sences obtained by this procedure. These essences can also be evaluated in the same manner described for orange essences.

Registry No. 1, 75-07-0; 2, 64-17-5; 3, 105-57-7; 4, 1576-87-0; 5, 66-25-1; 6, 105-54-4; 7, 6728-26-3; 8, 544-12-7; 9, 111-27-3; 10, 124-13-0; 11, 99-87-6; 12, 138-86-3; 13, 11063-78-8; 14, 111-65-9; 15, 11063-77-7; 16, 124-19-6; 17, 78-70-6; 18, 2305-25-1; 19, 562-74-3; 20, 98-55-5; 21, 112-31-2; 22, 1197-07-5; 23, 106-26-3; 24, 106-24-1; 25, 141-27-5; 26, 2111-75-3; 27, 54889-48-4; 28, 89-83-8; methanol, 67-56-1.

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Improved High-Performance Liquid Chromatographic Analysis of Phenolic Acids and Isoflavonoids from Soybean Protein Products

A. Seo and C. V. Morr*

An improved analytical high-performance liquid chromatographic (HPLC) method was developed for recovering, fractionating, and quantitating individual phenolic acids and isoflavonoids from soybean products. This improved method includes ethanol extraction of the phenolics, mild hydrolysis of phenolic acid esters, separation of phenolic acids from isoflavonoids by C_{18} Sep-PAK, and C_{18} reverse-phase HPLC analysis of the two phenolics fractions using a water–acetic acid and methanol–acetic acid gradient. This developed method provides substantially greater recovery of phenolic compounds, requires much less time, separates phenolic acids from isoflavonoids, and improves the resolution of individual phenolic compounds. Defatted soy flakes contained a total of 4 mg of total phenolics/g of sample, which was distributed as about 28% phenolic acids and 72% isoflavonoids. Genistin was the major isoflavonoid, accounting for about 75% of the total isoflavonoids. Major phenolic acids in defatted soy flakes were syringic, ferulic, and sinapic acids. Control and commercial soy protein isolates contained substantially lower concentrations of phenolics. Ion-exchange and activated carbon treatments both removed ≥90% of the total phenolics from defatted soy flakes and were equally effective for removing both phenolic acids and isoflavonoids.

Soybeans contain a number of important phenolic compounds including free phenolic acids, phenolic acid esters, isoflavones, and their glucosides. Maga (1978) and Sosulski (1979) reviewed the literature dealing with the composition and chemistry of these compounds in foods and oilseed protein products. They also discussed the key relationship that these compounds exhibit to flavor and color defects in oilseed proteins and related food products.

The isoflavonoid compounds have been isolated and quantitated by gas–liquid chromatography (GLC) and high–performance liquid chromatography (HPLC). For example, Naim et al. (1974) reported on the use of GLC and Murphy (1981) and Eldridge (1982) reported on the use of HPLC for quantitating phenolic compounds in soybeans. Maga and Lorenz (1974) also used GLC to study the phenolic acids in soybean flakes and reported a total of 256 μg of phenolic acids/g of defatted soy flakes.

How and Morr (1982) used an HPLC method that was slightly altered from that developed by Wulf and Nagel (1976) to study the effectiveness of several processing treatments to remove phenolic compounds from soy protein isolates. HPLC patterns of How and Morr (1982) revealed about 40 peaks, 25 of which were distinct and well resolved, for unfractionated phenolic compounds, e.g., phenolic acids and their esters and isoflavonoids.

The major objectives of the present study were (a) to fractionate the phenolic acids from neutral phenolic compounds prior to HPLC analysis and (b) to improve the resolution and quantitate the important phenolic compounds in defatted soy flakes and soy protein isolates by HPLC. These efforts included (a) mild alkaline hydrolysis of extracted phenolic compounds to free phenolic acids from their esters, (b) development of an improved recovery method based upon alcohol extraction and subsequent

Department of Food Science, Clemson Universtiy, Clemson, South Carolina 29631.

