Fractionation and Characterization of Alcohol Extractables Associated with Soybean Proteins. Nonprotein Components

A. M. NASH, A. C. ELDRIDGE, AND W. J. WOLF

Isolated soybean proteins extracted with 86% (v./v.) ethanol yielded 3.7% of a light brown semisolid. The alcohol-extractable materials were fractionated by chromatography on diethylaminoethylcellulose columns with nonaqueous solvents and preparative thin-layer chromatography on silicic acid. Phosphatidyl choline, phosphatidyl ethanolamine, saponins, sitosterol glycoside, and genistein were identified. About one fourth of the alcohol extract was a yellow oil containing triglycerides and other neutral compounds.

In 1946, Smiley and Smith (*22*) reported that extraction of soybean proteins with ethanol yielded a brown sirup like material. Acid hydrolysis of this sirupy material gave fatty acids, choline, sugars, and phosphoric acid, which indicated the presence of phospholipids.

The authors have shown that alcohol washing of isolated soybean proteins markedly improves their foaming properties by removing phospholipids and other alcohol-soluble materials (*7*, *8*). Effective removal of these materials depends upon alcohol concentration; for ethanol, the optimum concentration is 86% (v./v.). Here we describe the fractionation and characterization of materials extracted from soybean proteins with 86% (v./v.) ethanol.

Experimental

Preparation of Soybean Proteins. Undenatured, hexane-defatted flakes were extracted twice with water, first at a solvent–meal ratio of 10 to 1 and then at a ratio of 5 to 1. After centrifugation, the extracts were combined and adjusted to pH 4.5 with hydrochloric acid. The precipitated curd was concentrated by decantation of the whey and by centrifugation. The curd was suspended at pH 6.6 in 10% sodium chloride and dialyzed against changes of the salt solution for a week. After dialysis against water for 1 to 2 weeks, the salt-free protein was precipitated at pH 4.5 with acetic acid. After two washings with water to remove residual whey proteins, the curd was freeze-dried. The freeze-dried protein was extracted with hexane and desolventized in vacuo.

Preparation of Alcohol-Extractables. Soybean protein, prepared as described, was extracted on a magnetic stirrer with 25 volumes of 86% (v./v.) ethanol for 17 hours at 38° C. The alcohol-solubles were concentrated to dryness in vacuo with the exclusion of light.

Thin-Layer Chromatography. Fractions were analyzed by thin-layer chromatography according to Stahl (*23*). Glass plates were coated with silica gel G with a Stahl applicator (*12*). The solvent system of Wagner, Horhammer, and Wolff (*24*), chloroform–methanol–water (65:25:4), was used as a reference system during fractionation studies.

Diethylaminoethylcellulose Chromatography. The alcohol extract was fractionated by ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose with a modification of the nonaqueous solvent system of Rouser *et al.* (*20*). DEAE-cellulose, Selectacel type 40 (Brown Co., Berlin, N. H.) with a capacity of 0.86 meq. per gram, was prepared in the acetate form as described (*20*) and equilibrated with chloroform. From 300 to 500 mg. of alcohol solubles suspended in chloroform were applied to a 2.2 × 40 cm. column. The column was then eluted with chloroform as the first solvent, followed by chloroform–methanol (7 to 1) and the other solvents used by Rouser *et al.* (*20*). Maximum flow rate was 3 ml. per minute. Samples of 25 ml. were collected, evaporated to dryness, and combined according to the appearance of the solids and the nature of the eluting solvent. For subsequent chromatographic runs the resin was equilibrated with chloroform and the column repacked in that solvent.

Solids Determination. Solids were determined by evaporating column effluents almost to dryness and then drying 2 hours at 50° C. in vacuo.

Iodine Value. Iodine values were determined by the method of Rosenmund-Kuhnhenn as reported by Yasuda (*31*) with a 3-hour reaction time.

Saponification Number. The microsaponification method of Marcali and Rieman (*13*) was modified by changing the hydrolysis time to 3 hours.

Methanolysis. Methanolysis of alcohol-extractable materials and fractions thereof was usually for 4 hours at 100° C. with anhydrous 1.7*N* methanolic hydrochloric acid in a sealed tube (*5*).

Gas-Liquid Chromatography. Methyl esters of the alcohol solubles from methanolysis of the total mixture were applied in ether solution to a glass column of 10% Craig polyester succinate coated on Chromosorb W and run at 190° C. in a Pye A 3101 instrument.

