Lipids Extracted from Soy Products by Different Procedures

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ABSTRACT

Lipids in soybean defatted meal, concentrate and isolate were extracted by four procedures: (a) a Soxhlet extract by chloroform-methanol; (b) a Soxhlet extraction by benzene/ethanol; (c) a short extraction by chloroform/methanol; and (d) a short extraction by hexane/ethanol. Procedure 2 extracted more lipid than Procedure 1 from the isolate and meal. Both Soxhlet procedures extracted more lipid than Procedure 3 from the meal only, and more lipid than Procedure 4 from all products. Percent lipid on dry matter basis ranged for the meal, 1.56 to 4.52; concentrate 0.90 to 1.44; and isolate, 0.28 to 0.96. Lipids extracted from each product by Procedures 3 and 4 were fractionated quantitatively into (a) neutral lipids, (b) polar lipids except lecithin, and (c) lecithin. Fatty acid (C12-C20) composition of each lipid fraction was determined, and attempts were made to identify lipids. The larger amount of lipid in any product extracted by either Procedure 3 or 4 was in Fraction 1. Linoleic acid was the most abundant acid found in any lipid fraction. Significantly more oleic acid was found in Fractions I and II Concentrate lipids than in the same fractions of meal or isolate lipids. A number of lipids were found in Fractions I and II, but the only lipid in Fraction III was lecithin.

INTRODUCTION

Many solvent systems have been tried in the extraction of total lipids from different tissues. The most successfully and extensively used solvent system is chloroform/methanol, 2:1, v/v (1). Privett et al. (2) recently extracted lipids from whole soybeans with chloroform/methanol, 2:1, v/v. Zhukov and Verschagin (3) reported a procedure for quantitative extraction of total polar lipids from soybeans in which full fat soy flour was first extracted by chloroform/methanol/water, 3:2:0.17, v/v/v, and then by chloroform/methanol/nitric acid, 2:1:0.01, v/v/v. The extraction and/or removal of nonlipid contaminants from the extracts make the above methods too time-consuming to be used for routine lipid analysis.

Honig et al. (4) extracted lipids from soybean flakes by stirring the flakes 6 hr in a 79:21, v/v, hexane/absolute ethanol azetrope. Maga and Johnson (5) modified this method by blending the sample with the azetrope 5 min in a Waring blender. This method (5) is very rapid, but it is doubtful the lipid extraction is complete, and nonlipid contaminants are probably present in the extract.

Bligh and Dyer (6) reported a lipid extraction procedure in which codfish tissue was blended with chloroform/methanol/water, 1:2:0.8, v/v/v, a monophasic system, and then diluted with chloroform and/or water to a biphasic system. The advantages of this method were that it was quantitative, the lipid components were concentrated in the chloroform layer, and the nonlipid contaminants were concentrated in the methanol/water layer. Palmer (7) pointed out disadvantages of this method by showing that the acidic phospholipids (phosphatidyl serine, phosphatidyl inositol and phosphatidic acid) were absorbed on proteins when the homogenate was diluted to a biphasic system. He showed that low concentrations of divalent ions (100 μ equivalents) suppressed the readsorption of phosphatidyl serine and phosphatidic acid, but that higher concentrations of divalent ions (500 μ equivalents) were required to suppress readsorption of phosphatidyl inositol. Palmer recommended that the proteins be removed from the homogenate before it was diluted to a biphasic system.

Ostrander and Dugan (8), who modified the procedure of Bligh and Dyer (6), helped correct the problems pointed out by Palmer (7). Their method (8) adds zinc acetate to the monophasic homogenate to aid in precipitating the proteins, and the residue is separated from a monophasic homogenate and reextracted with chloroform. The final filtrate is a biphasic system with the advantages of the original Bligh and Dyer method (6). The addition of zinc acetate should also help suppress readsorption of acidic phospholipids.

The methods of Folch et al. (9), Maga and Johnson (5), and Ostrander and Dugan (8) were examined for rapid lipid extraction from defatted soy meal and soy isolate. In preliminary investigation, addition of zinc acetate did help precipitate the soy proteins; the chloroform/methanol/water, 1:1:0.8, v/v/v, ratio in the monophasic homogenate of the Ostrander and Dugan method (8) could be altered to 1:1:0.5 without loss of lipid quantity extracted; and the methanol/water layer contained no lipid components. Also, it was found that the hexane/absolute ethanol extract of soy protein products (5) was biphasic. In this extract, the quantity of the lower phase (ethanol/water) varied greatly with different soy products. It is possible, however, this procedure could be adapted to a method in which the lipid is concentrated in the hexane layer, and the ethanol/water layer could be discarded.