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Table I. HPLC Retention Times, Integrator Response Factors, and Sources for Reference Phenolic Compounds[a]

phenolic compounds	source	HPLC retention time, min	response factor $\times 10^{-7}$ at wavelength 254 nm	280 nm	300 nm
phenolic acids					
protocatechuic	J. T. Baker Chemical Co.[c]	5.8		224	
p-hydroxybenzoic	Sigma Chemical Co.[d]	11.7	574	1119 ± 22.6	
gentistic	Sigma Chemical Co.[d]	11.7	5787	5764 ± 134	7753
vanillic	Sigma Chemical Co.[d]	18.1		1926 ± 474	
caffeic	Sigma Chemical Co.[d]	18.1		1149	
syringic	Eastman Organic Chemicals[e]	21.6		1035 ± 394	
p-coumaric	K & K Laboratories, Inc.[f]	25.1		651 ± 86	
ferulic	ICN Pharmaceutical, Inc.[g]	27.9		1096 ± 182	
sinapic	ICN Pharmaceutical, Inc.[g]	28.8		3253 ± 102	
salicylic	Eastman Organic Chemicals[e]	31.9	6899	5608 ± 1052	2542
isoferulic	ICN Pharmaceutical, Inc.[g]	31.9		1295 ± 9	
o-coumaric	ICN Pharmaceutical, Inc.[g]	32.5		791 ± 33	
isoflavonoids					
daidzin	USDA[h]	25.56	*b*	*b*	*b*
genistin	USDA[h]	29.05		2155 ± 219	
daidzein	ICN Pharmaceutical, Inc.[g]	36.41		1329 ± 178	
genistein	ICN Pharmaceutical, Inc.[g]	40.18		1199 ± 104	

[a] Two to seven trials. [b] Insufficient sample was available to determine response factors. [c] Phillipsburg, NJ. [d] St. Louis, MO. [e] Rochester, NY. [f] Plainview, NY. [g] Plainview, NY. [h] Dr. L. C. Wang, U.S. Department of Agriculture, NRRC, Peoria, IL.

fractionation of acidic and neutral phenolic compounds by Sep-PAK, and (c) quantitation of phenolic peaks by an automatic integrator.

EXPERIMENTAL SECTION

Soy Products. Commercially defatted soy flakes, processed to minimize protein denaturation and related heat damage, and Supro 620 soy protein isolate were obtained from Ralston Purina Co., St. Louis, MO. Acid precipitated control, activated carbon treated, and ion exchange treated soy protein isolates were prepared in the laboratory by the procedures of How and Morr (1982) and Brooks and Morr (1982).

Chemicals. Sources of reference phenolic compounds are given in Table I. HPLC-grade distilled water and methanol were purchased from Fisher Scientific. All other chemicals were reagent grade and were purchased from Fisher Scientific.

HPLC Analysis. HPLC analysis was conducted with a Model 332 liquid chromtograph (Altex Scientific, Inc., Irvine, CA) equipped with a Model 420 microprocessor-controlled dual-pump system, a Model 100-40 variable-wavelength detector, and a Model C-RIA integrator. An Altex 4.6 × 150 mm Ultrasphere-ODS C_{18} reverse-phase column was used throughout the study.

Solutions of reference and extracted phenolic compounds were injected onto the HPLC column by using a 20-μL sample loop from Altex Scientific, Inc. Solvent gradients were formed by the dual pumping system by varying the proportion of solvent A [95:5 (v/v) water–acetic acid] to solvent B [95:5 (v/v) methanol–acetic acid]. The first 5 min of the elution schedule was with 0% solvent B, and the proportion of solvent B was increased linearly to 100% during the subsequent 55 min. The total solvent flow rate was 1.0 mL/min.

Eluted phenolic compounds were monitored at 280 nm, and recorder peaks were automatically quantitated by the above-described integrator by using the external standard method. The integrator window was programmed for predetermined retention times ±3% of the reference phenolic compounds. The integrator automatically provided concentrations of these phenolic compounds by being programmed with the appropriate response factors determined for each reference phenolic compound (Table I). An integrator response factor of 1035×10^{-7} was used for all unknown phenolic acid peaks, since this factor was determined for syringic acid, one of the predominant phenolic acids found in soy products (Arai et al., 1966; Maga and Lorenz, 1974; How and Morr, 1982). The response factor of 2155×10^{-7}, determined here for genistin, was also used for daidzin, since an insufficient amount of this latter reference compound was available for the study to permit its determination.

Recovery of Phenolics. One gram of defatted soy flakes or soy protein isolate was refluxed with stirring in a round-bottom flask by using two 100-mL aliquots of 80% ethanol for 1 h each. The extracts were combined and centrifuged at 17600*g* for 20 min. The supernatant was evaporated to dryness in a rotary evaporator and the dry residue was dispersed in 50 mL of 2 N NaOH and hydrolyzed 6 h at room temperature. The hydrolysate was adjusted to pH 7 with HCl and centrifuged as above. An aliquot of the supernatant was passed through a methanol-distilled water preconditioned C_{18} Sep-PAK as described below to adsorb the neutral phenolic compounds. The effluent fraction was adjusted to pH 2.5 with HCl and centrifuged as described above. This supernatant was passed through a second Sep-PAK that had been preconditioned to pH 2.5 by passing HCl as described below to adsorb acidic phenolic compounds. The recovered neutral and acidic phenolic fractions were eluted from their respective Sep-PAKs with 2–3 mL of 75% methanol as described below and analyzed by HPLC.

