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Note

Determination of isoflavones in soy bean by high-performance liquid chromatography with amperometric detection

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Soy beans contain several biologically active components including isoflavones such as daidzin, daidzein, genistin, genistein, etc. These isoflavones possess oestrogenic[1], antibacterial[2], antioxidative[3] and spasmolitic[4] activities. Because soy bean protein products are widely used in food products such as infant formulas, health foods and feeds for farm animals, it is necessary to know the concentration of these biologically active components in soy beans. The isolation and quantitation of isoflavones in soy beans have been reported[5–9]. For example, Naim *et al.*[8] developed a gas chromatographic method to isolate and quantitate genistein and daidzein as their trimethyl derivatives, and Murphy[9] reported the separation of daidzin and genistin, and their aglycones, by high-performance liquid chromatography (HPLC) with a gradient of methanol–water. The use of an amperometric detector for HPLC was reported to be useful for the analysis of phenolic compounds such as butylhydroxyanisole, dibutylhydroxytoluene and *tert.*-butylhydroquinone, which are oxidizable[10].

The amperometric determination of daidzin, daidzein, genistin and genistein in defatted soy bean was therefore investigated by HPLC.

EXPERIMENTAL

Genistein was purchased from K & K Labs (Plainview, NY, U.S.A.). Daidzin and daidzein were isolated from *Puerariae radix*[11]. Genistin was isolated as follows. Commercial defatted soy bean flakes were extracted with ethanol for 3 h under reflux, and the extract was partitioned with *n*-butanol and water. The butanol fraction was evaporated under reduced pressure on a rotary evaporator, and subjected to silica gel column chromatography (600 mm × 60 mm I.D., 74–149 μm; Wako, Osaka, Japan) with chloroform–methanol–water (6:1:0.1) in order to obtain a crude genistin fraction. Then, the fraction containing genistin was subjected to preparative HPLC, on a C_{18} column (7 μm, 250 mm × 20 mm I.D.; Yamamura Kagaku, Kyoto, Japan)

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with 30% aqueous acetonitrile as mobile phase. The genistin was recrystallized from 80% ethanol solution: m.p. 256–257°C; $C_{21}H_{20}O_{10}$. IR spectrum in KBr ($\nu_{max.}$, cm^{-1}): 3450, 1661, 1622, 1582, 1180, 1090, 1044, 830. UV spectrum in methanol [$\lambda_{max.}$ (log ε)]: 261 (4.58). Mass spectrum (*m/e*): 284, 270, 166. NMR spectrum in [$^2H_6$]dimethyl sulphoxide (δ, ppm): 12.92 (1H, bs), 9.60 (1H, bs), 8.40 (1H, s), 8.37 (2H, d, J = 7.7 Hz), 7.44 (2H, d, J = 7.7 Hz), 7.34 (1H, d, J = 1.7 Hz), 6.71 (1H, d, J = 1.7 Hz), 5.42 (1H, d, J = 2.2 Hz), 5.30–4.90 (3H, m), 4.30–4.05 (1H, m).

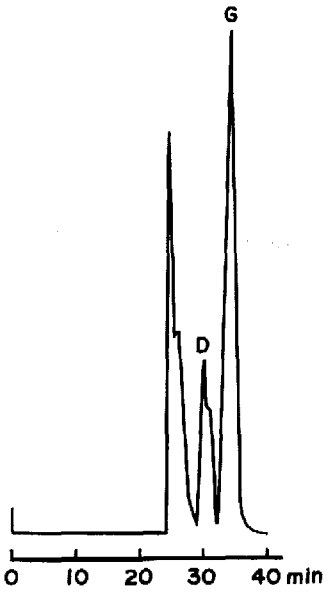
The liquid chromatograph comprised a Shimadzu LC-3A pump (Shimadzu Seisakusho, Kyoto, Japan) equipped with a column oven (Shimadzu CTO-2A) thermostatted at 50°C. The separation of the isoflavones was performed on a reversed-phase column, LiChrosorb RP-8 (5 μm, 250 mm × 4 mm I.D.; Merck) using acetonitrile–0.05 *M* potassium dihydrogenphosphate solution acidified with phosphoric acid to pH 2.0 (15:85) as a mobile phase. The flow-rate was 1.2 ml/min. A Shimadzu SPD-1 spectropohotometer at a wavelength of 260 nm and an IRICA E-502 amperometer (IRICA-Kogyo, Kyoto, Japan), with a glassy carbon working electrode operated at a potential of +0.90 V *vs.* Ag/AgCl, were used in series for the detection.

