云 南 植 物 研 究 1994; 16 (4): 424—430
Acta Botanica Yunnanica

反相高效液相层析法测定新鲜植物材料中的植物雌激素含量

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摘要 通过适当的样品处理方法，游离的和结合的植物雌激素[大豆素（daidzein），雌马酚（eguol），染料木素（genitein），芒柄花素（formononetin），香豆雌酚（coumestrol）和美皂异黄酮（biochanin A）]被从新鲜植物材料的提取物中分离出来。并在不同的紫外光波长下，可被HPLC法定量测定。根据滞留时间和标准品的添加，而鉴别出植物雌激素的层析波峰。本方法的测定灵敏度为2ppm。白三叶草（*Trifolium repens*）样品的加样回收率在80%—100%之间（平均回收率变异系数为5.4%）。通过比较游离植物雌激素的含量测定，本方法的提取率比经典的甲醇浸提法更为有效。

关键词 植物雌激素，高效液相层析；新鲜植物材料

THE QUANTIFICATION OF PHYTOESTROGENS IN FRESH PLANT MATERIALS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract By using a suitable sample preparation, free and bound phytoestrogens (daidzein, equol, genistein, formononetin, coumestrol and biochanin A) can be isolated from extracts of fresh plant material and subsequently quantified by HPLC at different wave lengths. Chromatographic peaks assigned to phytoestrogens were identified by retention time and by comparison to the addition of standards. The method is sensitive to about 2 ppm of phytoestrogens. Recoveries of phytoestrogens in spiked samples of white clover (*Trifolium repens* L.) ranged between 80% and 100% (average 5.4% R.S.D). This method also showed more efficient extraction for some phytoestrogens than the traditional soaking method by comparison with their free phytoestrogen concentrations.

Key words Phytoestrogens, HPLC, Fresh plant materials

1993-08-20 收稿, 1993-09-20 修回

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INTRODUCTION

Phytoestrogens are isoflavones which are produced by legumes and other plants (Fig.1). They can mimic the biological effects of estrogens in mammals [1,2]. In latge doses, phytoestrogens have beenassociatd with infertility in animals [1,2]. The decreased risk of certain hormone-dependent cancers in vegetarians has been related to their increased consumption of phytoestrogens [3]. Therefore, precise methodologies for monitoring phytoestrogens are needed.

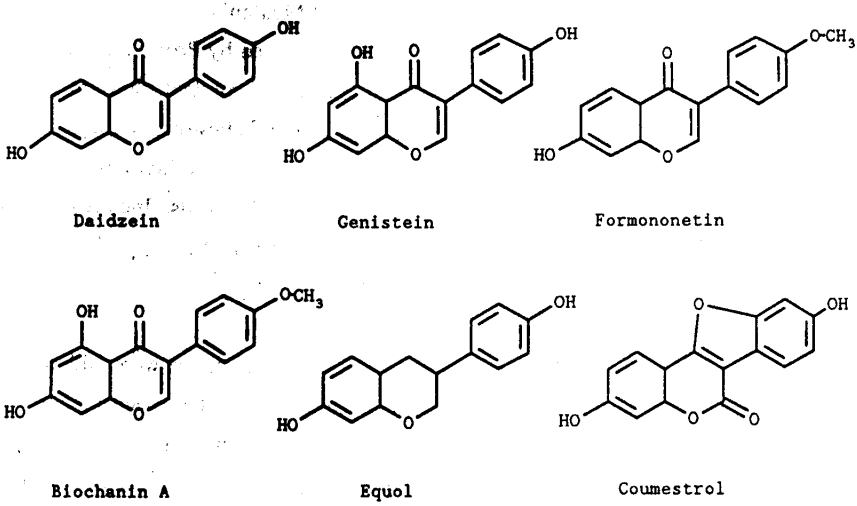

Fig. 1 Chemical structures of the principal phytoestrogens.

Methods involving thin-layer chromatography [4], gas chromatography-mass spectrometry [5], radioimmunoassay [6] and high-pressure liquid chromatography [7,8] have been used for the determination of phytoestrogens in plant materials. HPLC methods, with high resolution and sensitivity, are usually preferred. Some previous HPLC quantifications for phytoestrogens did not use fresh plant samples [8], and some methods do not give both free and bound concentrations of phytoestrogens [7].

