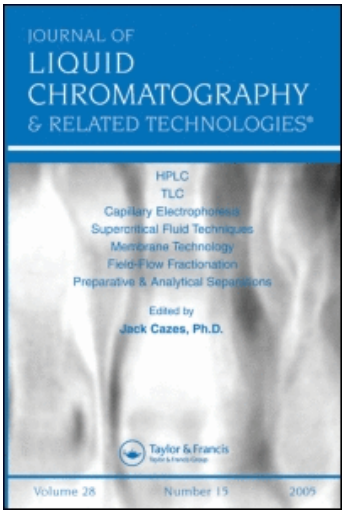

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ANALYSIS OF ISOFLAVONES IN NATURAL SOURCES AND NUTRITIONAL SUPPLEMENTS BY LIQUID CHROMATOGRAPHY AND MULTI-CHANNEL ELECTROCHEMICAL DETECTION

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ABSTRACT

A simple gradient reverse-phase liquid chromatographic method has been developed for the separation of daidzin, genistin, daidzein, and genistein in various natural sources and dietary supplements. Peak assignment at various retention times was confirmed by comparison of the simultaneous response of sample peaks to standards, at glassy carbon electrodes held at different oxidation potentials, using a multi-channel electrochemical detector. Identification was substantiated by ESI MS/MS fragmentation patterns. The analytes of interest were extracted by sonicating raw materials in cold 80% methanol/water for 10 minutes. The concentration of the named

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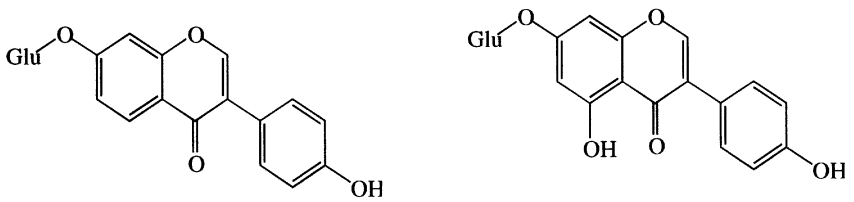
isoflavones in various dry food and nutritional supplements was determined using the present LCEC procedure.

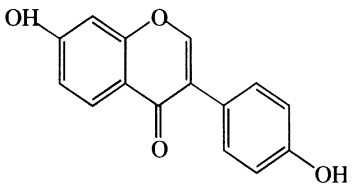
INTRODUCTION

Isoflavones, a subclass of flavonoids, are a group of plant polyphenolic compounds. They have attracted a great deal of public attention because of their potential in the prevention and treatment of a number of chronic diseases, such as cardiovascular disease, osteoporosis, and hormone-related cancers. Human dietary exposure to isoflavones is achieved mainly by ingesting legume family (Fabaceae) plants and their products, among which soybean products are the richest source of isoflavones (1). Western diets traditionally contain few soy products, however, various soybean extracts are now available as nutritional supplements. Many manufactures of these supplements indicate that the extracts are standardized regarding the content of isoflavones. Two of the most abundant isoflavones (Figure 1) are daidzein and genistein, which occur both in free state and as glycosides. They are the proposed bioactive ingredients and their estrogenic and antiestrogenic activity, anticarcinogenic effect, and antidipsotropic properties have been discussed (2–7).

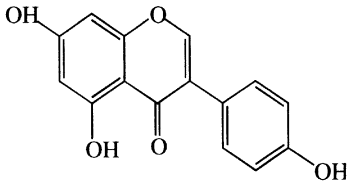


Daidzin (DIN)

Genistin (GIN)



Daidzein (DE)



Genistein (GE)

Figure 1. Structures of the isoflavones studied.

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A number of liquid chromatography methods (LC) have been reported for the determination of isoflavones. Absorbance detectors, UV and fluorescence, have been used in most systems (8–11). Recently, mass spectrometry has been used for quantitation (12–15) and identification (16). Electrochemical (EC) detection has been demonstrated as a sensitive and selective alternative (17–22) to both of the above detection schemes. In this study, a four-channel EC detector with a radial flow cell has been utilized. Peak profiling has been achieved by applying a different oxidation potential at each electrode, allowing for peak rationing and minimizing baseline noise (routinely less than 0.005 nA).