Northern Regional Research Laboratory, Peoria, Ill.

Results

Isolation of Alcohol Extractables. Crude protein preparations were used in our previous study of alcohol washing of soybean proteins (*8*). In this study we removed salts, sugars, amino acids, and other low molecular weight components from the proteins by dialysis followed by hexane extraction. Because only traces of materials (~0.01%) were removed from the dialyzed, freeze-dried proteins by hexane, the absence of loosely held lipids was indicated. Subsequent extraction of the freeze-dried soybean proteins with 86% (v./v.) ethanol in the absence of light and air yielded 3.7% of a light brown semisolid. When the alcohol extractables were exposed to air, a reddish brown color and a rancid odor developed, indicating autoxidation of unsaturated fatty acids. Sucrose, stachyose, and raffinose, normal soybean meal constituents, were not detectable in the alcohol extractables by thin-layer chromatography. After alcohol extraction the protein contained 17.04% N and 0.18% P, compared to 16.24 and 0.20% before extraction.

Properties of Alcohol Extractables. Properties of the alcohol extractables and their subfractions after chloroform extraction are summarized in Table I. Also included are data for soybean oil and lecithin for comparison. The presence of phospholipids was indicated, but the low phosphorus and nitrogen values and the insolubility in chloroform suggested that nonlipid components were also present.

The alcohol-extractable materials gave positive Molisch and orcinol tests for carbohydrates. Carbohydrate content was 6.9% (expressed as glucose) as determined by the phenol–sulfuric acid colorimetric procedure (*6*). The sugars present were isolated as follows: A 165-mg. sample of alcohol solubles was heated in 25 ml. of anhydrous methanolic hydrochloric acid for 4 hours at 100° C. in a sealed tube, neutralized with silver carbonate, and filtered; methanol was removed in a rotary evaporator. The residue was extracted with 1- to 2-ml. portions of benzene until extracts were colorless, and then refluxed with 1*N* hydrochloric acid (5 ml.) for 1.5 hours. After refluxing, crystals were present which were removed by centrifuging. The crystals (identified as genistein, as indicated below) were washed with 1*N* hydrochloric acid, and the washings were added to the hydrolyzate which was neutralized with silver carbonate, filtered, saturated with hydrogen sulfide, and refiltered. The filtrate was dried in a rotary evaporator, redissolved in a small volume of water, and refiltered to remove residual silver chloride. The filtrate was then passed through a 5 × 60 mm. column of BioRad AG 1-X4, 200- to 400-mesh (acetate form), followed by 3 ml. of distilled water to yield neutral sugars. The column was then eluted with 5 ml. of 8*N* acetic acid and the effluent was freeze-dried to yield the acidic sugars.

Descending paper chromatography (Schleicher and Schuell, 589 Blue Ribbon-C paper) of the acidic fraction [ethyl acetate–acetic acid–water (3:1:3) v. /v., identified as solvent A] for 44 hours indicated glucuronic acid and excluded mannuronic and galacturonic acids. Paper chromatography of the neutral sugars [1-butanol–pyridine–water (6:4:3), solvent B] with two descents of 23 to 26 hours each, indicated galactose, glucose, arabinose, xylose, rhamnose, and glycerol. No inositol was detected. Rhamnose and glycerol had identical R_f values in solvent B but were well separated in 22 to 24 hours with solvent A. The sugars on the chromatograms were detected by spraying with ammoniacal silver nitrate and heating at 90° to 95° C.

The presence of fatty acids in the alcohol extractables was confirmed by gas chromatography. The ether-soluble portion of the methanolysis products from the alcohol extractables contained components having retention times corresponding to methyl esters of linoleic, palmitic, oleic, stearic, linolenic, C_{20}-saturated and -unsaturated acids, and C_{16}-unsaturated acids, listed in the order of decreasing relative amounts. The ether-soluble portion of the methanolysis mixture also gave a positive Liebermann-Burchard test for sterols.

Mutual solubility effects of the components present in the alcohol-extractable mixture prevented a clear-cut separation by solvent fractionation procedures, such as the separation of lecithin and cephalin, or isolation of fatty acids after saponification. Because of these difficulties we used chromatographic methods to fractionate the complex mixture.

