Alcohol Washing of Soybean Protein

A. C. ELDRIDGE, W. J. WOLF, A. M. NASH, and A. K. SMITH
Northern Regional Research Laboratory, Peoria, Ill.

Soybean protein isolated by acidification of an aqueous extract of hexane-defatted soybean meal contains 2 to 4% of a phospholipide-like material. These lipides cannot be removed by isoelectric precipitation, washing with water, dialysis, or ammonium sulfate precipitation. However, they are extractable with aqueous alcohols. The concentration of alcohol used to wash the protein has a pronounced effect on the amount of material removed and on the nitrogen content of the extracted protein. Optimum concentrations of alcohol are: methyl, 95 to 97% (v./v.); ethyl, 84 to 88% (v./v.); and isopropyl, 78 to 88% (v./v.). The effects of extraction time, temperature, pH, and solvent to protein ratio were also studied. The washed protein has improved color and flavor and produces extremely stable, low density foams and whips similar to egg white and commercially available soy products.

FOOD UTILIZATION of soybeans in the United States is still expanding, and the potential is much greater than its present use. Extensive reviews (*1*, *25*, *30*) on the use of soybean protein products in foods have been published. They indicate that color and flavor of soybean protein are important considerations. In some foods, these properties can be masked by other flavorings or colorings. In other foods, however, it is more desirable for these properties to be altered or greatly reduced.

One approach to the problem is to wash soybean meal with alcohol (*3*, *4*, *26*). Beckel, Belter, and Smith (*5*, *7*) reported that this treatment reduces flavor and color of the meal or of isolated protein. The associated problem of denaturation of soybean protein with alcohols has been investigated by Nagel, Becker, and Milner (*15*) and by Smith, Johnsen, and Derges (*23*) in studies on Gelsoy (*6*, *9*), a whipping agent from soybeans.

Many researchers have also used combinations of alcohol and diethyl ether to dry soybean proteins. Perhaps one of the first such reports is the often quoted, classical work of Osborne and Campbell (*20*) who obtained a protein of 17.6% nitrogen. Smiley and Smith (*22*) prepared soybean protein by various procedures and measured the nitrogen content of the isolated proteins. Highest nitrogen values in the proteins were obtained either by using ethyl alcohol-washed meals or by ethyl alcohol extraction of the dried protein. In the latter method, 4 grams of brown syrup-like material were isolated from 100 grams of protein. Hydrolysis of the material indicated the presence of phospholipides.

The authors have confirmed that washing soybean protein with alcohols removes a phospholipide-like material reported earlier (*22*). This article reports the conditions for removal of lipide-like materials from soy protein with aqueous alcohols. The resulting protein has less color and beany flavor, and it will form extremely stable foams and thermo-reversible gels. Detailed descriptions of the foaming and gelling properties will be published elsewhere.

Materials and Methods

Preparation of Crude, Acid-Precipitated Soybean Protein. Laboratory-prepared, undenatured hexane-defatted flakes were extracted twice with water, first at a solvent-to-meal ratio of 10:1 and a second time at 5:1. After centrifugation, the supernatant solutions of the two extracts were combined and adjusted to pH 4.5 with hydrochloric acid; the precipitated curd was centrifuged, collected, and freeze-dried. After drying, the material was ground in a hammer mill using a 100-mesh screen.

Preparation of a Partially Purified Acid-Precipitated Soybean Protein. Crude, acid-precipitated soybean protein was dissolved in 10% sodium chloride–potassium phosphate buffer, pH 7.6, and dialyzed at 4° C. for 1 week against several changes of the buffer. The second week the protein was dialyzed in the cold against water. The protein then was precipitated with acid at pH 4.5; the curd was thoroughly washed with water and freeze-dried.

Preparation of Other Purified Soybean Proteins. Other soybean protein preparations were isolated. The cold-insoluble fraction was prepared by the method of Briggs and Wolf (*8*). Its preparation is essentially a precipitation of the 11S ultracentrifugal component from the water extract by cooling, dialysis, and freeze-drying.

The supernatant solution remaining after removal of the cold-insoluble fraction was adjusted to pH 4.5, and the precipitate was isolated, dispersed in phosphate buffer pH 7.6, and dialyzed against water at 4° C. for 6 days. The pH was adjusted to 4.5, and the precipitate was isolated, washed with water, and freeze-dried.