Procedures for preparation of plant samples affect the concentration of phytoestrogens. Phytoestrogen concentrations were decreased by 30 to 50% after plant samles are dried [9], compared to fresh plant samples by the methanol soaking method [10,11]. However, only free forms of phytoestrogens were detected by this method [11]. Naturally occurring phytoestrogens exist mainly bound to carbohydrates, e.g. glucosides [12]. Both free and bound forms of phytoestrogens can be exchanged with each other, depending in the stage of plant growth and environmental conditions. It would be useful to be able to detect both free and bound phytoestrogens.

This paper reports a suitable method for the determination of free and bound phytoestrogens in fresh plant materials using HPLC. Results of this proposed method are compared with that of the traditional methanol soaking method [10,11].

MATERIALS AND METHODS

Instrumentation The HPLC system consisted of a Shimadzu Model SIL-6A high performance liquid chromatograph pump equipped with an automatic sample injector and an SPD-6AV UV-VIS spectrophotometric detector (Kyoto, Japan). A Partisphere C18 (5 μm), 4.7 mm i.d. × 23.5 cm length, with guard column packed with C18 (37—50 μm) was used (Whatman Inc., NJ).

Materials Plant samples were collected from two University of Hawaii experiment stations Waimanalo (elevation 21 m) on the Island of Oahu and Mealani (elevation 853 m) on the Island of Hawaii. The equol standard (4′, 7-dihydroxy-isoflavan) was donated by Prof. H. Adlercreutz, Department of Clinical Chemistry, University of Helsinki, Finland and the coumestrol standard was donated by Dr. Adrian Franke, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI. The other standard phytoestrogens, biochanin, genistein, daidzein (4′, 7-dihydroxyisoflavone) and formononetin (7-hydroxy-4′-methoxyisoflavone) were purchased from ICN Biochemicals, Inc. (Costa Mesa, CA). Acetonitrile and methanol used for HPLC analyses were obtained from Fisher Scientific, Inc. (Fair Lawn, NJ).

Sample preparation

A. Procedure for free phytoestrogens Ten grams of fresh plant leaf-tissue were added to 30 mL of 1 mol / L HCl (Fisher Scientific, Fair Lawn, NJ) and homogenized in a high-speed blender (Brinkmann Homogenizer) for 15 min. Twelve mL of acetonitrile were added to four grams of the blended sample (equivalent to 1.00 g of the original sample), and shaken for 1 min. The mixture was allowed to settle for 5 min and then the supernatant fluid was filtered with Gelman Nylon Acrodisc size 0.2 μm syringe filter (Ann Arbor, MI). This filtrate was kept at 4 ℃ until used for detection by HPLC.

B. Procedure for total phytoestrogens Four grams of the blended sample received 4 mL of 1 mol / L HCl, and were heated inside an Isotemp Oven (Fisher Scientific, Model 750G) at 98℃ for 2 h [8]. Twelve mL acetonitrile were added and shaken for 1 min. The mixture was allowed to settle for 5 min and then tissue was further processed as in (A).

C. Methanol soaking method The determination of free phytoestrogens using methanol soaking method was performed as described by Nicollier *et al* [10], slightly modified by Smith *et al* [11]. Two grams of plant material were placed in tightly capped vials and soaked in fifteen mL of methanol at 4 ℃ for seven days. The plant materials were then removed by filtering with Gelman Nylon Acrodisc size 0.2 μm syringe filter. The filtrate was stored at 4 ℃ until analysis for phytoestrogens by HPLC.

Assay conditions The LC column was equilibrated at ambient temperature. Separtions were performed using an initial mobile phase of 55% methanol in 1 mmol / L ammonium acetate (EM Science, Cherry Hill, HJ), rising to 65% at 10 min until biochanin A was eluted. The flow rate was 1 mL / min. The UV detector was set at 254 nm to detect daidzein, formononetin, genistein and biochanin A; at 214 nm to detect equol; and at 345 nm to detect coumestrol, respectively. Twenty μL of mixed standard methanol solution or sample were injected and the chromatogram peak areas were calculated using Axxi-Chrom Model 727 software (Calabasas, CA).

RESULTS AND DISCUSSION

All six phytostrogens in the mixed standard solution eluted within 25 min with a return to base-line resolution (Figure 2).