Table I. Properties of Alcohol Extractables and Fractions from Chloroform Extraction

Fraction of Alcohol Extractables	Total, %	N, %	P, %	Molar N:P Ratio	Iodine Value[a]	Saponification No.[b]
Total	100	0.87	0.63	3.06	86	185
Chloroform-soluble	85	0.78	1.28	1.35	...	...
Chloroform-insoluble	15	1.21	0.76	3.52	...	...
Soybean oil[c]	...	...	...	...	103–152	189–195
Soybean lecithin[d]	...	1.75	3.79	1.02	...	...

[a] Grams of iodine absorbed per 100 grams of alcohol-extractable material.
[b] Mg. of potassium hydroxide to saponify 1 gram of alcohol-extractable material.
[c] Data of Bauer (*2*).
[d] Data of Noda and Song (*17*).

Thin-Layer Chromatography. The complexity of the alcohol extractables was indicated by thin-layer chromatography (Figure 1). Eight major fractions designated A through H were observed. A number of minor components were also present; gray bands were sometimes observed in the areas below groups C, E, and G when 50% sulfuric acid was used as the spray reagent. Color reactions of thin-layer fractions A through H with other spray reagents are summarized in the caption for Figure 1.

DEAE-Cellulose Chromatography. The alcohol-extractable mixture was fractionated by ion exchange chromatography on DEAE-cellulose (*20*). This system offered a method of separating components of an interacting nature with reasonably quantitative recoveries. Factors affecting fractionation and quantitation in this system were: partial solubility of DEAE-cellulose in the solvents; differences in densities of the eluting solvents, which caused mixing in the adsorbent matrix; and low solids per unit volume of effluent and salt content of the eluents, which made the salt and solvent nonvolatiles content greater than that of the alcohol-extractable components in some effluents.

After removal of the solvent used to elute the fractions from DEAE-cellulose, the samples were dispersed in chloroform. Since several of the column fractions were partially insoluble in chloroform, additional fractionation was carried out. The subfractions are designated by the letter I to indicate insolubility in chloroform and by S to indicate solubility.

Results of chromatographing the alcohol-extractable materials on DEAE-cellulose are summarized in Table II. The sample was applied as a suspension in chloroform to the column equilibrated with chloroform. On washing the column with additional chloroform about

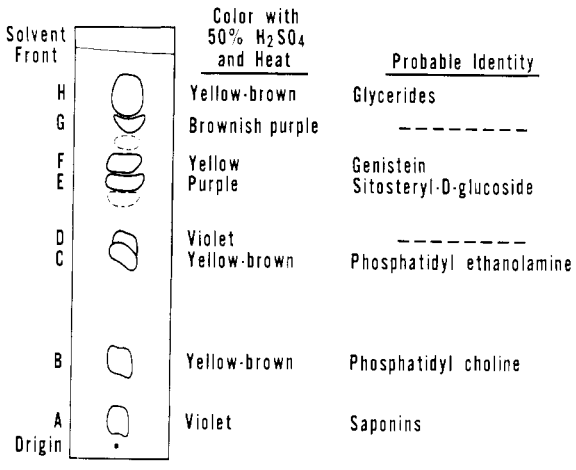

Figure 1. Schematic diagram of silica gel thin-layer chromatogram of alcohol-extractable materials from soybean proteins

Plate load 980 μg.
Solvent system. Chloroform–methanol–water (65:25:4). Spots detected by spraying with 50% sulfuric acid and heating. Only major spots designated by letters. Streaking between spots also observed but not indicated
Positive tests. Phosphomolybdate (*21*), *A*, *B*, *C*, *G*, and *H*; Dragendorf reagent (*21*), *B*; ninhydrin (*9*), *C*