EXPERIMENTAL

Apparatus

The LC system consisted of a gradient pump (PM-80, BAS, West Lafayette, IN), an in-line filter (0.5 μm, Rheodyne) before the analytical column, a C_8 5 μm column (150 × 2.0 mm, BAS, West Lafayette, IN), a CMA/200 refrigerated autosampler (Stockholm, Sweden) equipped with a 50 μL loop. An epsilon™ electrochemical detector (BAS, West Lafayette, IN) was coupled to a four-channel glassy carbon working electrode utilizing a radial flow configuration (23). Applied potentials were +1100, 950, 850, and 750 mV vs. Ag/AgCl. Data was acquired and integrated using BAS ChromGraph version 9.35 chromatography software.

LC/MS/MS used the above LC coupled to a Finnigan LCQ Deca ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Samples were injected manually, using a 50 μL loop. The mass spectrometer was operated in ESI positive ion mode. Nitrogen was used as both the sheath and auxiliary gas at a pressure of 100 units and 20 units, respectively. The spray voltage was set at 5.0 kV and the capillary temperature at 300°C.

Chemicals and Reagents

Daidzein, daidzin, genistein, and genistin standards were purchased from Indofine (Somerville, NJ). Methanol and acetonitrile were of HPLC grade (Burdick & Jackon, Muskegon, MI). Reagent grade water was prepared by in-house deionization using a NANOpure system (Barnstead/Thermolyne, Dubuque, IA). Ammonium acetate and ethylenedinitrilotetraacetic acid disodium salt (EDTA) were of analytical reagent grade (Mallinckordt, Paris, KY). Dried Puraria sample was imported from China. Dried soybean, soy flour, and nutritional

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supplements were randomly purchased from local retail stores. Regular rat food was a LabDiet® product (Purina Mills, Inc., St. Louis, MO).

Sample Preparation

A nutritional supplement sample consisted of 15 tablets or capsules randomly chosen and pooled. The various dry foods or supplements were ground into a fine powder using a mortar and pestle. A 0.25 g portion of each powdered sample was added to 10 mL of extraction solvent (methanol : water, 8 : 2, v/v) and immediately sonicated in ice water for 30 minutes. The sample was vortexed and a 200 μL aliquot of solution was transferred into a microcentrifuge filter (0.45 μm) and centrifuged at 6000 *g* for 2 minutes. Following centrifugation, a 50 μL aliquot of clear solution was diluted appropriately to make sure its concentration fell into the range of the standard curve. A 20 μL volume was then injected into the LC system.

Chromatography

Mobile phase A was composed of 9.3% acetonitrile, 5.9% methanol, and 84.8% aqueous buffer (25 mM ammonium acetate, pH 4.3, 0.25 mM EDTA). Mobile phase B was composed of 19.6% acetonitrile, 12.0% methanol, and 68.4% aqueous buffer (25 mM ammonium acetate, pH 4.3, 0.25 mM EDTA). The gradient cycle was as follows: 100% A for 1 min, 100% A to 80% A over 8 min, 80% A to 0% A over 1 min, 0% A for 10 min, 0% A to 100% A over 1 min, 100% A for 6 min. The flow rate was 0.6 mL/min.

RESULTS AND DISCUSSION

Voltammetric Characterization of Isoflavones

The multi-channel electrochemical detector allows for the simultaneous application of different oxidation potentials on four separate channels. Therefore, the time needed to construct a hydrodynamic voltammogram (HDV) can be reduced substantially. The normalized response of the isoflavone standards at the individual electrodes was plotted against oxidation potential and is presented in Figure 2. Based on the HDV's of the standard isoflavones, the four channels were set at oxidation potentials of +1100, 950, 850, and 750 mV, respectively, for all subsequent determinations carried out in this study (Figure 3). Peak height ratios between different oxidation potentials of a standard can be used for peak