Glycinin was prepared by the method of Osborne and Campbell (*20*), which consists of a salt extraction followed by precipitation with ammonium sulfate and dialysis. The material was precipitated with acid, washed thoroughly with water, and freeze-dried.

The highly purified 11S fraction (*28*) was obtained by ammonium sulfate fractionation of the cold-insoluble fraction.

Aqueous Alcohols. All alcohols used were ACS grade, and the concentrations are given on a v./v. basis—e.g., 86% (v./v.) ethyl alcohol was prepared by mixing 86 volumes of alcohol and 14 volumes of water.

Alcoholic Washings. The extractions with alcohol were carried out at room temperature (23°–26° C.) either in a Waring Blendor or by mechanical shaking in screw-capped test tubes.

Soluble Solids. Aliquots of the alcoholic washes were evaporated to dryness on a hot plate and dried at 50° C. in a vacuum oven for 2 hours.

Soluble Esters. Alcoholic washes were analyzed for esters by the hydroxamic acid procedure described by Antonis (*2*). Diethyl ether was successfully substituted for isopropyl ether. Values are given as microequivalents of ester per gram of protein with triolein as the standard.

Foam Stability. Five per cent dispersions of the alcohol-washed protein were made at pH 7.5. The samples,

Table I. Foam Stability of Soybean Protein after Various Treatments

Protein and Treatment	Foam Stability, Min.
Untreated, acid-precipitated protein	6
86% Ethyl alcohol–washed, acid-precipitated protein	~200
86% Ethyl alcohol–washed, acid-precipitated protein with alcohol solubles added	6
Untreated, acid-precipitated protein suspended in alcohol, alcohol removed in vacuo	6
Glycinin	18
Dialyzed, acid-precipitated protein untreated with alcohol	6
Acid-precipitated protein from diethyl ether extracted soybean meal	6–10

usually 200 ml., were heated in a boiling water bath for 5 minutes. (Terminal temperature 80°–85° C.) The suspensions were whipped for 15 minutes in a Hobart mixer (Model G) at high speed using a Type D wire whip.

The stability of the foam is given as the time in minutes required for one drop of liquid to fall from a 60° glass funnel 70 mm. in diameter, having a stem 8 mm. in diameter and 150 mm. long. In the stability tests, four to six funnels were filled uniformly with foam. Reproducible foam-stability values were obtained only when the funnel stems were filled with a solid column of foam and liquid at the time of dripping.

Results and Discussion

Effects of Alcohol Concentration on Properties of Acid-Precipitated Protein. Preliminary experiments showed that acid-precipitated soybean protein dried by ethyl alcohol and ethyl ether had more foam stability than commercial, edible-grade soybean protein or freeze-dried, laboratory-prepared protein. When 86% ethyl alcohol–washed protein was whipped and a concentrate of the alcohol solubles was added to the foam, the foam collapsed. When crude, acid-precipitated protein was suspended in ethyl alcohol and the alcohol removed in vacuo, foam stability was similar to that of the untreated protein. These results are shown in Table I. They indicate that increased foam stability is the result of removing an alcohol-soluble foam inhibitor, rather than the result of alcohol denaturation of the protein. The data in Table I also show that the foam inhibitor is not removed by ammonium sulfate precipitation and dialysis in the preparation of glycinin and is also present in protein isolated from ethyl ether–defatted flakes. Earlier work (*22*) showed that hydrolyzates of alcoholic extracts of crude, acid-precipitated protein contain carbohydrate, phosphoric acid, fatty acids, and choline thus indicating that part of the impurity consists of phospholipides.