The sample solution was prepared according to the method of Pettersson and Kiessling[12]. Defatted soy bean flakes (1 g) pulverized with a coffee mill in 25 ml of 80% methanol solution were heated on a water-bath at 80°C for 4 h, and then cooled. A 1-ml volume of the extract was diluted in 3 ml of water and subjected to chromatography on a Waters Sep-Pak C_{18} cartridge (Millipore), which was pre-wetted with methanol and water (each 5 ml). The cartridge was washed with 2 ml of 20% methanol solution and eluted with 2 ml of 80% methanol solution. This eluate was filled up to 5 ml with the HPLC mobile phase for analysis.

RESULTS AND DISCUSSION

Farmakalidis and Murphy[13] reported the use of semi-preparative HPLC with a non-linear gradient of methanol–water for the isolation and purification of the soy bean isoflavones, daidzin and genistin. Fig. 1 shows that an isocratic elution with 30% aqueous methanol is most suitable for separating genistin from other isoflavones. Preliminary experiments showed that the crude soy bean extract could not be directly fractionated by HPLC. Therefore, purification by silica gel column chromatography was necessary before fractionation by preparative HPLC.

Amperometric detection was more effective than ultraviolet (UV) and fluorimetric detection for isoflavones in *Puerariae radix*[10]. In this study, amperometric and UV detectors were used in series in order to cover the wide range of concentration of soy bean isoflavones. The wavelength of the UV detector was set at 260 nm, corresponding to the maximum absorption of genistin and genistein in the mobile phase. To determine the optimum voltage for the amperometric detector, the peak heights of isoflavones were measured at various potentials in the range between +0.60 and +1.10 V *vs.* Ag/AgCl. The peak heights of these compounds increased with increasing potential (Fig. 2). Based on a consideration of the intensity of the background current and the stability of the baseline, the potential of the amperometric detector was set at +0.90 V *vs.* Ag/AgCl.



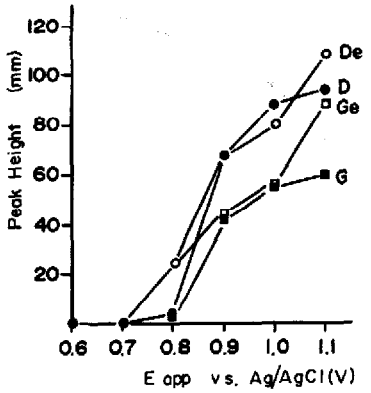


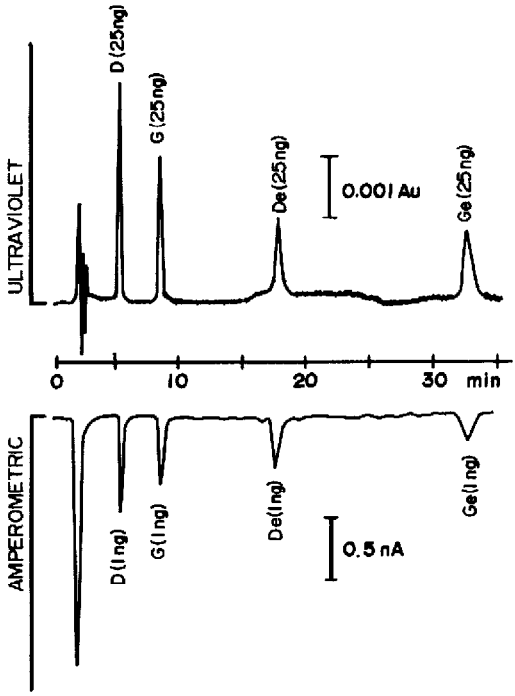
Fig. 1. HPLC of the crude genistin defatted soy bean flakes on a reversed-phase C_{18} semi-preparative column: D = daidzin; G = genistin. Conditions: column, YMC-PACK ODS (7 μm, 250 mm × 20 mm I.D.); mobile phase, water–acetonitrile (70:30); flow-rate, 2.0 ml/min; column temperature, 55°C; detection, UV 260 nm.