Table II. Results of Fractionating Alcohol Extractables on DEAE-Cellulose

Column Fraction	Eluting Solvent	Volume of Solvent, Ml.	Appearance of Eluted Material[a]	Chloroform Fractionation		Thin-Layer Fractions Present[c]
				Soluble, %[b]	Insoluble, %[b]	
Holdup volume	$CHCl_3$	125	...	...	...	...
1	$CHCl_3$	75	Yellow oil	26	...	H
2	$CHCl_3$	250	Yellow waxy semisolid	3	...	H
3	$CHCl_3$:CH_3OH 7:1	35	Viscous, orange oil	16	...	B, E, G
4	$CHCl_3$:CH_3OH 7:1	125	Orange, wax like semisolid	11	...	B, D, E, G
5	$CHCl_3$:CH_3OH 7:1	125	Yellow semisolid	5	...	D, E, G, (C)
6	$CHCl_3$:CH_3OH 7:1	25	White fibrous solid	Trace	Trace	D, F, (C)
7	$CHCl_3$:CH_3OH 7:1	200	Yellow solid	2	3	C, F
8	$CHCl_3$:CH_3OH 7:1	75	Yellow resin	2	1	C, F
9	$CHCl_3$:CH_3OH 7:3	75	Orange oil	1	Trace	(C) (F)
10	CH_3OH	125	Tan solid	1	1	
11	$CHCl_3$:CH_3COOH 3:1	150	Brown resin	2	Trace	
12	Glacial CH_3COOH	175	Brown resin	1	10	A
13	$CHCl_3$:CH_3OH 4:1, 1% NH_4OH	175	Tan resin	3	2	A
14	$CHCl_3$:CH_3OH 7:1	125	Yellow resin	1	Trace	

[a] Appearance of fraction after removal of solvent.
[b] Expressed as percentage of total sample applied to column.
[c] Letters in parenthesis indicate minor constituents.

one fourth of the total sample was eluted at or near the column holdup volume. The yellow oillike material corresponded to thin-layer fraction H (Figure 1). Further elution with chloroform yielded a semisolid material (column fraction 2) having an R_f value similar to thin-layer fraction H. The next solvent, chloroform–methanol (7 to 1), eluted about 40% of the sample in column fractions 3 through 8. Each of these column fractions contained two or more thin-layer fractions, as indicated in Table II. Column fractions 7 and 8 contained nonlipid material, as indicated by their partial solubility in chloroform. Solubility in chloroform served as a means for separating thin-layer fraction F (chloroform-insoluble) from thin-layer fraction C (chloroform-soluble). Elution with chloroform–methanol (7 to 3) followed by methanol yielded column fractions 9 and 10, respectively, both in small amounts. Another minor fraction was separated by changing the solvent to chloroform–acetic acid (3 to 1) (column fraction 11). Elution with glacial acetic acid yielded thin-layer fraction A, which was largely insoluble in chloroform. Additional material having an R_f on thin-layer chromatography similar to fraction A was obtained with chloroform–methanol (4 to 1) containing 1% ammonium hydroxide (column fraction 13).

On thin-layer chromatography of column fractions 10, 11, 13, and 14, a significant amount of material streaked from the origin to the solvent front. That the alcohol extractables also contained this material, possibly polymers of oxidized lipids, was indicated by streaking between the major spots.

The fractions from DEAE-cellulose chromatography and chloroform fractionation were used as starting materials to identify some of the major components observed on thin-layer chromatograms.

Thin-Layer Fraction A. This group of substances was found largely in column fraction 12, which eluted with glacial acetic acid; this behavior indicated components of an acidic nature (*20*). The bulk of column fraction 12 was insoluble in chloroform, and the chloroform-insoluble fraction is henceforth designated as fraction 12-I to differentiate it from the parent fraction and from the portion soluble in chloroform, fraction 12-S. Fraction 12-I was crystallized from 80% ethanol and had a melting point of 251° to 254° C. Although insoluble in chloroform, fraction 12-I dissolved in water to form solutions that foamed when shaken. These properties, plus a carbohydrate content of 26% determined as glucose by the phenol–sulfuric acid procedure, suggested the presence of saponins.

Soybean saponins prepared by the procedure of Birk *et al.* (*3*) contained 24 to 27% carbohydrates; carbohydrate contents were similar after methanolysis. Chromatography of soybean saponins and fraction 12-I on Whatman 3MM filter paper with butanol–ethanol–water (6:2:3) and detection with saturated antimony trichloride in chloroform (*3*) revealed no differences between the two samples. Both preparations gave two major spots having R_f values of 0.45 and 0.55. Thin-layer chromatography on silica gel G with chloroform–methanol–water (65:25:4) or methanol–water–7*N* ammonium hydroxide (6:3:1) revealed several poorly separated spots in soybean saponins and fraction 12-I. The components in fraction 12-I moved with a higher R_f value than the components in soybean saponins, but the shapes of the spots and color reactions with 50% sulfuric acid were similar.

Methanolysis of fraction 12-I confirmed its identity as soybean saponins. In a typical experiment 5 mg. of sample were heated in a sealed tube at 100° C. for 4 hours in 1 ml. of 1.7*N* anhydrous methanolic hydrochloric acid. After evaporation of the methanolic hydrochloric acid, the residue was suspended in 2 ml. of water and extracted three times with 2-ml. portions of benzene. The benzene extracts were evaporated and the residues redissolved in methanol for spotting on thin-layer plates.