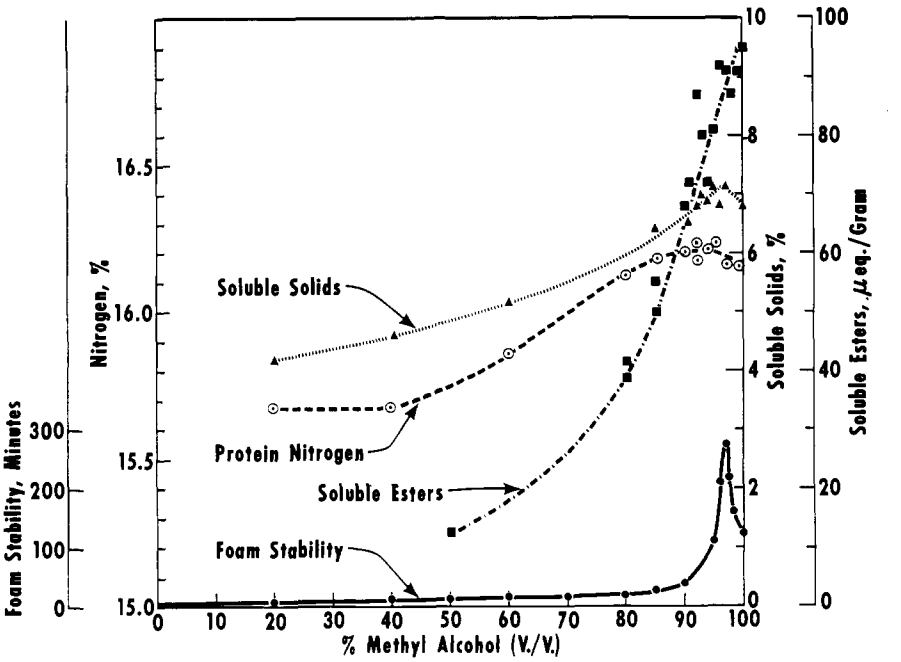

Figure 1. Effect of methyl alcohol concentration on the washing of crude, acid-precipitated soybean protein at room temperature

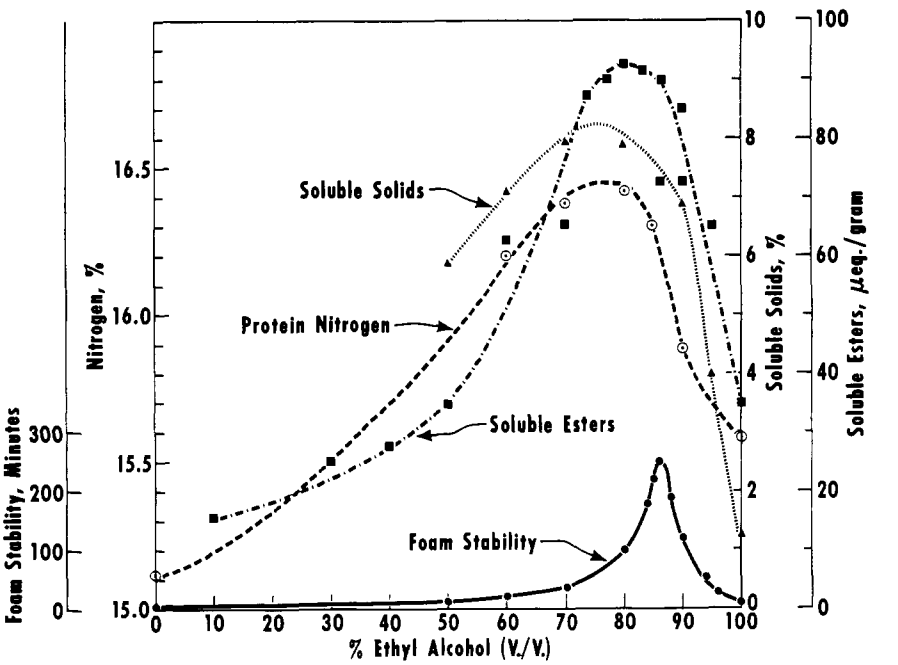

Figure 2. Effect of ethyl alcohol concentration on the washing of crude, acid-precipitated soybean protein at room temperature

The authors' studies show that removal of the impurity is very dependent upon the concentration of water present and the alcohol used, and also that aqueous alcoholic extracts of the crude protein give a positive test for esters with hydroxamic acid which is further support for the presence of fatty acid esters.

Effects of washing crude, isolated soybean protein with different concentrations of methyl alcohol, ethyl alcohol, and isopropyl alcohol are shown in Figures 1, 2, and 3. Values are given for the amount of solids and esters removed, and for the foam stability and nitrogen content of the protein after washing. All washings were carried out with a solvent to protein ratio of 25 ml. per gram.