Sep-PAK Recovery of Phenolics. C_{18} Sep-PAK cartridges, purchased from Waters Associates, Milford, MA, were preconditioned by sequentially passing 5 mL each of methanol and distilled water at a dropwise flow rate. The flow rate through the Sep-PAKs was controlled by applying vacuum to their outlet stems from a water aspirator. This was accomplished by fitting the Sep-PAK's outlet stem into a disposable plastic pipet tip that was inserted into a single-hole rubber stopper that was itself inserted into the mouth of a 250-mL vacuum filter flask. Sep-PAKs preconditioned as above were used to adsorb neutral phenolic compounds. Those to be used to adsorb phenolic acids were further preconditioned by passing an additional 5 mL of 0.01 N HCl. Phenolic extracts and solutions were passed through the preconditioned Sep-PAKs at a dropwise flow rate to provide efficient adsorption of the phenolic compounds. The adsorbed phenolics were eluted

Table II. Comparison of Alcohol-Ether and Alcohol-Sep-PAK Recovery Methods for Recovering Reference Phenolic Compounds

method	recovery, %[a]		
	syringic acid	genistin	genistein
alcohol-ether	69.3 ± 11.7	35.7 ± 14.4	79.6 ± 14.4
alcohol-Sep-PAK	88.8 ± 16.4	95.5 ± 6.1	82.8 ± 8.3

[a] Sample sizes ranged from 20 to 400 μg/determination. Average of three to five determinations.

from the Sep-PAKs by passing 3 mL of 60–75% (v/v) methanol–water solution at a dropwise flow rate. After the first 3 drops of effluent was discarded, the remainder was collected in a 10-mL graduated cylinder to determine the final volume and analyzed by HPLC.

RESULTS AND DISCUSSION

Retention times and integrator response factors for reference phenolic acids and isoflavonoids are listed in Table I. The HPLC method was unable to separate *p*-hydroxybenzoic acid from gentistic acid, vanillic acid from caffeic acid, or salicylic acid from isoferulic acid. Thus, concentrations of these phenolic acids were reported as their respective sums.

Integrator response factors determined at 280 nm ranged from 651×10^{-7} to 5764×10^{-7}. Each group of phenolic compounds exhibit different absorption maxima. For example, isoflavonoids have their maximum absorption at 260 nm, and cinnamic acids have their absorption maxima at 310 nm with a shoulder that extends to 270–290 nm and also have a minimum absorption at 250–260 nm. Most benzoic acids have their absorption maxima at 250–260 nm with a small peak at 290 nm. Salicylic and gentistic acids have their absorption maxima at 300–320 nm and also below 250 nm. Smaller and more favorable integrator response factors could have been attained for certain of the phenolic compounds by monitoring at wavelengths nearer their absorption maxima. However, a compromise value was required, and it was chosen at 280 nm because this value resulted in less recorder base-line shift during the elution schedule due to changing solvent composition and also because most of the phenolic compounds show reasonably high absorbance values at this wavelength (Table I). Due to the small size of the salicylic acid peak at 280 nm, the wavelength chosen for its quantitation was 300 nm, which provided a response factor of 2542×10^{-7} (Table I).

Although most phenolic compounds were tentatively identified on the basis of their retention times, which were remarkably stable and reproducible, one major acidic phenolic compound with retention time of 33 min was unidentified (Figure 1). It is important to indicate that additional work would be required to more precisely confirm the identity of each of these individual phenolic compounds.

Relative recoveries of reference syringic acid, genistin, and genistein were determined for both the alcohol and ether extraction method (Ribereau-Gayon, 1972; How and Morr, 1982) and the developed alcohol–Sep-PAK method (Table II). The alcohol–Sep-PAK recovery method was superior to the alcohol–ether recovery method in terms of recovery of syringic acid, genistin, and genistein. For example, the alcohol–Sep-PAK method recovered from 83 to 96% of these compounds compared to values from 36 to 70% for the alcohol–ether recovery method. Results of additional experiments not shown here demonstrated that the alcohol–Sep-PAK method recovered about 3 times as much total phenolics per gram of defatted soy flakes

