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Note

Separation and quantitation of estrogenic isoflavones from clovers by high-performance liquid chromatography

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Phytoestrogens have been isolated from many species of plants, particularly those in the Leguminosae family[1]. Uterotropic activity in many clover species appears to be the result of the isoflavones genistein, biochanin A, daidzein and formononetin (Fig. 1), either singularly or in combination[2–4]. Leaves and leaflets have higher accumulations of uterotropic compounds than stems. Variation in isoflavone content in leaves from different strains of subterranean clover are due primarily to genetic factors[3]. The estrogenic isoflavones are insoluble in water but extractable with alcohol. Beck[5] has developed a thin-layer chromatography (TLC) method which separates the isoflavones and permits a visual semiquantitative estimation of each. Gourley[6] has improved the TLC technique to include quantitation by UV absorption.

West *et al.*[7] separated two isoflavones from soybeans by high-performance liquid chromatography (HPLC) but dit not quantitate them. We were mainly interested in developing a HPLC technique that would separate and quantitate multicomponent isoflavones isolated from new genetic lines of clover.

EXPERIMENTAL*

Authentic samples of isoflavones: genistein, biochanin A, daidzein and formononetin (K & K Labs, Plainview, NY, U.S.A.) were obtained and dissolved in methanol.

Several concentrations of the standards were injected separately into the high-performance liquid chromatograph (Waters Model 6000) equipped with a variable-wavelength detector (Model 450). The peak areas were measured with an integrator (Hewlett-Packard Model HP 3390A) and standard curves prepared (0.5–20 μg).

Samples of each variety of subterranean clover (see Table I) were prepared by removing the top leaves and soaking at 5°C in methanol (1 g/5 ml) for a week.

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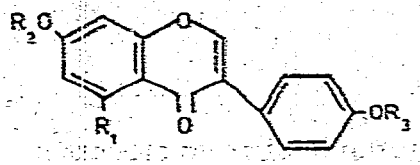
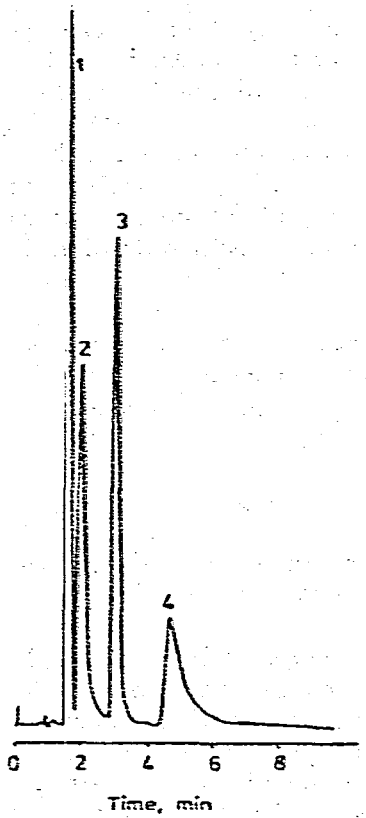


$R_1 = R_2 = R_3 = H$ daidzein
$R_1 = OH, R_2 = R_3 = h$ genistein
$R_1 = R_2 = H, R_3 = CH_3$ formononetin
$R_1 = OH, R_2 = H, R_3 = CH_3$ biochanin A

$R_1 = OCH_3, R_2 = R_3 = H$ genistein-5-methylether
$R_1 = OH, R_2 = glucosyl, R_3 =$ genistin
$R_1 = H, R_2 = glucosyl, R_3 = CH_3$ formononetin 7-O-glucoside
$R_1 = OH, R_2 = glucosyl, R_3 = CH_3$ biochanin 7-O-glucoside

Fig. 1. Structures of isoflavonoids.

Separations were carried out with a mobile phase of methanol–water (2.1:1) at flow-rates of 2.0 ml/min for the standard (Fig. 2) and 1.0 ml/mn for the clover's extract (Fig. 3). The column 25 cm × 4.6 mm I.D. stainless steel and packed with