Figure 1 shows that the greatest amount of solubles (7.1%) was removed and the greatest foam stability (270

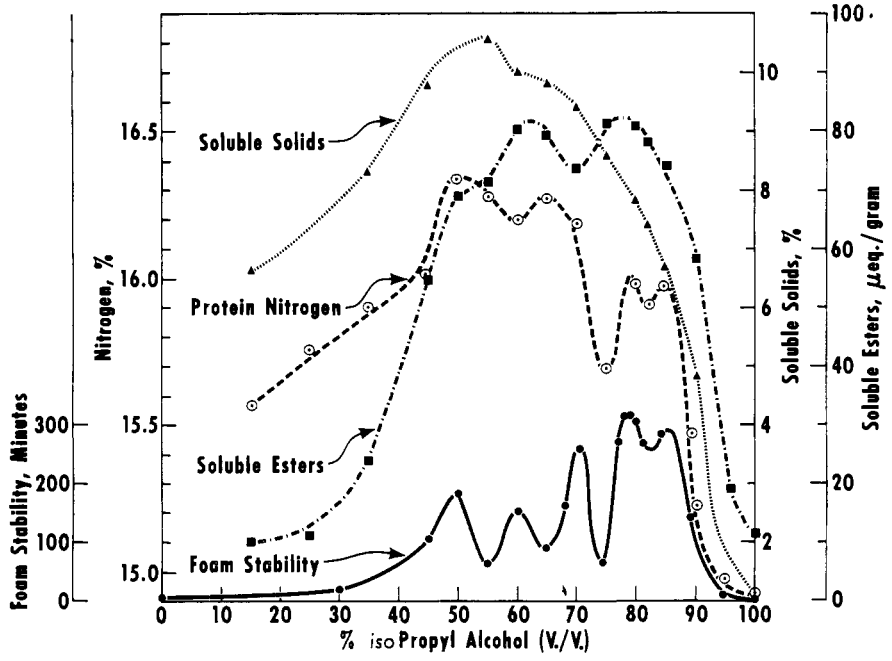

Figure 3. Effect of isopropyl alcohol concentration on the washing of crude, acid-precipitated soybean protein at room temperature

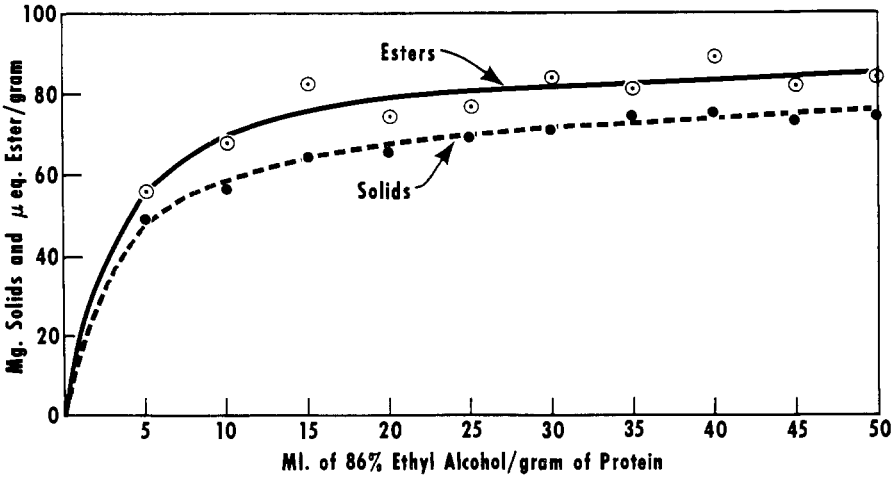

Figure 4. Effect of the ratio of solvent to protein on extraction of solids and esters from soybean protein

minutes) was obtained when 95 to 97% methyl alcohol was used to wash the protein. Foam stability for the unextracted protein was only 6 minutes. The soluble esters reached a maximum when absolute methyl alcohol was used to wash the protein. The nitrogen content of the protein reached a maximum of 16.2% when 85 to 100% methyl alcohol was used to wash the protein.

Figure 2 indicates that washing the crude protein with 70 to 80% ethyl alcohol removes the most solubles and gives the highest nitrogen value (16.5%). The most soluble esters were removed with 78 to 85% ethyl alcohol, and the greatest foam stability (250 minutes) was obtained when 86% ethyl alcohol was used to wash the protein.

Figure 3 shows the effect of washing crude soybean protein with aqueous isopropyl alcohol. All the properties measured vary considerably with alcohol concentration. The most esters were removed and maximum foam stability of the protein was obtained by washing with 77 to 82% isopropyl alcohol. The most solubles were removed and the nitrogen content of the protein was highest when 52% isopropyl alcohol was used for the washing.

Soybean phosphatides reduce stability of gliadin foams (*12*). Thus, the fluctuations in the foam stability curve in Figure 3 suggest that the crude protein contained either a mixture of phosphatides with different solubilities in aqueous isopropyl alcohol or a single phosphatide interacting with the proteins to different degrees.