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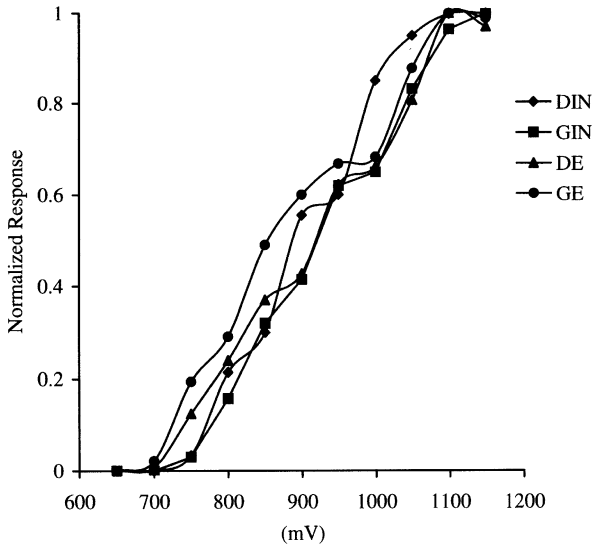

Figure 2. Hydrodynamic voltammograms (HDV) of the isoflavone standards.

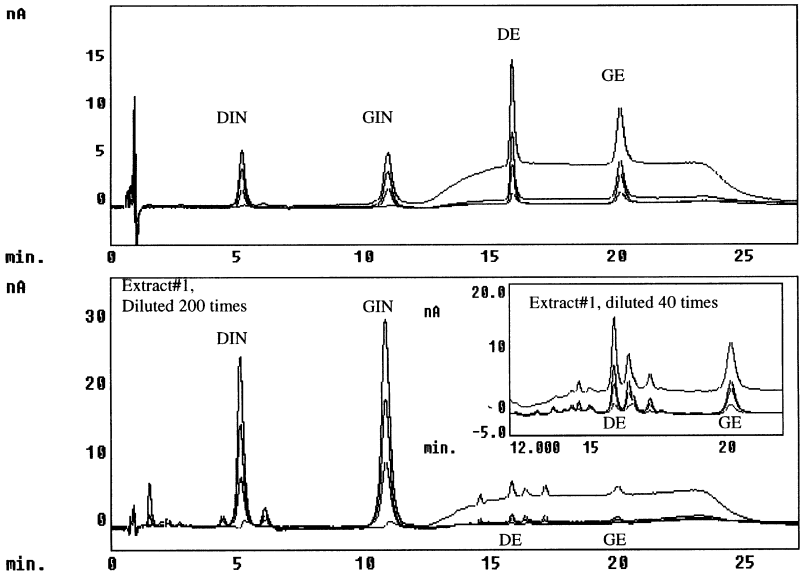

Figure 3. Chromatograms of standard mixture and representative extract. Traces with decreasing intensity are for oxidation potentials of +1100, 950, 850 and 750 mV, respectively.

Table 1. Comparison of Peak Height Ratios* for Standards and Retention Equivalent Peaks in Extract #1

	750 mV/850 mV		850 mV/950 mV		950 mV/1100 mV	
	Standards	Extract #1	Standards	Extract #1	Standards	Extract #1
DIN	0.10	0.11	0.49	0.47	0.64	0.63
GIN	0.10	0.09	0.53	0.52	0.68	0.65
DE	0.32	0.32	0.59	0.61	0.62	0.63
GE	0.38	0.38	0.74	0.74	0.67	0.67

*Data from Figure 3.

identification of the retention-equivalent peak in an unknown sample. Such peak height ratios of standard and sample from Figure 3 are listed in Table 1. The close correlation confirms peak assignment in the sample.

Identification of Isoflavones by LC/MS/MS

To further confirm the peak assignment, a standards mixture and extracts were injected into an LC/MS/MS system. The parent ions (m/z 417 for DIN, m/z 433 for GIN, m/z 255 for DE, and m/z 271 for GE) were mass-selected. Full scan MS/MS experiments were conducted in order to detect all product ions of the selected parent ions. The scan event was divided into four segments, which were tailored to the compounds of interest and specific analysis windows around their retention time. Collision-induced dissociation of the parent ions with helium gas was performed at 20% and 36% collision energy for glycosides and aglycones, respectively. Figure 4 shows the full scan MS/MS ion chromatograms and corresponding mass spectra for standard solution and extract. The match of the MS/MS spectra of both the standard solution and extract confirms the identification of four isoflavones in soy products.