Thin-layer chromatography on silica gel G with benzene–ethyl acetate (1 to 1) showed the products from soybean saponins and fraction 12-I to be identical; both benzene-soluble fractions separated into four components corresponding to soyasapogenols (Table III). The component of R_f 0.30, present in small amounts, presumably corresponds to soyasapogenol A; we did not have an authentic sample for reference purposes. Components of R_f 0.44 and 0.46 appear to correspond to the respective soyasapogenols B and E. Our reference sample of soyasapogenol B contained two compounds, a major component (R_f 0.43) believed to be soyasapogenol B and a minor component (R_f 0.46) corresponding to soyasapogenol E on the basis of its green color reaction with antimony trichloride (*25*). The spots of R_f 0.49 to 0.50 correspond to soyasapogenol C or soyasapogenol D, or both; the R_f's of the two components were so similar that it was impossible to differentiate between the two.

Since soyasapogenol C contains two double bonds and soyasapogenol D contains only one double bond, it was possible to separate them by thin-layer chromatography on silica gel G impregnated with silver nitrate (plates were coated with 30 grams of silica gel G mixed with 70 ml. of 15% silver nitrate solution) as used for separating fatty acids according to degree of unsaturation (*14*). In the presence of silver nitrate, soyasapogenol D apparently forms a silver complex having an R_f of 0.0, whereas the complex of soyasapogenol C has an R_f of 0.30 (Table III). This technique showed soyasapogenol D but little or no soyasapogenol C in the sapogenins of soybean saponins and fraction 12-I. The presence of soyasapogenol B in both preparations was also confirmed; the component of R_f 0.11 observed in trace amounts in fraction 12-I presumably corresponds to soyasapogenol A. The minor component (soyasapogenol E?) observed in our soyasapogenol B reference sample by thin-layer chromatography without silver nitrate was not detected on the silver nitrate–impregnated plates.

The benzene-insoluble portions of the methanolysis products from soybean saponins and fraction 12-I were refluxed with 1*N* hydrochloric acid for 1.5 hours and then treated as described for isolation and identification of sugars in the alcohol solubles. The acidic sugar fractions from soybean saponins and fraction 12-I both contained only glucuronic acid, while the neutral

Table III. Thin-Layer Chromatography of Benzene-Soluble Methanolysis Products from Soybean Saponins and Fraction 12-I and of Soyasapogenols[a]

Sample	Silica Gel G			Silica Gel G–$AgNO_3$	
		Color Reaction with			Color reaction with
	R_f	50% H_2SO_4	$SbCl_3$	R_f	50% H_2SO_4
Soybean saponins after methanolysis	0.30	Violet	Violet	0.0	Pink
	0.44	Violet	Violet	0.24	Pink
	0.46	Bluish purple	Green		
	0.49	Violet	Rose		
Fraction 12-I after methanolysis	0.31	Violet	Violet	0.0	Pink
	0.44	Violet	Violet	0.11	Pink
	0.47	Bluish purple	Green	0.24	Pink
	0.50	Violet	Rose		
Soyasapogenol B	0.43	Violet	Violet	0.24	Pink
	0.46[b]	Bluish purple	Green		
Soyasapogenol C	0.52	Violet	Greenish yellow	0.30	Pink
Soyasapogenol D	0.50	Brown	Rose	0.0	Pink

[a] Chromatographic solvent was benzene–ethyl acetate (1:1) for both types of thin-layer plates. [b] Probably soyasapogenol E (*25*) and present in small amounts.

sugars consisted of galactose, glucose, arabinose, rhamnose, and xylose. Our results on the sugars of soybean saponins are in agreement with other findings (*1*). Identifying the sugars in fraction 12-I provides additional evidence that fraction 12-I contains soybean saponins.

