The chromatogram is shown in Figure 2, and some of the peaks were identified by LC-MS and the standard chemicals of genistein and daidzein.

In the column (0.39 × 30 cm) packed by 15 μm preparative packings, the amount of sample size injected and flow rate of mobile phase were 10 μL and 1 mL/min, respectively, the same as in the μ-Bondapak column. With the same mobile phase and gradient conditions, the peak of genistin was coeluted with the following 6″-o-malonyldaidzin and 6″-o-molonylglycitin caused by larger packing sizes (refer to Figure 3). The other isoflavones of daidzin, glycitin, daidzein, and glycitein were relatively well resolved, although the column efficiencies were deteriorated.

To obtain the isoflavones on a preparative scale, the two larger columns (1 cm and 1″ dia. with 25 cm length) were packed by the 15 μm preparative packings and used for experiments. The gradient conditions need to be precisely tuned to separate the isoflavones. The controllable experimental variables were the gradient times and the numbers of gradient steps, injection volumes and flow rates of mobile phase. In the smaller column (1 × 25 cm), initially the total run time of 100 min was set at the flow rate of 4.0 mL/min. From the several experiments, we changed the numbers of gradient steps and mobile phase compositions. The injection volume was set at 0.2 mL. To decrease the total elution time, the numbers of linear gradient modes and the acetonitrile contents were adjusted. So the four gradient steps and the corresponding gradient times were experimentally obtained. The resulting chromatogram is shown in Figure

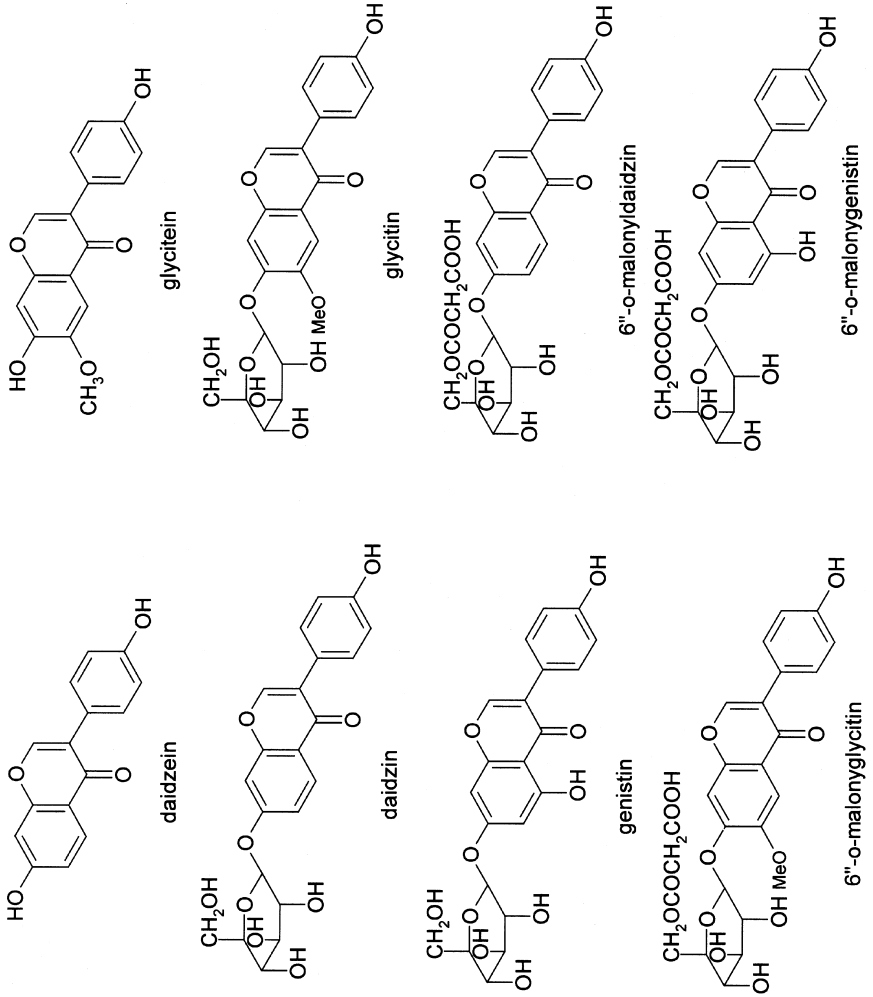

Figure 1. Structures of isoflavones.