Quantitative Determination of Isoflavones

Under the described chromatography conditions, all analytes of interest are separated from other components in the extract (Figure 5). Peak heights at +950 mV were plotted against varying concentrations of standards. The calibration curves were obtained by linear regression on the data sets. Linearity was found in the range of 125–4000 nM for daidzin and genistin, and 62.5–2000 nM for daidzein and genistein. Correlation coefficients (r^2) were greater

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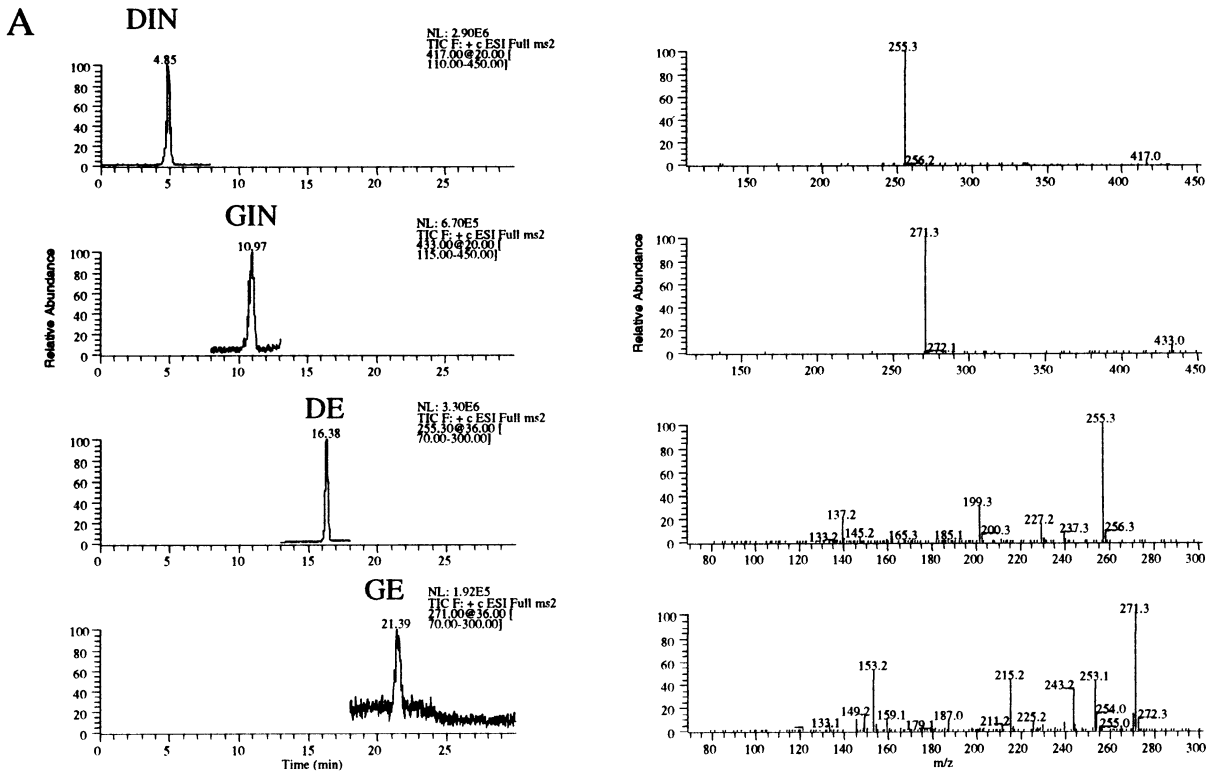

Figure 4. Chromatograms and MS/MS spectra of the standard mixture (panel A) and representative extract (panel B).