This paper compares the quantity and type of lipids found in the hexane layer of the Maga and Johnson procedure (5) with the quantity and type of lipids found in the chloroform layer of the modified Ostrander and Dugan (8) procedure. It also compares the quantity of lipid with two more stringent lipid extraction procedures.

MATERIALS AND METHODS

Defatted soy meal (Cherokee Mills, Rome, GA), soy concentrate (Promosoy 100) and soy isolate (Promine D), both from Central Soya, Chicago, were extracted by four different lipid extraction procedures. Procedure 1 was a 24 hr Soxhlet extraction with chloroform/methanol, 2:1, v/v. Folch et al. (1) used this method to measure completeness of lipid extraction from brain tissues. Procedure 2 was an 18 hr Soxhlet extraction with benzene/ethanol, 80:20, v/v (10), a solvent used by Central Soya for quantitative determination of lipids in soy products.

Procedure 3 was a modified procedure of Ostrander and Dugan (8). This method was as follows.

1. Blend 50 g defatted soy meal, concentrate or isolate with 130 ml methanol for 5 min on a Waring blender at medium speed.
2. Add 65 ml chloroform; reblend 5 min.
3. Add 65 ml chloroform; reblend 20 sec.
4. Add 65 ml distilled water containing 1.5 g zinc acetate and reblend 10 sec.
5. Filter through Whatman No. 1 filter paper in a Buchner funnel with suction. Maintain lipid extract under an atmosphere of nitrogen at all times (this homogenate had a chloroform/methanol/water, 1:1:0.5, v/v/v ratio).

TABLE I

Lipid Extractable from Soy Protein Samples by Different Procedures

Soy sample	Procedure[a] 1	2	3	4
	% Lipid, dry matter basis[b]			
Defatted meal	4.04[c]	4.55[d]	2.88[e]	1.56[f]
Concentrate	1.38[c]	1.44[c]	1.25[c]	0.90[d]
Isolate	0.60[c]	0.96[d]	0.87[d]	0.29[e]

[a]1 = chloroform/methanol Soxhlet extraction; 2 = benzene/ethanol Soxhlet extraction; 3 = modified chloroform/methanol method (8); and 4 = modified hexane-ethanol method (5).
[b]Each value is a mean of 8 determinations.
[c,d,e,f]Means in a row bearing like superscript letters are not significantly different at the $P < .05$ level.

6. Reblend filter paper, residue and facial tissue (used for wiping funnel after first filtration) with 100 ml chloroform for 2½ min.

7. Filter as in Step 5; rinse blender jar contents into funnel with 75 ml chloroform.

8. Transfer filtrate to 500 ml graduate cylinder with 25 ml methanol for rinsing.

9. Let stand in cold (6 C) under nitrogen until sharp interface appears between methanol/water and chloroform layers.

10. Record volume of chloroform layer; find weight of solids in 10 ml portion of the chloroform layer and calculate lipid content. Percentage lipid in the sample is equal to (ml $CHCl_3$ layer) (g solid/10 ml) (100)/50 g sample

Procedure 4 was the procedure of Maga and Johnson (5). The extract, however, was put into 500 ml graduated cylinder and kept at -18 C until a sharp interface between the two phases appeared. The volume of the hexane layer was recorded and the ethanol/water layer was discarded. The quantity of lipid in the hexane layer was determined as described for lipid in the chloroform layer of Procedure 3.

Eight samples from homogenous lots of defatted soy meal, soy concentrate and isolate were extracted by Procedures 1, 2, 3 and 4. The quantity of material extracted was determined for each sample.

Lipid quantitites extracted by Procedures 1, 2, 3, and 4 from each soy product were analyzed by analysis of variance, and the means which were significantly different were separated by the Student-Newman-Keul's test (14).

Two samples from the same homogeneous lots of defatted soy meal, soy concentrate and isolate were extracted by Procedures 3 and 4 and stored in chloroform and in hexane, respectively, under nitrogen at -18 C for further analyses. Each lipid sample was dried on a flash evaporator at 35 C and redissolved in chloroform/methanol, 20:1, v/v. The lipid sample was separated into three lipid fractions by elution from silicic acid column by (I) chloroform/methanol, 20:1, v/v, (II) chloroform/methanol, 1:1, v/v and (III) methanol (II). The percent of lipid separated in to each fraction and the percent recovery of lipid after silicic acid column fractionation were determined.