For maximum foam stability of the protein the optimum alcohol concentrations are shown in Figures 1 to 3 to be 95 to 97% for methanol, 86% for ethanol, and 77 to 82% for isopropanol. These alcohol concentrations have been used, therefore, to study other variables which may be involved in the washing of soybean protein with alcohols.

Effect of Solvent to Protein Ratio. The ratio of solvent to protein for optimum removal of alcohol-soluble solids and esters was determined for the three aqueous alcohols. The alcoholic washings were made at the isoelectric point of the protein (pH 4.5) at room temperature for 18 hours on a reciprocating shaker for the various solvent:protein ratios. The results obtained with 86% ethyl alcohol are shown in Figure 4.

Maximum extraction of alcohol-soluble solids and esters occurred with approximately 25 ml. of solvent per gram of protein, but only about 75% of the soluble solids and esters were removed with a 10:1 extraction ratio. Similar results were obtained with 95% methyl alcohol and 82% isopropyl alcohol.

Effect of Time on Washing. Figure 5 indicates the effect of time on the soluble solids and soluble esters removed by extraction with 25 ml. of 86% ethyl alcohol per gram of crude, acid-precipitated protein.

The protein was extracted at room temperature, pH 4.5, on a reciprocating shaker. The curves show that the alcohol-soluble solids and soluble esters increased very rapidly the first 15 minutes and gradually reached maximum values in 4 hours.

Effect of Temperature on Washing. The effect of temperature on washing of crude, soybean, acid-precipitated protein with 86% ethyl alcohol was investigated. The crude protein was extracted with 25 ml. of 86% ethyl alcohol per gram for 20 hours at pH 4.5. The data show that an increase in temperature resulted in an increase in both soluble esters and solids removed.

Temperature, °C.	Soluble Esters, μEq./Gram	Soluble Solids, %
−13°	59	5.0
25°	81	6.8
52°	90	7.4

Effect of pH on Washing. The effect of pH on the washing of crude, acid-precipitated protein with aqueous ethanol was studied. The protein was washed for 20 hours with 20 ml. of 86% ethyl alcohol per gram of protein. The pH was changed by adding various amounts of ammonium hydroxide to the aqueous alcohol. The data following show the effects and indicate that the washing can be improved if a pH of 6.0–7.0 is used.

pH	Soluble Esters, μEq./Gram	Soluble Solids, %
4.5	80	6.5
5.2	119	8.1
6.0	128	8.3
6.7	133	8.4
7.0	119	8.6

Comparison of Aqueous Alcohols with Other Solvents. Other solvents, some of which have been used to extract phospholipides from soybean meal and protein, were also studied to note how much material could be extracted from crude, acid-precipitated soybean protein. These results are shown in Table II. The table also includes an estimate of the amount of lipide (designated as chloroform solubles) extracted by the different solvents. Lipide was estimated by extracting the dried, soluble solids with chloroform and determining the weight of dissolved material by evaporating the chloroform and drying for 2 hours at 50° C. in vacuo. This determination was made to differentiate between the lipide and nonlipide materials extracted from the crude protein by the different solvents. Crude, acid-precipitated protein is contaminated with soybean whey solids which contain 28 to 36% sugars, plus salts, nonprotein nitrogen materials, and other unidentified constituents (*24*). These whey solids may be extracted from the protein to varying degrees by the different solvents; hence, values for total solids do not represent true values for the amount of lipide extracted. The data in Table II indicate that aqueous alcohols and 80% acetone are better extraction solvents than ethyl alcohol mixed with hexane, benzene, or ethyl ether. It is also apparent that dry alcohols and acetone are poor solvents. Thus, water appears to be an important component of the solvent mixtures. The effectiveness of water-saturated butyl alcohol for extracting lipides (chloroform solubles) is in agreement with the results of others in extracting lipides from wheat flour (*13*).

Alcohol is known to disrupt lipide-protein interactions in blood lipoproteins and to facilitate extraction of the lipide by nonpolar solvents (*10*). From Table II, it is apparent that ethyl alcohol mixed with lipide solvents such as hexane, benzene, or ethyl ether extracts only a fraction of the lipide extracted by aqueous alcohols. Thus with soybean protein, aqueous alcohols appear necessary to dissolve the lipide rather than to disrupt lipide-protein complexes. It is also observed that an 86% ethyl alcohol extract of soybean protein (5 ml. solvent per gram of protein) becomes turbid upon dilution to 60 to 70% ethyl alcohol, again indicating that maximum extraction is the result of maximum solubility.