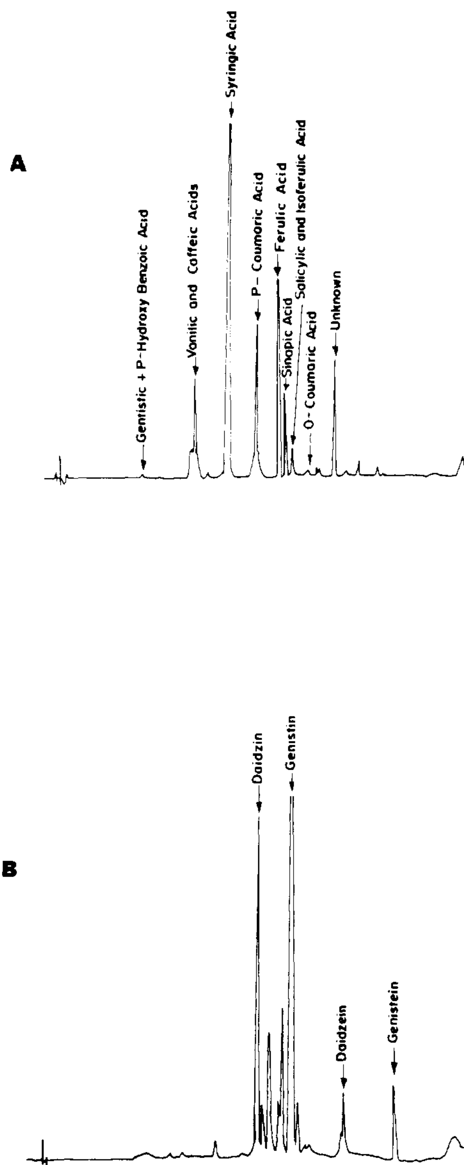

Figure 1. HPLC patterns of (A) phenolic acids and (B) neutral phenolics (isoflavanoids) recovered from defatted soy flakes by the developed method.

as by the alcohol–ether method.

A series of experiments was conducted to determine the optimum time and temperature for hydrolysis of phenolic acid esters with NaOH and HCl, using the general approach of Fenton et al. (1980). The total phenolics were extracted from defatted soy flakes and hydrolyzed by holding them at room temperature for 6 or 16 or heating on a steam bath from 2 to 4 h in 2 N NaOH or 1 to 2 h in 1 N HCl. Acid hydrolysis resulted in good recovery of isoflavonoids (about 2500–2900 μg/g), but poor recovery of phenolic acids (184–390 μg/g) from defatted soy flakes. Heating in the presence of NaOH resulted in poor recovery of isoflavonoids (about 270–660 μg/g) but good recoveries of phenolic acids (about 940–965 μg/g) from defatted flakes. Optimum hydrolysis was achieved by holding for 6 h at room temperature in 2 N NaOH. This latter condition resulted in recoveries of about 3100 μg of isoflavonoid/g and 920 μg of phenolic acids/g of defatted flakes. On the basis of these experiments, all subsequent hydrolysis was conducted in 2 N NaOH for 6 h at room temperature.

HPLC patterns for phenolic acids and neutral phenolics recovered from defatted soy flakes following hydrolysis for 6 h at room temperature in 2 N NaOH are given in Figure

Table III. Recovery of Phenolics from Soy Protein Products by the Developed Procedure, μg/g[a]

phenolic compounds	retention time, min	DFSI[b]	DPI[c]	APPI[d]	CPI[e]	ACPI[f]	IEPI[g]
phenolic acids							
p-hydroxybenzoic and gentistic	11.7	7.5 ± 6.2	<0.5	0.8 ± 2.8	3.2 ± 2.8	<0.5	<0.5
vanillic and caffeic	18.1	129 ± 15.7	21.6 ± 1.4	7.7 ± 0.1	46.7 ± 13.6	5.5 ± 1.4	6.8 ± 0.6
syringic	21.6	221 ± 24.3	57.4 ± 15.8	40.5 ± 1.4	131 ± 43.9	14.0 ± 1.9	5.9 ± 0.6
p-coumaric	25.1	122 ± 5.5	12.8 ± 6.2	16.5 ± 2.8	44.5 ± 17.2	22.3 ± 1.3	17.6 ± 1.3
ferulic	27.9	215 ± 11.4	63.3 ± 21.4	45.1 ± 7.1	115 ± 36.8	10.0 ± 1.4	19.7 ± 4.3
sinapic	28.8	216 ± 21.6	47.7 ± 10.9	21.7 ± 4.9	76.8 ± 21.4	19.9 ± 2.9	<1.6
salicylic and isoferulic	30.0	162 ± 48.5	29.6 ± 14.4	18.1 ± 6.0	50.2 ± 20.7	8.2 ± 1.7	11.6 ± 4.6
o-coumaric	31.9	5.2 ± 3.4	4.9 ± 2.7	<0.4	2.1 ± 1.8	0.4 ± 0.3	<0.4
unknown	33	57.2 ± 1.7	31.8 ± 6.4	17.3 ± 12.7	14.5 ± 0.5	4.2 ± 0.3	5.2 ± 0.3
total		1134 ± 63.2	269 ± 37.5	168 ± 16.8	467 ± 77.9	79.9 ± 4.7	66.8 ± 6.5
isoflavonoids							
daidzin	26.4	596 ± 80.6	61.0 ± 6.6	226 ± 45.7	134 ± 19.4	2.0 ± 0.4	10.6 ± 1.8
genistin	31.4	2150 ± 87.7	270 ± 11.9	1410 ± 67.9	672 ± 41.9	52.0 ± 8.3	50.4 ± 7.7
daidzein	36.9	55.5 ± 7.3	77.3 ± 15.6	215 ± 31.7	55.8 ± 14.7	2.7 ± 0.4	14.0 ± 1.5
genistein	41.6	66.7 ± 7.8	120 ± 50.9	177 ± 24.7	103 ± 12.7	3.4 ± 0.5	7.5 ± 0.7
total		2870 ± 120	529 ± 54.9	2030 ± 91.2	965 ± 50.1	60.1 ± 8.3	82.5 ± 8.0