Thin-Layer Fraction B. This fraction had the R_f and color reactions corresponding to phosphatidyl choline and was concentrated in column fraction 3. Isolation was performed by preparative thin-layer chromatography of column fraction 3. The fraction B area was detected with iodine vapor, the silicic acid scraped from the glass plate, and fraction B eluted with chloroform–methanol–water. After desolventization, the material was subjected to a sealed-tube methanolysis. The reaction mixture was evaporated to dryness, dissolved in water, and chromatographed on Whatman No. 1 filter paper with butanol–acetic acid–water (10:3:10). A single rose-colored spot with R_f 0.26 appeared on spraying with Dragendorf's reagent. Choline and a hydrolyzate of synthetic phosphatidyl choline gave similarly colored spots with respective R_f values of 0.27 and 0.24. Fraction B was therefore concluded to be phosphatidyl choline. These results confirm the earlier observations of Smiley and Smith (*22*) concerning the presence of phosphatidyl choline in isoelectrically precipitated soybean proteins.

Thin-Layer Fraction C. Fraction C was concentrated in column fraction 8 (Table II). On subfractionating with chloroform, fraction F, the major contaminant, was removed, leaving fraction 8-S. This fraction yielded only a single ninhydrin-positive spot (R_f 0.57) on silica gel G with chloroform–methanol–water (65:25:4) and (R_f 0.61) with chloroform–methanol–7*N* ammonium hydroxide (35:60:5). R_f values were similar for synthetic phosphatidyl ethanolamine. Spraying the thin-layer plates with 50% sulfuric acid showed components migrating faster and slower than the ninhydrin-positive material. Fraction C was then isolated by preparative thin-layer chromatography of fraction 8-S on silica gel G and hydrolyzed with methanolic hydrochloric acid. Chromatography of the hydrolyzate on Whatman No. 1 filter paper with 88% phenol–water–ammonium hydroxide (100:20:0.3) showed the presence of ethanolamine, R_f 0.81 (authentic ethanolamine, R_f 0.78). With the upper phase of butanol–acetic acid (9 to 1) saturated with water, a ninhydrin-positive spot of R_f 0.20 was obtained for the hydrolyzate and R_f 0.21 to 0.23 for authentic ethanolamine. Thin-layer fraction C is therefore believed to be phosphatidyl ethanolamine.

Thin-Layer Fraction E. Fraction E was eluted in column fractions 3, 4, and 5, with highest concentration in 5. Fraction E was further purified by preparative thin-layer chromatography on silica gel G. It gave a positive test for sterols with Liebermann-Burchard reagent; its R_f and color with 50% sulfuric acid on silica gel G were identical with those of β-sitosterol-D-glucoside isolated from peanut flour (*15*). The sitosterol moiety was identified by methanolysis and thin-layer chromatography of the benzene-soluble portion of the methanolysis mixture; R_f and color reaction with 50% sulfuric acid agreed with those for authentic β-sitosterol. Carbohydrate content of purified fraction E determined with phenol–sulfuric acid was ~30% (as glucose). A portion of fraction E crystallized as rosettes from pyridine had a melting point of 290° to 291° C.; Morris and Lee (*15*) reported a melting point of 285° to 289° C. for β-sitosterol-D-glucoside. The infrared spectrum (potassium bromide disk method) of the crystalline compound was identical with that of authentic β-sitosterol-D-glucoside. Although the glucose moiety was not isolated and identified, the evidence strongly indicates that fraction E is β-sitosterol-D-glucoside.

Thin-Layer Fraction F. Fraction F occurred in

column fractions 6 to 9. It was separated from column fraction 7 as a chloroform-insoluble subfraction which, upon crystallization from methanol, had a melting point of 296° C. Ultraviolet absorption spectra, a positive test with ferric chloride, and the characteristic yellow color with sulfuric acid suggested an isoflavone structure. In Table IV the melting point and ultraviolet absorption maxima of thin-layer fraction F are compared with similar properties of genistein, daidzein, and 6,7,4′-trihydroxyisoflavone. The presence of genistein and daidzein as glycosides in soybeans is well authenticated (*18*); the occurrence of 6,7,4′-trihydroxyisoflavone in tempeh, a fermented soybean food product, has been reported (*10*), but its existence in unfermented soybeans is still unknown. Data of Table IV indicate that fraction F is identical with genistein. Infrared analysis of fraction F by the potassium bromide disk method gave a spectrum indistinguishable from authentic genistein.

Chromatography on Whatman No. 1 paper with benzene–acetic acid–water (125:72:3) (*30*); chromatography on Whatman No. 4 paper saturated with borate buffer and developed with butanol–ethanol–borate buffer (1:1:1) (*4*); and thin-layer chromatography on silica gel G with chloroform–methanol–water (65:25:4) also confirmed that fraction F was genistein.

