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Note

Semi-preparative high-performance liquid chromatographic isolation of soybean isoflavones

E. FARMAKALIDIS* and P. A. MURPHY
Department of Food Technology, Iowa State University, Ames, IA 50011 (U.S.A.)
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Soybeans are known to contain the estrogenic isoflavones genistein and daidzein, however, the glucosides genistin and daidzin are the major forms of the isoflavonoids in soybeans. Previous reports indicate significant carryover of these forms into soybean protein products intended for human use[1,2].

Methods currently in use for the separation and purification of the flavonoids include paper chromatography, thin-layer chromatography (TLC) and column chromatography utilizing adsorbents such as alumina[3], silica gel and Sephadex LH20[4]. Gas–liquid chromatography has also been used on a preparative scale, but its use is limited to volatile samples[5]. The direct analysis of non-volatile and thermally unstable phenolics is impossible.

It is noteworthy that in the isoflavonoid field, high-performance liquid chromatography (HPLC) has been mainly used as an analytical technique; *e.g.*, for checking the purity of natural samples, for quantitative determination of plant constituents[6–9] and for chemotaxonomic comparisons. The use of HPLC on a preparative scale has not been investigated. Our paper reports how HPLC can be used on a preparative scale for the isolation and purification of the soybean isoflavones, namely genistin and daidzin.

MATERIALS AND METHODS

Toasted defatted soyflakes were obtained from A. E. Staley Manufacturing Co. (Des Moines, IA, U.S.A.). The flakes were extracted overnight with acetone–0.1 *M* hydrochloric acid in the ratio of 5 to 1 (ml/g flakes). This solvent system was previously found effective in extracting the isoflavones and gives low extraction of other interfering material[10]. After filtration, the extract was concentrated by using a rotary evaporator at 40°C. The reddish brown residue was suspended in chloroform–methanol (9:1) and passed through a 50 × 2.5 cm silica gel column (60–80 mesh, J. T. Baker, Jackson, TN, U.S.A.) equilibrated with chloroform–methanol (9:1). The column was washed with 300 ml of chloroform–methanol (9:1). This gets rid of some of the contaminating phospholipids as well as most of the free forms of isoflavones, *i.e.* daidzein and genistein. The isoflavones (mainly genistin and daidzin) were eluted from the column with 250 ml of chloroform–methanol (50:50). After the solvent was evaporated, the residue was redissolved in methanol, filtered through a 0.45-μm filter, and fractionated by using semi-preparative HPLC.

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The chromatographic system consisted of a reversed-phase 250 × 9.4 mm Partisil ODS-3 C_{18} (Whatman) semi-preparative olumn with a 2-cm LiChrosorb RP-18 guard column (Brownlee Labs). Separation of the peaks was performed with a non-linear methanol–water gradient, utilizing a Beckman/Altex microprocessor/controller and two model 110A pumps. Monitoring of the peaks was achieved by utilizing a fixed-wavelength UV detector (Beckman/Altex) at 254 nm equipped with a preparative flow cell. The combined flow-rate was 5 ml/min. Samples were injected by using a 2-ml loop. Total chromatographic analysis time was 16.5 min. Peak areas were integrated by using a Varian CDS-111 computer.

Purity of the compounds collected with this method was determined using silica gel TLC (solvent system, chloroform–methanol–water, 65:25:4), analytical HPLC[10], and melting points. The results of these experiments were compared with results obtained with compounds isolated by utilizing conventional chromatographic methods[4].

RESULTS AND DISCUSSION

A non-linear gradient of methanol–water was found the most suitable solvent system in separating the soybean isoflavones, as can be seen in Fig. 1. Preliminary experiments showed that an isocratic elution of the compounds would not be effective for adequate separation of the compounds on a preparative column. An analytical method for separating the isoflavones developed earlier in our laboratory[10] was used as a reference point for the development of the gradient used.

Preliminary experiments indicated that the crude soybean extract could not be fractionated by using HPLC directly, mainly because of the large concentration of

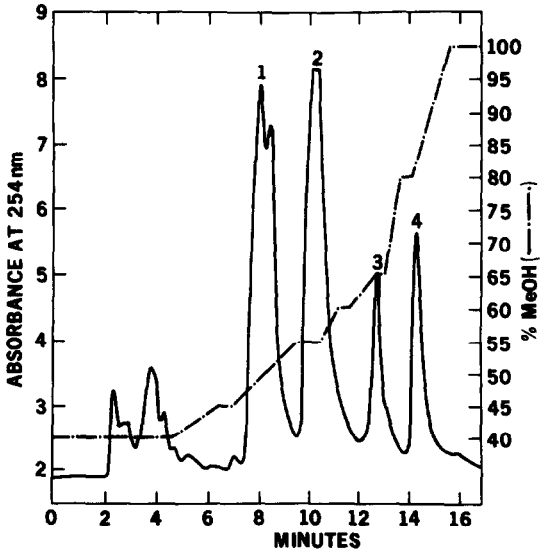

Fig. 1. HPLC fractionation of crude soybean extract, on a reversed-phase C_{18} semi-preparative column (Whatman) utilizing a non-linear methanol–water gradient. The technique of "heart-cutting" was used for the collection of daidzin. Peaks: 1 = daidzin; 2 = genistin; 3 = daidzein; 4 = genistein. Each division on the absorbance axis is equal to 0.4 absorbance units.

phospholipids in the extract. This contamination of the sample was not present in the analytical method because the sample size used was small. On a preparative scale, however, this contamination made it necessary to employ an initial silica-gel purification step before fractionation using preparative HPLC. Retention times for daidzin and genistin were 8.2 and 10.5 min, respectively.