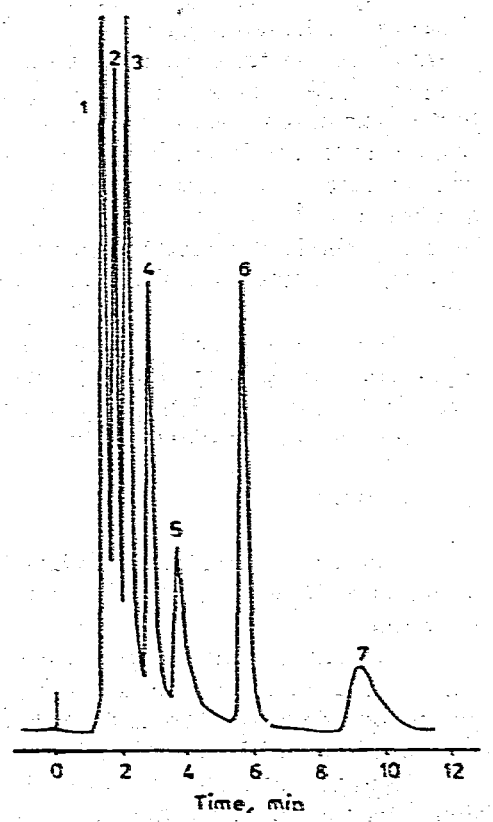


Fig. 2. HPLC chromatogram of estrogenic isoflavones. Peaks: 1 = daidzein, 2 = genistein, 3 = formononetin and 4 = biochanin A.

Fig. 3. HPLC chromatogram of Tallarook methanol extract. Peaks: 1 = genistin, 2 = genistein-5-methyl ether, 3 = formononetin 7-O-glucoside, 4 = biochanin A-7-O-glucoside, 5 = genistein, 6 = formononetin and 7 = biochanin A.

TABLE I

QUANTITY (μg/g OF FRESH WEIGHT) OF GENISTEIN, FORMONONETIN AND BIOCHANIN A OF THE FIVE VARIETIES OF SUBTERRANEAN CLOVER

Varieties	*Genistein*		*Formononetin*		*Biochanin A*	
	Free	*Total*	*Free*	*Total*	*Free*	*Total*
Nangeela leaves	212	439.74	43	50	2215	2532
Woogenellup leaves	2322	4483	119	130	326	459
Tallarook leaves	1367	2025	3376	3534	1181	1970
Mt. Baker leaves	173	173	—	—	1481	1658
Miss. Ecotype leaves	1002	2090	1583	2938	859	1279

Partisil-10 ODS-2 (Whatman, Clifton, NJ, U.S.A.). Detection was at a wavelength of 250 nm. Each peak was collected and the UV spectrum with shift reagents ($AlCl_3$, $AlCl_3$ + HCl, $NaOCH_3$ and $NaOOCCH_3$) was run and compared to the literature[8] and to the standards. Chemical ionization spectra, using an HP 5985B gas chromatograph–mass spectrometer with methane as ionization gas were taken to confirm our results. A volume of 1 ml of methanol extract from each clover was hydrolyzed with 4 *N* HCl at 70°C (2 h) and the neutralized extract reinjected to determine the total amount of genistein, formononetin and biochanin A in each clover.

RESULTS AND DISCUSSION

The estrogenic isoflavones found in subterranean clover can be satisfactorily separated with HPLC as illustrated in Fig. 2. Several other isoflavones (aglycon and glucosyl types) were isolated from the methanol extracts (Fig. 3). The separation of estrogenic isoflavones with HPLC can be very useful as an analytical tool in the screening of varieties of subterranean clover.

The amount of free genistein, formononetin and biochanin A in each clover was quantified and a portion of the sample then hydrolyzed to determine the total amount of estrogenic isoflavones of genistein, biochanin A and formononetin that were present in the glucosyl form. We did not find daidzein in any of the clovers. Table I gives the amounts of the estrogenic isoflavones in five varieties of clover. Note that the ratio between free genistein, formononetin and biochanin A related to the total amount of each one found after hydrolysis, is different in each case, but almost double for genistein in most clover species (Table I). It is likely that some hydrolysis occurred during the extraction procedure. Several hydrolysis conditions were tried to determine the transformation of bound genistein, formononetin and biochanin A to free isoflavones. The best conditions were 4 *N* HCl for 2 h at 70°C. The bound isoflavones were not hydrolyzed fully in 1 *N* HCl at 70°C after 1 h. This experiment shows that the plant has free and bound isoflavones. The leaves of Nangeela and Mt. Baker varieties (with the same parent line) contained the greatest concentration of

free biochanin A and the lowest amount of formononetin (Table I). Tallarook and Mississippi Ecotype are from the same parent line but differ from Mt. Baker and are high in both formononetin and biochanin A (Table I). Genistein is the major isoflavone in Woogenellup. The genetic variance appears to be responsible for a significant portion of the isoflavone variation.

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* *Editor's Note:* A similar separation was submitted by J. J. Patroni, W. J. Collins and W. R. Stern (*J. Chromatogr.*, 247 (1982) 366) and was in press when this paper reached us.