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Note

Separation of genistin, daidzin and their aglucones, and coumesterol by gradient high-performance liquid chromatography

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Soybeans and soybean protein foods contain the phytoestrogens genistein and daidzein, their glucosides genistin and daidzin, and coumesterol. These isoflavonoid compounds are capable of producing an estrogenic response in a number of diverse species[1]. The content of genistein and daidzein in whole soybeans has been reported[2]. However, very little is known concerning the carryover of these compounds into soybean protein products[3,4].

Naim *et al.*[2] have developed a method to quantitate the trimethylsilyl derivatives of genistein and daidzein by using gas chromatography. West *et al.*[5] reported a method for high-performance liquid chromatographic (HPLC) analysis of genistein and 4′,6,7-trihydroxyisoflavone but gave no data for daidzein or the glucosides, genistin and daidzin. A linear gradient HPLC procedure has been published by Ohta *et al.*[6] for most of the phytoestrogens in soybeans. They did not report recovery data for their method, however.

Lookhart[7] reported that the maximum recovery he could achieve for added coumesterol in defatted soybeans was 65%. Water considerably reduced the efficiency of the methanol extraction of coumesterol. Bowman[8] reported an extraction efficiency of 85–90% for diethylstilbesterol, a synthetic non-steroidal estrogen, added in ppb (10^9) amounts to animal feed for an HPLC work-up procedure.

We are interested in developing a method to maximize extraction efficiency and increase the speed of separation for the glucosides and aglucones of isoflavones from soybeans and their protein products.

EXPERIMENTAL

Authentic samples of genistein, daidzein (K & K Rare and Fine Chemicals, ICN, Plainview, NY, U.S.A.), and coumesterol (Pfaltz and Bauer, Stanford, CT, U.S.A.) were obtained as standards. Genistin and daidzin were isolated according to Walter[9] and Naim *et al.*[2], respectively. Toasted defatted soy flakes were obtained from A. E. Staley Manufacturing Co. (Des Moines, IA, U.S.A.). The flakes were extracted with one of several solvents systems (Table I) with a wrist-action shaker for 2 h, filtered, and evaporated to dryness on a rotary evaporator.

Separation was performed by using a non-linear methanol–water gradient (Fig. 1) produced by a program utilizing a Beckman-Altex microprocessor/controller

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and two Model 110A pumps. The combined flow-rate was 1.0 ml/min. Samples were dissolved in methanol and injected by using a 20-μl sample loop. The column was a 250 × 4.6 mm stainless-steel Ultrasphere octadecysilane (Beckman-Altex). Peaks were monitored at 254 nm with use of a Beckman-Altex UV monitor and fluorescence with an Aminco fluoromonitor. Total chromatography time was 15 min. Peak areas were integrated with a Varian CDS-111 computer.

RESULTS AND DISCUSSION

The phytoestrogens, genistin, daidzin and their aglucones, and coumesterol, can be efficiently separated on a non-linear methanol–water gradient in 15 min with peak widths for all compounds of 1 min (Fig. 1). The fluorescent properties of daidzein and coumesterol were used to detect these compounds in soy flakes. The fluorescent sensitivity for coumesterol is much greater than its absorbance at 254 nm. This allows detection of coumesterol at levels typically found in soybeans[10]. The fluorescence of daidzein was used in soy extracts because another substance closely chromatographs with the daidzein peak and was detectable at 254 nm. Daidzein added as an internal standard could not account for all absorbance in the peak pair.

In preliminary experiments, toasted defatted soy flakes were spiked with genistin, genistein, daidzein and coumesterol and extracted with methanol by using mechanical agitation or by using a Goldfisch or a Soxhlet extraction apparatus. Recoveries of added phytoestrogens from Soxhlet and Goldfisch extractions were about 70% with methanol and not more efficient than mechanical agitation. The procedure reported by Lookhart[11] for coumesterol in soybeans was examined but yielded very low extraction efficiencies for the phytoestrogens other than coumesterol.