[a] Results from two or three replicate trials. [b] Defatted soy flakes. [c] Dialysis soy protein isolate. [d] Acid-precipitated soy protein isolate. [e] Commercial soy protein isolate. [f] Activated carbon treated soy protein isolate. [g] Ion exchange treated soy protein isolate.

1. The HPLC retention times of these latter components matched those of the reference phenolic components much better than those from unhydrolyzed phenolic extracts. Also, several of the unknown phenolic compounds separated from unhydrolyzed phenolic extracts were missing from hydrolyzed phenolic extract patterns. This simulaneous loss of unknown phenolic components and the increase in syringic acid content of phenolic extracts from defatted soy flakes following hydrolysis are circumstantial evidence that upward of 75% of the syringic acid exists in soybeans as esters or bound phenolic compounds.

The major neutral isoflavonoid compounds recovered from defatted soy flakes was genistin, at about 2.15 mg/g, which compared well with values of 1.64 mg/g by Naim et al. (1974) using GLC and 1.885 mg/g by Eldridge and Kwolek (1983) using HPLC. Genistin accounted for about 75% of the total isoflavonoid fractions and 54% of the total phenolics recovered from defatted soy flakes. The amounts of daidzein and genistein recovered were greater than those reported by Naim et al. (1974) Coumesterol, reported by Murphy (1981), was not detected in the present study, presumably due to its low concentration. In addition, HPLC elution patterns exhibited several unknown neutral phenolic (isoflavonoid) components with retention times ranging from 26 to 30 min and low concentrations, which may be due to glycitein and its glucoside (Huang et al., 1981).

The main objective of this study was to develop and use the HPLC method for quantitating the individual phenolic acids in defatted soy flakes and to determine their removal from soy protein isolates by several processing treatments. Phenolic acid concentrations in defatted soy flakes account for only about 28% of the total phenolics. Removal of isoflavonoids by Sep-PAK and alkaline hydrolysis of phenolic extracts as in the present study greatly facilitated the separation and identification of phenolic acids compared to results of How and Morr (1982). Major phenolic acids from defatted soy flakes were syringic, ferulic, and sinapic acids.

As expected, soy protein isolates contained substantially lower levels of phenolic compounds than defatted soy flakes (How, 1981; How and Morr, 1982). Activated carbon and ion-exchange treated soy protein isolates contained the lowest concentrations of both acidic and neutral phenolic compounds (Table III). Dialysis, acid-precipitated, and commercial soy protein isolates all contained substantially less total phenolic acids and isoflavonoids than defatted soy flakes. Activated carbon and ion-exchanged soy protein isolates had ≥90% reduction of both phenolic acids and isoflavonoids compared to defatted soy flakes. These results generally agree with the qualitative results of How (1981) and How and Morr (1982). However, the ion-exchange treatment was more effective for removing isoflavonoids in the present study than was previously shown.

Registry No. Genistin, 529-59-9; p-hydroxybenzoic acid, 99-96-7; gentisic acid, 490-79-9; vanillic acid, 121-34-6; caffeic acid, 331-39-5; p-coumaric acid, 7400-08-0; salicylic acid, 69-72-7; isoferulic acid, 537-73-5; o-coumaric acid, 583-17-5; syringic acid, 530-57-4; ferulic acid, 1135-24-6; sinapic acid, 530-59-6; daidzin, 552-66-9; daidzein, 486-66-8; genistein, 446-72-0.

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