As can be observed in Fig. 1, peak 1 consists of two compounds. Efficient resolution of the two peaks could not be achieved with the gradient used for fractionation. The compound eluting closely to daidzin was not identified but was believed to be glycitin. Several reports on the presence of glycitin in soybean extracts denote the elution time of the compound to be close to that of daidzin[2,6]. That the two peaks eluted so closely made it necessary to employ the technique of "heart-cutting"[11] for collection of daidzin. The technique involves collection of the center portion of the peak of daidzin and ignoring the leading and tailing portions. This reduced the amount of daidzin that could be collected in the original fractionation. Recycling of the daidzin peak was necessary. We utilized a different methanol–water gradient to allow further separation of the two peaks and further purification of the fraction (Fig. 2) collected.

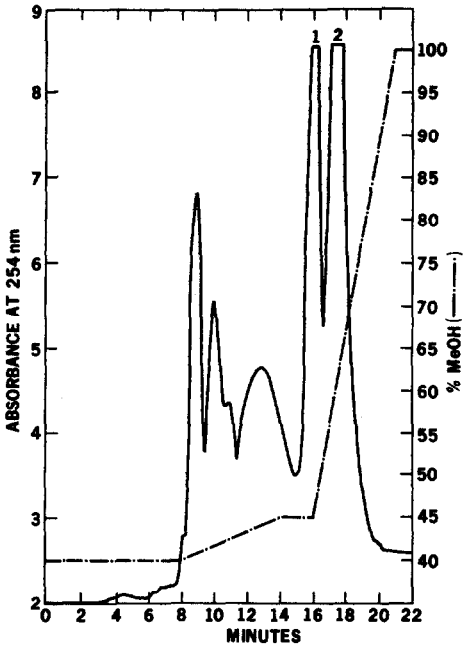

Fig. 2. Purification of the daidzin peak collected by "heart-cutting", in Fig. 1 and utilizing a new methanol–water gradient. Peaks: 1 = daidzin; 2 = not identified. Each division on the absorbance axis is equal to 0.4 absorbance units.

Collection of the genistin peak was easier because it only contained one compound, but recycling was required to get rid of contaminating material. The methanol–water gradient was changed to suit the purification of the compound (Fig. 3).

After recycling twice through the column, daidzin was crystallized from 50%

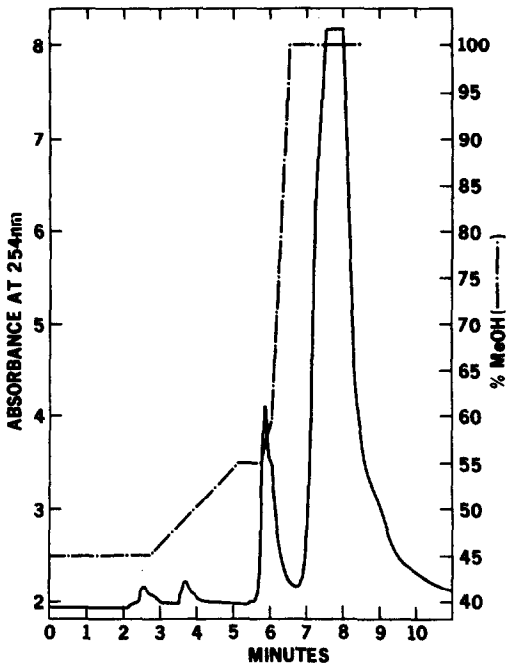

Fig. 3. Purification of genistin fraction collected from Fig. 1 and utilizing a different methanol–water gradient. Each division on the absorbance axis is equal to 0.4 absorbance unit.

methanol and genistin was precipitated out of a super-saturated solution in 100% ethanol. The results of the various tests performed on the compounds are shown on Table I. TLC revealed one spot for each of the compounds. Analytical HPLC revealed only one peak. Melting points were identical with those reported in the literature[12]. The main limitation of the method was the amount of mixture that could be applied to the column. The maximum amount that could be applied was only 10 mg of a mixture of genistin and daidzin. The maximum solubility of the compounds

TABLE I

COMPARISON OF COMPOUNDS ISOLATED BY USING CONVENTIONAL AND HPLC METHODS

Compound	*Conventional**	*HPLC*
R_F value		
Genistin	0.61	0.60
Daidzin	0.51	0.51
Melting point (°C)		
Genistin	256 ± 2	256 ± 2
Daidzin	230 ± 2	230 ± 2
Retention times with analytical HPLC (min)		
Genistin	8.3	8.3
Daidzin	7.4	7.4

* Use of silica gel and Sephadex LH-20 column chromatography according to Ohta *et al.*[4].

in methanol was only 5 mg/ml. The recovery of pure sample was 30% of the original sample fractionated.

The time required to purify large amounts of the compounds is comparable to the time required for the final fractionation of a mixture of daidzin and genistin by use of conventional chromatographic[4] methods (Table II). Therefore, the method offers some advantages over the conventional methods of isolation because the purification steps required are fewer. Apart from the original purification of the sample through silica gel, no other purification was necessary before application of the sample on the column. Use of conventional methods requires several purification steps before fractionation of the mixture into its components, genistin and daidzin.

Even though the solubility of the compounds in methanol is low, the use of preparative HPLC still is more rapid than the conventional chromatographic methods presently reported in the literature.

TABLE II

COMPARISON OF TIMES REQUIRED FOR SEPARATION OF DAIDZIN AND GENISTIN

Method	*Maximum load (mg)*	*Time required*	*Recovery (mg)*
HPLC	10	16.5 min	3
Sephadex LH-20 fractionation	50	12 h	45

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