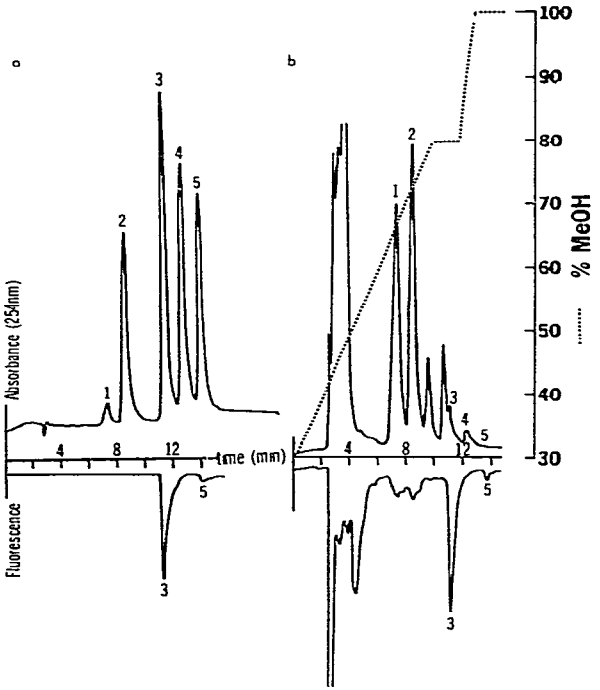

Fig. 1. Gradient chromatogram of phytoestrogens from soybeans: a, phytoestrogen standards: (1) daidzin, (2) genistin, (3) daidzein, (4) genistein, (5) coumesterol; b, toasted, defatted soy flakes extracted with acetonitrile and deactivated with 0.1 *N* hydrochloric acid.

TABLE I

PHYTOESTROGEN EXTRACTION FROM TOASTED SOY FLAKES

*Solvent**	*Deactivation***	*Residue (mg/g)*	*Genistin (μg)*	*Genistein (μg)*	*Daidzein (μg)*	*Coumesterol (ng)*
Methanol	None	14 ± 1	522 ± 12	31 ± 1	12 ± 2	30
	Water	97 ± 13	596 ± 41	21 ± 2	11 ± 2	0
	HCl	92 ± 6	1190 ± 85	31 ± 1	40 ± 9	0
Chloroform–methanol (90:10)	None	12 ± 2	213 ± 5	27 ± 3	13 ± 2	14 ± 14
	Water	4 ± 1	72 ± 22	43 ± 1	5 ± 1	12
	HCl	5 ± 2	43	32 ± 8	10 ± 1	9 ± 4
Acetonitrile	None	1 ± 0.0	21 ± 8	0	1 ± 0.4	6
	Water	12 ± 0.1	2581 ± 80	50 ± 4	28 ± 14	76 ± 81
	HCl	14 ± 0.3	2578 ± 106	48 ± 4	29 ± 6	106 ± 20
Acetone	None	5 ± 2	14 ± 2	8	4 ± 1	0
	Water	36 ± 1	647 ± 54	28 ± 4	76 ± 8	133 ± 84
	HCl	34 ± 2	2242 ± 69	46 ± 3	17	75

* Triplicate samples (5 g of soy flakes) were extracted with 25 ml of solvent.

** Samples were extracted with and without deactivation. Water (5 ml) or 0.1 *N* hydrochloric acid (5 ml) was used to deactivate the dried samples.

Because the glucosides were more soluble in aqueous mixtures, deactivation with water and 0.1 *N* hydrochloric acid was examined as a means to increase extraction efficiency and minimize coextractives. The results of the various extraction solvents with and without deactivation are presented in Table I. All combinations of the chloroform–methanol mixture and other solvents without deactivation gave very low extraction data. The addition of water or acid to methanol, acetone, and acetonitrile greatly improved the extraction efficiency of all the phytoestrogens examined. In terms of coextractives, acetonitrile with water or acid was superior to all other solvent systems examined.

The efficiency of recovery was examined in all 12 combinations. Data are reported in Table II for the best systems in terms of maximum recovery and minimum coextractive residues. Typically, minimal coextractives gave the cleanest HPLC chromatograms (Fig. 1).

TABLE II

RECOVERY OF PHYTOESTROGENS FROM SOY FLAKES

Recovery data were generated by adding standards dissolved in methanol to dry sample, redrying sample and extraction with the appropriate solvent. Recovery and blank samples were run in triplicate.

Extraction solvent	*Extraction residue (mg/g)*	*Recovery (%)*			
		Genistin	*Genistein*	*Daidzein*	*Coumesterol*
Acetonitrile + Water	12	81	125	63	34
Acetonitrile + HCl	14	91	86	57	55
Acetone + HCl	34	218	81	60	26

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