Fig. 2. Hydrodynamic voltammograms of daidzin (D), genistin (G), daidzein (De) and genistein (Ge). Conditions: column, LiChrosorb RP-8 (5 μm, 250 mm × 4 mm I.D.); mobile phase, 0.05 *M* potassium dihydrogenphosphate (pH 2.0)–acetonitrile (85:15); flow-rate, 1.2 ml/min; column temperature, 50°C.

To examine the effect of the pH of the phosphate buffer on the peak heights and the capacity factors of these compounds, the pH was changed in the range 2–6. Each peak height was constant. The capacity factors of daidzin and genistin were constant in the pH range studied, while those of daidzein and genistein decreased at pH > 6. On this basis, an acidic phosphate buffer of pH 2.0 was used as the mobile phase. The effect of the concentration of the phosphate buffer on the peak heights and the capacity factors of these isoflavones was also examined in the range 0.01–0.10 *M*. The peak heights and the capacity factors were constant, and the concentration of the phosphate buffer was set at 0.05 *M*.

Under the conditions described above, these compounds were well separated and completely eluted within 32 min. Genistein was detectable at a level of 0.05 ng and other components were detectable at an even lower level. The signal-to-noise ratio (S/N) was 3, and the injection volume was 5 μl. In the range 0.5–75 ng the detector response was linear. As seen in Fig. 3 the detector response towards each compound was higher in amperometric detection than in UV detection. From a chromatogram of a sample solution with a dilution factor of 125 (Fig. 4), the contents of daidzin, genistin, daidzein and genistein in the extract of defatted soy bean flakes were calculated to be 1695, 2935, 131 and 117 μg/g, and their coefficients of variation, from triplicate measurements, were 1.4, 2.5, 3.1 and 3.1%, respectively.

In conclusion, the simultaneous analysis of daidzin, genistin, daidzein and genistein in soy bean by HPLC was achieved. By using an amperometric detector, these



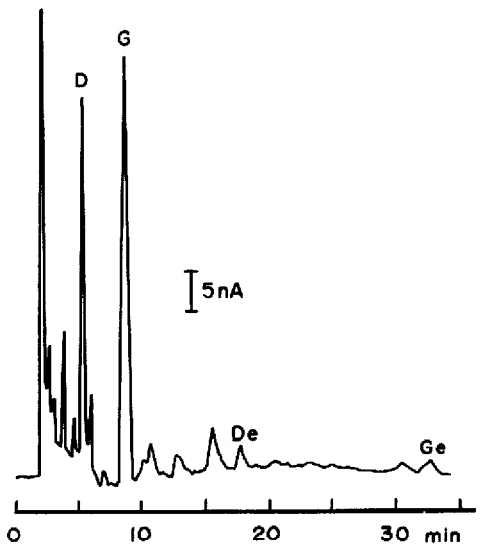


Fig. 3. Comparison of the sensitivity of the different methods of detection. Conditions: mobile phase, 0.05 *M* potassium dihydrogenphosphate (pH 2.0)–acetonitrile (85:15); UV detection, 260 nm (0.02 a.u.f.s.); applied voltage, 0.90 V *vs.* Ag/AgCl (10 nA f.s.).

Fig. 4. Chromatogram of a soy bean extract. Conditions: applied voltage, +0.90 V *vs.* Ag/AgCl; range, 160 nA f.s.

biologically active compounds were sensitively assayed in comparison with UV detection.

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