Recent nutritional studies (*14*, *16*) report that commercial soybean protein contains 6.7% of lipide material which is extractable with hot butyl alcohol. Findings here confirm the presence of lipide-like material in the isolated soybean protein. However, the authors' values obtained with butyl and isobutyl alcohols are considerably lower. This may be caused by differences in extraction temperatures. Also of interest is the work of Kratzer and coworkers (*11*) and Vohra and coworkers (*27*) who have found that a methyl alcohol extract of soybean meal contains growth and antiperotic factors. Soybean phosphatides have also been reported (*18*) to improve feed utilization, increase weight gains, and improve the quality of meat when fed to laying hens. All of these results emphasize the need for careful evaluation of nutritional studies with soybean meal and protein.

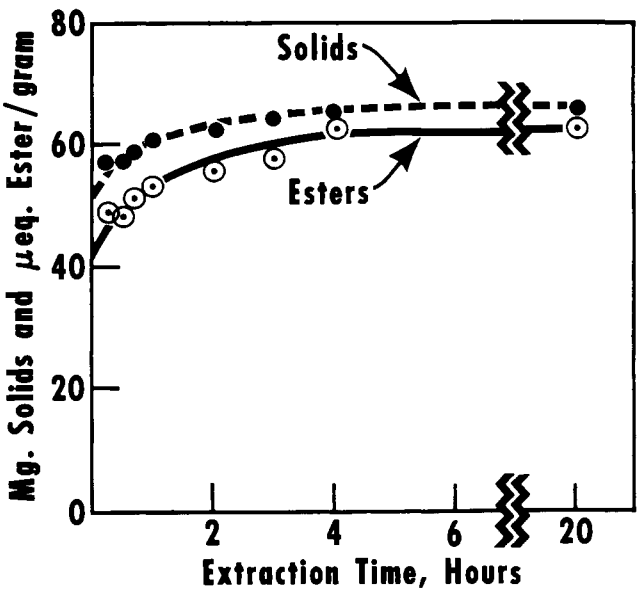

Figure 5. Effect of extraction time on the extraction of alcohol-soluble solids and esters

Table II. Effect of Extracting Crude, Acid-Precipitated Soybean Protein with Various Solvents with Respect to Soluble Solids, Crude Lipide, and Soluble Esters

Extraction Solvent	Amount of Material Extracted[a] Soluble solids, %	Chloroform solubles, %	Soluble esters, μeq./gram
95% Methyl alcohol	7.3	. .	81
97% Methyl alcohol	7.4	3.1	103
86% Ethyl alcohol	7.1	3.0	82
80% *n*-Propyl alcohol	8.4	3.5	113
82% Isopropyl alcohol	8.0	2.8	106
Water-saturated *n*-butyl alcohol	7.7	6.3	101
n-Butyl alcohol	0.5	0.4	6
Water-saturated isobutyl alcohol	6.0	5.5	101
Isobutyl alcohol	0.6	0.3	7
Water-saturated diethyl ether	1.8	0.3	11
Anhydrous diethyl ether	1.3	0.1	7
80% Acetone	7.0	2.7	83
Acetone	2.5	0.6	9
Hexane–benzene–ethyl alcohol (1:1:1)	2.0	0.6	18
Benzene–ethyl alcohol (4:1)	1.8	0.4	21
Hexane–ethanol (4:1)	1.2	1.3	12
Diethyl ether–ethyl alcohol (1:1)	1.5	1.1	6

[a] Extraction time was 18 hours using 25 ml. of solvent per gram of protein. All values are based on the weight of the crude protein.

Alcohol Washing of Purified Soybean Protein Fractions. Table III shows solubilities, ultracentrifugal compositions, and alcohol-extractable lipide contents of purified soybean protein fractions prepared as described earlier.