The lipids in the three fractions were further investigated by thin layer chromatography (TLC) on 250 μ Absorbosil 5 thin layer plates (Prekotes, Applied Science Lab, State College, PA) and solvent systems: chloroform/methanol/water, 65:25:4, v/v/v and ethyl ether/petroleum ether/acetic acid, 70:20:4, v/v/v. Identification of specific lipids in each fraction was made by comparison of R_f values with R_f values of appropriate standards and by the following detection tests (12): antimony trichloride test for steroids; molybdenum blue test for phospholipids; diphenylamine test for glycolipids, gangliosides and sulfatides, ninhydrin test for free amino groups (phosphatidyl serine, phosphatidyl ethanolamine and their lyso derivatives); and Dragendorff test for choline-containing phospholipids.

TABLE II

Lipid Fractions Extracted from Soybean Protein Products

Product	Extraction procedure[b]	Lipid fraction[a] I	II	III
		Percent of lipid[c]		
Defatted meal	3	50.99	27.44	21.07
		2.18	2.33	0.14
	4	66.83	21.79	11.28
		1.58	1.20	2.79
Concentrate	3	79.87	12.87	7.26
		2.51	2.80	0.32
	4	67.94	23.53	8.53
		0.49	0.37	0.12
Isolate	3	47.38	30.48	22.13
		5.70	6.52	3.56

[a]Fraction I = neutral lipids, Fraction II = polar lipids except lecithin, and Fraction III = lecithin.
[b]3 = modified chloroform/methanol extraction (8) and 4 = modified hexane/ethanol extraction (5).
[c]Values are the mean of two observations with the mean written above the standard deviation.

The relative percentages fatty acids (C_{12}-C_{20}) of lipids in Fractions I, II and III of each lipid sample extracted by Procedures 3 and 4 were determined by GLC analysis of methyl esters (13) on a 1.83m x 0.64 cm o.d. stainless steel column packed with 15% DEGS on 60-80 mesh Chromosorb W, acid washed (Applied Science, State College, PA). The analysis was done by a Bendix Model 2500 gas chromatograph equipped with dual ionization detectors and a recorder with a disc integrator.

Each fatty acid was analyzed in a split plot design with replication. A 2 x 3 factorial arrangement of two factors (procedure and product) was the whole plots and the split plots were the silicic acid column lipid fractions. Two replications were completed. Means which were significantly different were separated by the Student-Newman-Keul's test (14).

RESULTS AND DISCUSSION

The quantity of material extracted from defatted soy meal, concentrate and isolate by Procedures 1, 2, 3 and 4 are shown in Table I. For defatted soy meal, significant differences ($P<0.05$) in amounts of material extracted were found among all four procedures. The greatest amount of material was extracted by Procedure 2 and the least amount by Procedure 4. Procedures 1, 2 and 3 extracted more ($P<0.05$) material from soy concentrate than Procedure 4. Procedure 3 extracted more lipid from the meal and isolate than Procedure 4. For some unknown reason, Procedure 3 also extracted more ($P<0.05$) material from the isolate than Procedure 1. These results show that the modified Ostrander and Dugan procedure (8) extracts as much lipid from the concentrate and isolate as the more stringent extraction Procedures 1 and 2. The modified hexane/ethanol azeotrope method, however, did not remove as much lipid as any of the other methods. The fact that more lipid-containing material was removed by the boiling solvents from the soy meal was probably because of nonlipid components (sugars, amino acids, etc.) being extracted by Procedures 1 and 2. These nonlipid components are not present in the concentrate or the isolate to any great extent.

The mean percentages and standard deviation of the lipid samples which were eluted into Fraction I, II, or III from the silicic acid column are given in Table II. Recovery of lipids after fractionation by the column averaged 97.7±