The crystalline material from methanolysis and hydrolysis of the alcohol-extractable mixture described earlier was also identified as genistein. The crystalline material gave a gray violet color with ferric chloride when spotted on filter paper, and on thin-layer chromatography on silica gel G with chloroform–methanol–water (65:25:4), it gave an R_f value and lemon yellow color with 50% sulfuric acid characteristic of genistein. Paper chromatography with benzene–acetic acid–water (125:72:3) revealed a major spot corresponding to genistein (R_f 0.55) plus two minor spots (R_f 0.35 and 0.67). Ultraviolet spectral analysis of the crystalline material in methanol revealed an absorption maximum at 261 mμ which shifted to 273 mμ in the presence of 0.2% aluminum chloride and to 269 mμ in the presence of 1% sodium acetate. Recrystallization of the crude crystals from 60% ethanol yielded a small quantity of needles which had a melting point of 286° to 290° C. (dec.).

The presence of genistin, the glucoside of genistein, in soybeans is well known, but isolation of genistein from soybeans has not been reported. Although genistein has been found in frozen tofu, an Oriental food product made from soybeans, it was thought to have formed by hydrolysis of genistin during the manufacturing process (*16*).

Thin-Layer Fraction H. This group represented the major fraction of the alcohol-extractable materials. Isolation of this component group with the holdup volume of chloroform (fraction 1) on the DEAE-cellulose column indicated the nonpolar nature of the material. This fraction, which represented 26% of the total alcohol extractables, was a yellow oil, was optically inactive, and contained 0.01% phosphorus, 0.075% nitrogen, and 0.06% sulfur. Negative phosphomolybdate and ninhydrin spot tests and a negative

Table IV. Physical Properties of Thin-Layer Fraction F and Soybean Isoflavones

Compound	M.P., °C.	$\lambda_{max.}$ Mμ Methanol	Alcoholic sodium acetate	Alcoholic aluminum chloride
Fraction F	296	262	272	272
Genistein[a]	296	263	277[b]	275
Daidzein[a]	324	250	260[b]	250
6,7,4′-Trihydroxyisoflavone[c]	320	260	...	...
Acetate derivative of fraction F	205	...	...	...
Genistein triacetate[d]	202	...	...	...

[a] From Wong (*28*) and Wong and Flux (*29*). Spectral data as determined in ethanol.
[b] Value of Wong (*28*) as determined in ethanolic sodium hydroxide.
[c] From Gyorgy *et al.* (*10*).
[d] From Wong and Flux (*29*).

Liebermann-Burchard reaction eliminated phospholipid and sterol structures. The hydroxamate test for esters was positive and the saponification number was 181 mg. of KOH per gram; infrared and ultraviolet spectra were typical of triglycerides. Thin-layer chromatography on silicic acid with petroleum ether–diethyl ether–acetic acid (90:10:1) gave a spot with an R_f of 0.43 as compared to R_f 0.5 for triolein. However, a large portion of fraction H formed an elongated spot from the origin to R_f of ~0.3, the region in which monoglycerides and diglycerides migrate (*11*). Results were similar with petroleum ether–diethyl ether (90 to 10). Apparently fraction H contains triglycerides plus a number of other neutral components that may include mono- and diglycerides.

Discussion

Our results show that soybean proteins isolated by isoelectric precipitation from a water extract of hexane-defatted meal contain a number of nonprotein constituents extractable with aqueous alcohols. These components, including phosphatidyl choline, phosphatidyl ethanolamine, saponins, sitosterol glycoside, and genistein, have all been previously reported in soybeans. Evidence for the presence of phosphatides in isolated soybean proteins was presented by Smiley and Smith (*22*), but our report appears to be the first that saponins, sitosterol glycoside, and genistein are also associated with the proteins. Further work will be necessary to determine whether these nonprotein-protein complexes exist in vivo or are artifacts formed during isolation of the proteins. These complexes may be important in basic studies on soybean protein and in use of the proteins in food products. The presence in purified 11-S protein of only traces of materials extractable with aqueous alcohol indicates that the non-

protein components are not uniformly distributed among the proteins in the globulin fraction (*8*). Although the nonprotein constituents are extractable from the proteins with aqueous alcohols, some of the proteins are insolubilized by this treatment (*19*, *26*, *27*). Milder methods would be desirable for removing the nonprotein constituents without causing irreversible conformational changes in the proteins. Such methods would allow a study of the interaction between the proteins and the various nonprotein compounds under controlled conditions.

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