Alcohol-solubles content is lower than in Table II probably due to removal of low molecular weight materials (sugars, salts, etc.) by dialysis and precipitation in purification of the proteins. Values for alcohol solubles of the purified, acid-

Table III. Extractable Solids and Ultracentrifugal Distribution of Various Purified Soybean Protein Fractions

Protein Fraction	Protein Solubility, %[a]	Ultracentrifugal Distribution of Soluble Protein, %					Per Cent Solids Soluble in:	
		2S	7S	11S	15S	>15S	86% Ethyl alcohol	Water-saturated n-butyl alcohol
Acid-precipitated[b]	..	..	..	..	..	..	3.7	4.0
Acid-precipitated[c]	62	21	32	41	6	0	1.9	2.0
Cold insoluble[c]	97	5	11	78	7	0	0.9	1.1
Supernatant from cold insoluble[c]	97	29	39	29	3	0	1.9	2.4
Glycinin[c]	96	15	26	45	8	6	1.1	1.6
Purified 11S[d,e]	100	0	0	94	6	0	0.1	..

[a] Represents percentage of freeze-dried preparations soluble in potassium phosphate–sodium chloride, pH 7.6, 0.5 ionic strength containing 0.01*M* mercaptoethanol. Ratios of dry protein to buffer varied from 5 to 15 mg. of protein per ml. for various preparations. [b] Adams variety 1960 crop, same as used for study in Table II. [c] Clark variety, 1957 crop. [d] Hawkeye variety, 1957 crop. [e] Ultracentrifugal analysis was made before dialysis and freeze drying which cause aggregation of the 11S component (*28*).

Table IV. Nitrogen and Phosphorus Content of Various Preparations before and after Extraction with 86% Ethyl Alcohol

Preparation	%[a] Before Alcohol Extraction		%[a] After Alcohol Extraction	
	N	P	N	P
Acid-precipitated protein (crude)[b]	15.33	0.93	16.07	0.85
Acid-precipitated protein (partially purified)[b]	16.18	0.19	16.47	0.12
Acid-precipitated protein (partially purified)[c]	16.46	..	16.90	..
Cold-insoluble fraction[d]	17.24	0.17	17.66	0.16
Supernatant of cold-insoluble fraction[d]	16.28	0.62	16.52	0.62
Purified 11S fraction[e]	18.08	..	17.88	..
Glycinin[d]	16.52	0.10	17.09	0.05

[a] All percentages, dry basis. [b] Prepared from Adams soybeans, 1960 crop. [c] Prepared from Clark soybeans, 1958 crop. [d] Prepared from Clark soybeans, 1957 crop. [e] Prepared from Hawkeye soybeans, 1957 crop.

precipitated protein (Table III) approximate the chloroform solubles in Table II. All of the protein preparations are contaminated with the lipide impurities. However, extent of contamination appears to decrease with purification as noted for the 11S fraction which is precipitated three times with ammonium sulfate in its isolation.

Nitrogen and Phosphorus Contents of Various Protein Preparations. The effects of alcohol washing on the nitrogen and phosphorus content of the purified protein preparations used in this study are shown in Table IV. The nitrogen content of the proteins increased when the proteins were washed with 86% ethyl alcohol (25 ml. per gram of protein) presumably because of removal of phospholipide impurities. This, plus the high nitrogen content of the 11S component (Table IV) and its tendency to be concentrated during the preparation of glycinin (*19*), probably explains the long-standing discrepancy between the high nitrogen contents (17.14 to 17.72%) of glycinin reported by Osborne and Campbell (*20*) and the lower values obtained in more recent studies (*22, 29*). Osborne and Campbell used ethyl alcohol and ether to dry glycinin and undoubtedly removed a part of the phospholipide impurity in this manner. The nitrogen content of glycinin listed in Tables III and IV approaches the lower range of Osborne and Campbell's values. The glycinin was prepared by exhaustive dialysis and adjustment to the isoelectric point, thus no fractionation of the globulin mixture occurred and the ultracentrifugal composition (Table III) is similar to that of the acid-precipitated protein.

Nature of the Alcohol-Soluble Material. An 86% ethyl alcohol wash of crude, acid-precipitated soybean protein was concentrated to dryness and extracted with chloroform. Concentration of the chloroform extract yielded an oily, gummy material, reddish brown in color and having a rancid odor. It had a nitrogen to phosphorus ratio of approximately 1:1, and infrared analyses inchloroform or carbon disulfide indicated that the material was phospholipide. The isolated material absorbs iodine, is saponified with alcoholic potassium hydroxide, and gives a positive test for esters with hydroxamic acid. It gives a positive test for choline with the Dragendorff reagent and phosphomolybic acid, and also gives positive Molisch and orcinol tests for carbohydrate.

Nielsen (*17*) states that in the extraction of soybeans with hexane, only about half of the phosphatides present are soluble. The difficultly extractable phosphatides remain in the hexane-extracted meal and are soluble only in mixtures of polar and nonpolar solvents. Evidence presented here indicates that some of the phosphatides occur in the acid-precipitated protein.