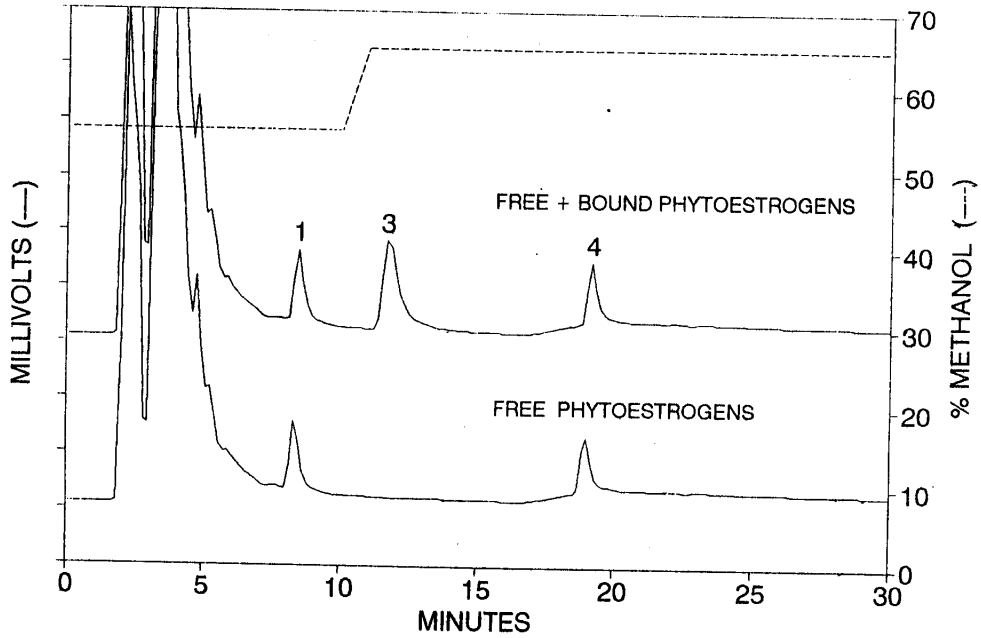

Fig.2. HPLC separation of standard phytoestrogens was monitored recording at 214 nm, 254 nm and 345 nm values. Peaks: 1 = daidzein; 2 = equol; 3 = genistein; 4 = formononetin; 5 = coumestrol and 6 = biochanin A, respectively.

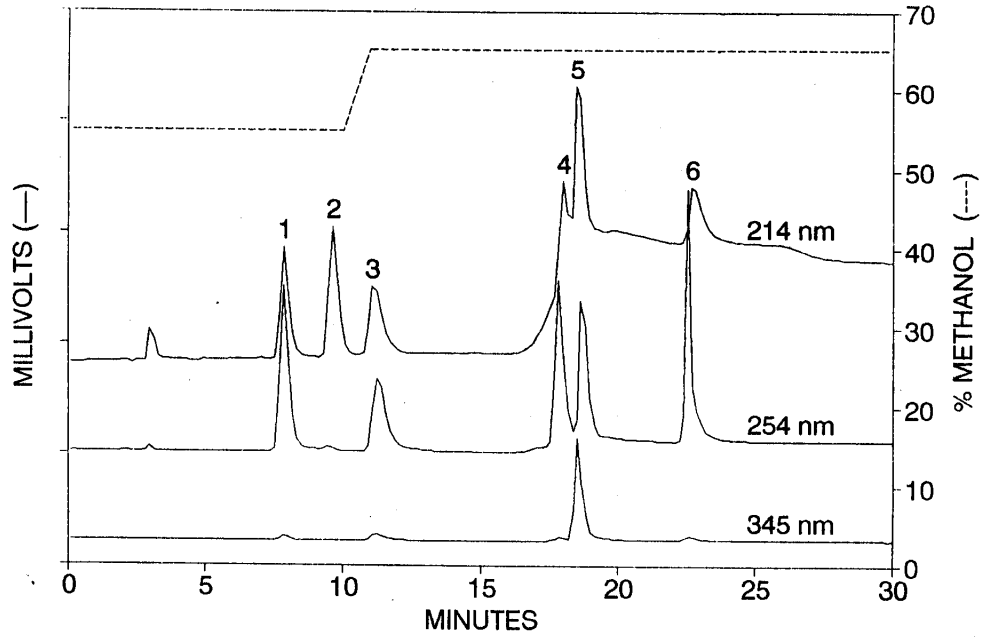

Fig.3. HPLC chromatograms for samples from White Clover. Signals 1,3 and 4 are daidzein, genistein and formononetin, respectively.