(continued)

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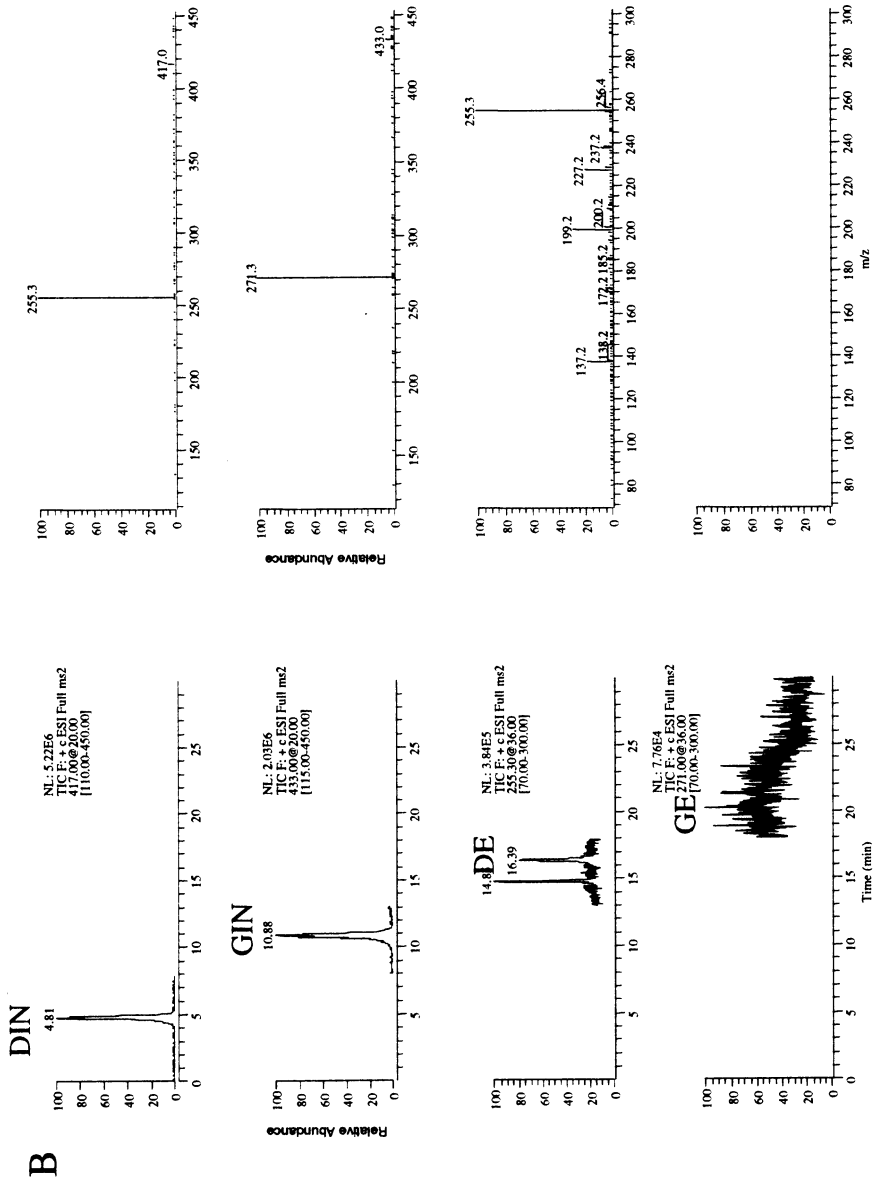

Figure 4. Continued.