The possibility that soybean protein contains a lipoprotein has been reported by Sagastume *et al.* (*21*). However, Sagastume's lipide-containing protein was isolated from full-fat meal, and it remains to be demonstrated that this complex exists in vivo. With wheat flour, mere wetting with water results in the formation of lipide-protein complexes (*16*), indicating the need for caution in postulating the existence of lipoproteins in plant materials.

Acknowledgment

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FOOD STORAGE EFFECTS

The Effect of Storage on the Total Lipides and the Fatty Acid Composition of Potatoes

NELL I. MONDY

New York State College of Home Economics, Graduate School of Nutrition, Ithaca, N. Y.

L. R. MATTICK and EMILY OWENS[1]

New York State Agricultural Experiment Station, Geneva, N. Y.

The total crude lipides and free fatty acid compositions of two varieties of potatoes were studied to determine changes during storage at 40° F. In both varieties, the total amount of lipide extracted did not change significantly with storage, but the fatty acid composition of each variety was altered. During storage, Pontiac potatoes showed a marked decrease in linoleic acid and an increase in palmitic acid, whereas Ontario potatoes showed a decrease in both palmitic and linolenic acids. Fatty acids containing more than 18 carbons were present in both varieties in significant quantity, and these increased in quantity during storage.

DURING POTATO STORAGE, many changes occur in the chemical composition of the potato. It is likely that fatty acids are among the compounds undergoing change. Relatively little information is available on the nature of the fatty acids of potatoes and the changes these undergo during storage.

Although the amount of lipide is small, approximately 0.10% on the fresh weight basis as found by Lampitt (*8*), its importance cannot be judged solely by its quantity.

Hendel (*5*) found that a portion of the potato lipide was bound to other materials. He obtained 0.15% on the dry weight basis with ether extraction, and an additional 0.20% with ethanol extraction. Highlands *et al.* (*6*) extracted approximately 0.17% fat on the dry weight basis from air-dried and from vacuum-dried potatoes using petroleum ether. The fatty acids in this material consisted of about 40% linoleic, 30% linolenic, 5% oleic, and 25% saturated acids. From these observations, one would expect potato fat to be relatively susceptible to oxidative deterioration, a fact confirmed by experience with potato flour (*1, 5*) and potato granules (*2*).

In this study, determinations were made for total lipides and fatty acid compositions of two varieties of potatoes stored for 2 weeks and 16 weeks.

Method

Two varieties of potatoes, Pontiac and Ontario, grown near Ithaca, N. Y., were used. The fatty acid composition of the lipides in these potatoes was determined after 2 weeks and again after 16 weeks of storage at 40° F. Approximately 8 pounds of each variety of potato were sampled at each storage period.

Potato slices including the peel were frozen, lyophilized in a Stokes freeze-dryer, and ground in a Wiley mill through a 40-mesh screen. The method of Lee and Mattick (*9*) for the extraction of lipides in peas was adapted for use with potatoes.

A 40-gram sample of dehydrated potatoes was combined with a solvent consisting of chloroform and methanol (2:1) and stirred for 3 hours under nitrogen with a magnetic stirrer. The solution was then filtered through sintered glass and the residue taken up in fresh solvent and stirred again for $1\frac{1}{2}$ hours and filtered. The filtrates from the two extractions were then combined. For the first extraction, 10 ml. of solvent per gram of sample was used and for the second, 5 ml. per gram.

The filtrate was freed of water-soluble impurities by a modification of the method of Folch, Lees, and Stanley (*3*). A solution of 0.034% magnesium chloride was added to the filtrate so that the ratio of chloroform, methanol, and magnesium chloride was 8:4:3. The solutions were shaken together and stored under nitrogen overnight in a freezer. The following day, the upper phase was drawn off by suction, discarded, and the interface washed twice with small amounts of "pure solvents' upper phase." This was obtained by shaking together chloroform, methanol, and magnesium chloride in the proportions 8:4:3 and collecting the upper phase.

The solvent was removed in a rotary evaporator, and the samples were stored under vacuum over phosphorus pentoxide until they reached constant weight.

The lipides were hydrolyzed using concentrated hydrochloric acid and the

[1] Present address: Food Quality Laboratory, Human Nutrition Research Division, U. S. Department of Agriculture, Beltsville, Md.