Calibration curves were constructed using peak area vs corresponding phytoestrogen concentration and changing response factors for the different wave lengths of the UV detection. Linear responses were obtained (r = 0.999) for 0.05 to 10 μg / mL.

Table 1 Recoveries of Phytoestrogens Added to White Clover

(ND: not detected; free and bound: free and bound concentrations of phytoestrogens, respectively)

Phytoestrogens		White Clover			
		content mg / kg wet	added mg / kg wet	recovery * (%)	R.S.D. (%)
daidzein	free	1.8	90.0	93.7 ± 4.45	0.47
	bound	6.9	70.0	94.3 ± 0.70	0.07
genistein	free	ND	30.0	96.7 ± 2.10	0.22
	bound	36.8	10.0	102.7 ± 13.8	13.44
formononetin	free	27.2	20.0	81.7 ± 4.73	5.79
	bound	44.4	90.0	96.6 ± 0.84	0.87
coumestrol	free	ND	25.0	89.5 ± 10.23	11.43
	bound	ND	80.0	96.4 ± 1.31	1.36
biochanin A	free	ND	10.0	87.0 ± 7.21	8.29
	bound	ND	30.0	88.7 ± 6.41	7.23
equol	free	ND	2.0	98.5 ± 12.73	12.92
	bound	ND	4.0	100.5 ± 3.11	3.09

* Three replicate samples measurements (mean ± S.D.).

Table 2 Phytoestrogen Concentrations With The Proposed Method And Soaking Method In Some Tropical Forage Cultivars

(ND: not detected; free and bound: free and bound concentrations of phytoestrogens, respectively)

Cultivars		Phytoestrogen concentration *, mg / kg wet tissue					
		daidzein	genistein	formononetin	coumestrol	equol	biochanin A
Trifolium repens							
proposed	bound	6.9 ± 0.1	36.8 ± 6.2	44.4 ± 1.4	ND	ND	ND
	free	1.8 ± 0.1	ND	27.2 ± 0.1	ND	ND	ND
soaking	free	0.8 ± 0.1	ND	7.9 ± 2.7	ND	ND	ND
Desmodium intortum							
proposed	bound	9.8 ± 4.6	17.9 ± 0.4	ND	ND	ND	ND
	free	7.5 ± 1.2	1.7 ± 0.3	ND	ND	ND	ND
soaking	free	3.9 ± 2.5	1.8 ± 0.3	ND	ND	ND	ND
Signalgrass							
proposed	bound	23.3 ± 2.1	ND	ND	ND	ND	ND
	free	5.0 ± 2.2	ND	ND	ND	ND	ND
soaking	free	5.3 ± 2.0	ND	ND	ND	ND	ND
Hosaka kikuyugrass							
proposed	bound	ND	ND	ND	0.6 ± 0.2	ND	ND
	free	ND	ND	ND	ND	ND	ND
soaking	free	ND	ND	ND	ND	ND	ND

* Three replicate samples measurements (mean ± S.D.).

Figure 3 shows the sample chromatogram for detection of free and total (free + bound) phytoestrogens in a White clover sample using the proposed method.

The recoveries were determined by adding the methanol solution of the phytoestrogen standards to White clover samples. These samples were extracted and analyzed by use of this procedure. Recoveries for daidzein, genistein, formononetin, coumestrol, equol and biochanin A are shown in table 1.

The results in Table 2 indicate phytoestrogen concentrations in some tropical pastures using the proposed method and soaking method, respectively. The proposed method permits the quantification of bound phytoestrogen concentrations, in addition to free phytoestrogen concentrations. Furthermore, the proposed method gives better estimates for some free phytoestrogen concentrations than the soaking method. The increased efficiency probably results from the homogenization before extraction, which cannot be simply compared by recoveries of both methods.

Equol and biochanin A were not identified in the plant samples of this study. In fact, equol is the dominant metabolite of phytoestrogens in mammals, and may be responsible for their reproductive problems [1]. The proposed method may be used to screen these phytoestrogens in tropical legumes and other grasses.

ACKNOWLEDGMENT This research was supported by the Governot's Agriculture Coordinating Committee Contract Grant No. 91-23 and a grant from the Project Development Fund of the University of Hawaii to C.W.W. and D.L.V..

We thand Dr. Stanley M. Ishizaki, Department of Animal Sciences, University of Hawaii, for the supply of plant cultivars used in this study.

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