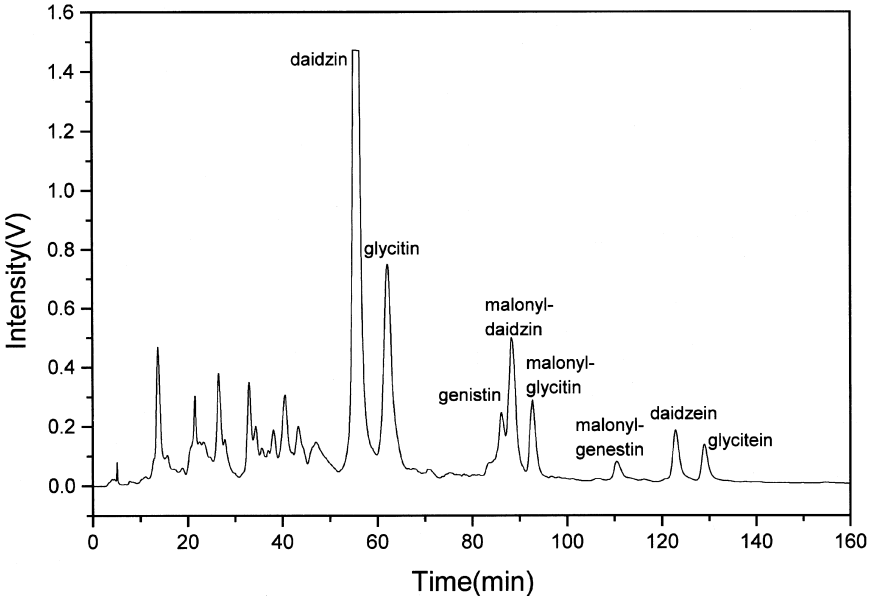

Figure 2. Separation of isoflavones from soybean (μ-Bondapak, 10 μL injection, 1 mL/min).

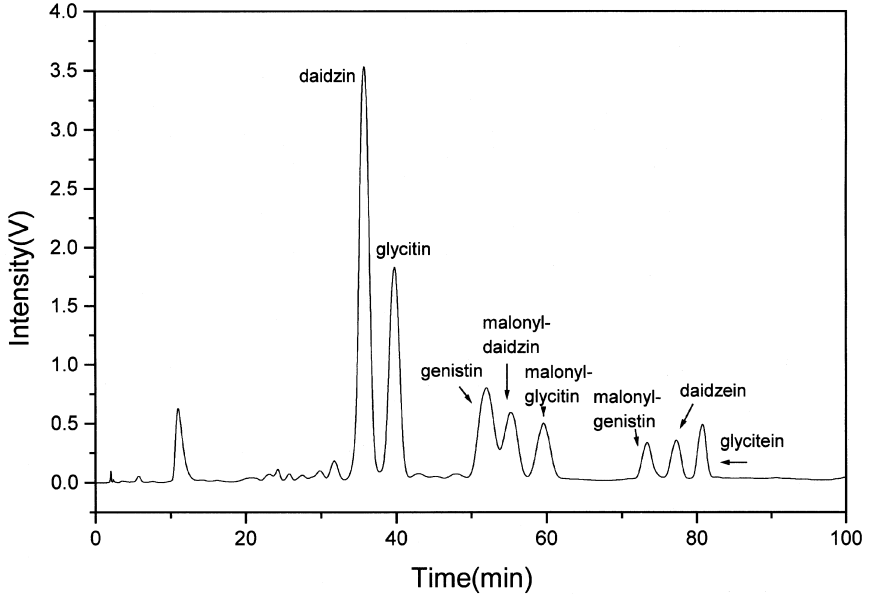

Figure 3. Separation of isoflavones from soybean (0.39 cm x 30 cm, 10 μL injection, 1 mL/min).

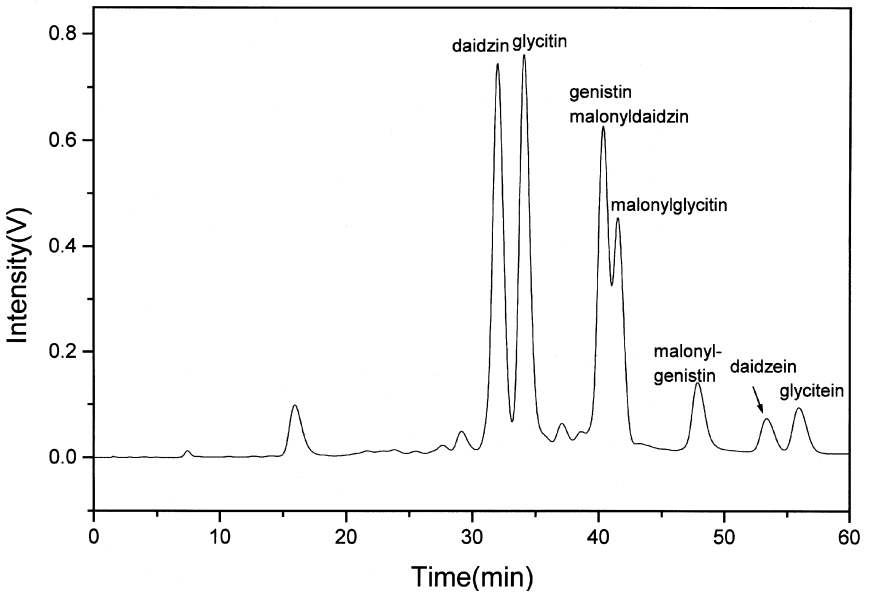

Figure 4. Separation of isoflavones from soybean (1 cm x 25 cm, 200 μL injection, 4 mL/min).