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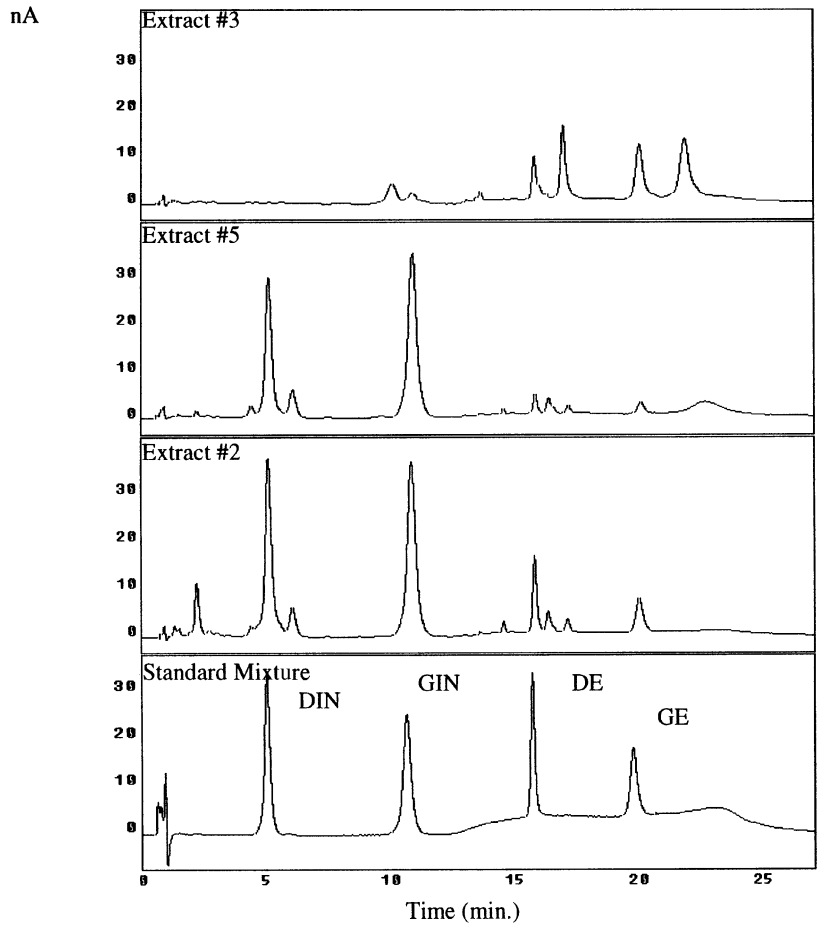

Figure 5. Chromatograms of standard mixture and representative extracts at an applied potential of +950 mV.

than 0.9996 in all cases. The detection limit was determined to be 33 nM for daidzin and genistin, and 16 nM for daidzein and genistein, based on a signal to noise ratio of 3 : 1.

The measured content of daidzein, genistein, daidzin, and genistin in various dry food and nutritional supplements is presented in Table 2. It is observed that dried soybean, soy flour, and regular rat food contain a significantly greater amount of glycoside than aglycone. This is also the case for the six nutritional supplements we sampled, four provide significantly more glycoside. Yet, one of them (NS#3) contains a significantly greater amount of aglycone.

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Table 2. Content of Isoflavones (μg/g) in Natural Sources and Nutritional Supplements

Sample	DIN	GIN	DE	GE
Puraria	212	14.0	182	33.2
	(6.7)	(1.5)	(2.2)	(1.1)
Dried soybean	138	158	2.8	4.6
	(13)	(9.7)	(0.5)	(0.3)
Soy flour	94.3	117	*	3.0
	(2.5)	(14)		(0.9)
Regular rat food	34.3	56.2	*	4.9
	(4.0)	(3.7)		(0.3)
NS#1	6343	10015	118	185
	(95)	(104)	(3.6)	(17)
NS#2	9809	11533	1210	1386
	(309)	(1160)	(91)	(78)
NS#3	67.1	199	663	1810
	(17)	(37)	(113)	(189)
NS#4	991	125	453	54.0
	(224)	(23)	(50)	(9.5)
NS#5	4814	7573	287	429
	(483)	(1764)	(49)	(36)
NS#6	3024	5327	209	366
	(428)	(223)	(28)	(23)

Standard deviations (n = 3, independent sampling and sample preparation) are listed in parentheses. NS: nutritional supplement. * Not detected.

As can be seen, there are large variations in the content of individual constituents within a supplement, as well as between brands for the same constituent (sometimes as much as 150 times).

CONCLUSIONS

It has been demonstrated that liquid chromatography, coupled with multi-channel electrochemical detection, is a sensitive and specific method for the identification and determination of isoflavones in various food and nutritional supplements.

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