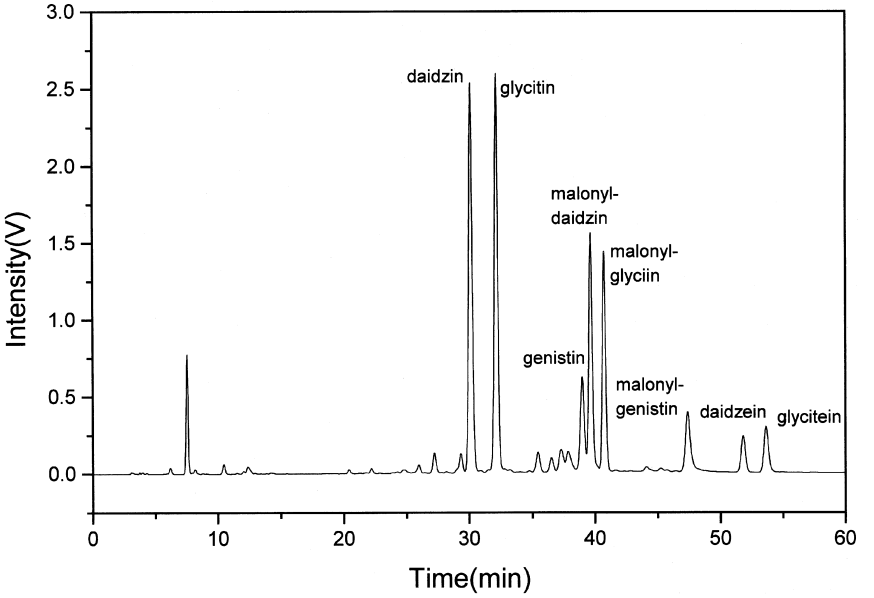

Figure 5. Separation of isoflavones from soybean (1″ x 25 cm, 2 mL injection, 18 mL/min).

Table 2

Reproducibility of Retention Times of Some Isoflavones*

Component	No. (min) 1	2	3	4	5	6	7	Standard Deviation
Malonyl diadzein	84.17	84.01	84.69	85.68	86.58	85.46	86.32	0.94
Malonyl glycitein	88.78	89.18	90.12	90.06	91.03	90.42	90.80	0.76
Malonyl genistein	108.97	108.79	109.61	109.38	109.33	110.13	110.12	0.68
Diadzein	120.68	121.23	121.73	121.62	122.01	122.72	122.58	0.67
Glycitein	126.93	127.93	127.89	127.75	127.87	128.46	128.55	0.49

* (1″ x 25 cm column, 2 mL injection, 18 mL/min).

4, and the experimental conditions in Table 1. The peak of genistin was partially resolved. In the larger column with 1″ diameter, to inject greater amounts of 2 mL, the run time was adjusted, and the number of gradient steps was decreased to 3. As shown in Figure 5, at the flow rate of 18 mL/min, the resolution was not worse, as compared to the smaller column. The gradients used in Figure 5 were quite complex, so the reproducibilities of the retention times of some isoflavones were listed, as in Table 2, which showed that the standard deviations were as small as below 1.0.

Table 3

Resolutions Between the Identified Peaks

	μ-Bondapak	0.39cm x 30cm	1cm x 25cm	1″ x 25cm
R_{12}[a]	3.74	1.21	1.55	1.78
R_{34}[a]	1.14	*	0.81	0.73
R_{56}[a]	9.70	3.25	4.69	5.78
R_{78}[a]	2.52	1.19	1.47	2.10

*: coeluted. [a] (1 denotes diadzin, 2 glycitin, 3 genistin, 4 6″-o-malonyldaidzin, 5 6″-o-malonylglycitin, 6 6″-o-malonylgenistin, 7 daidzein, and 8 glycitein).

The resolution is defined by the ratio of difference in residence times to the average peak width. The resolutions of daidzin and glycitin (R_{12}), genistin and 6″-o-malonyldaidzin (R_{34}), 6″-o-malonylglycitin and 6″-o-malonylgenistin (R_{56}), and daidzein and glycitein (R_{78}) with the types of columns, were listed in Table 3. It showed that the resolution was greatly influenced by the gradient condition and mobile phase composition. Therefore, some isoflavones could be purely collected, although the column dimensions were large.

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