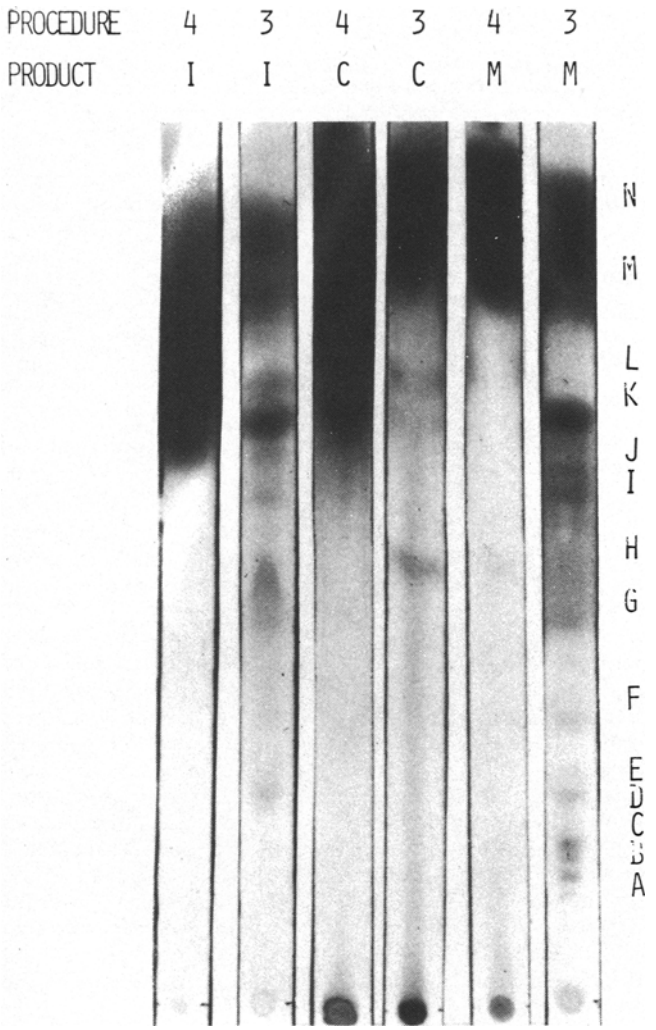

FIG. 1. Fraction I lipids extracted from soy products by chloroform-methanol (Procedure 3) and by hexane-ethanol (Procedure 4). Product I = isolate, C = concentrate and M = defatted meal. A through N are lipids.

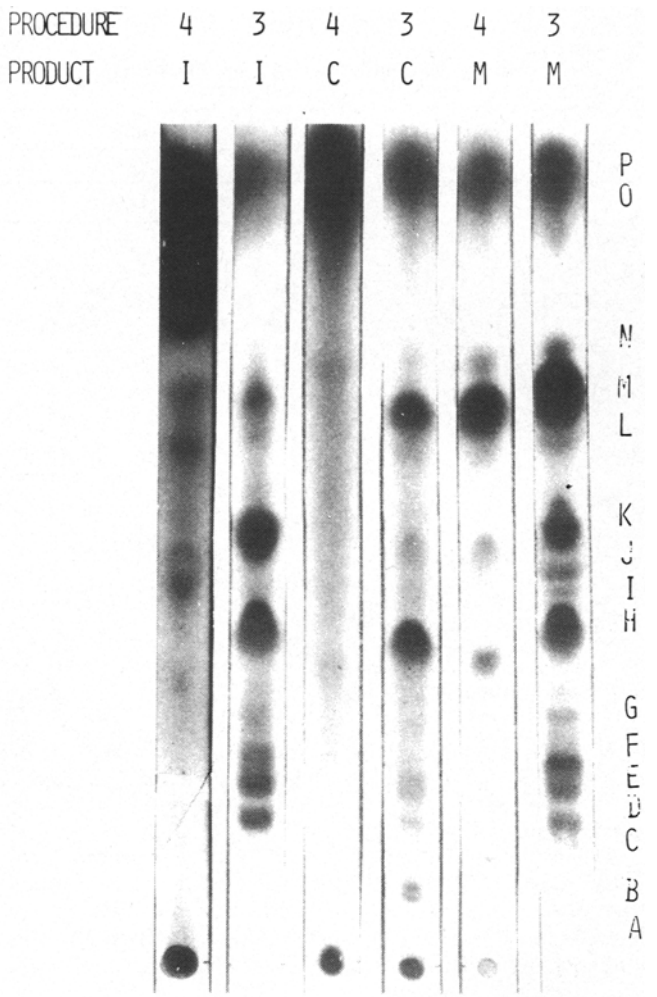

FIG. 2. Fraction II lipids extracted from soy products by chloroform-methanol (Procedure 3) and by hexane-ethanol (Procedure 4). Product I = isolate, C = concentrate and M = defatted meal. A through P are lipids.

8.0% across both extraction procedures for soy meal and concentrate and Procedure 3 for the isolate. The recovery of lipid extracted by hexane/ethanol (Procedure 4) from the isolate averaged 149%, and these data are not included in Table II. Little lipid was extracted from the isolate by this procedure (Table I), and the homogeneous supply of isolate was depleted before obtaining a silicic acid column lipid fractionation with acceptable recovery. These lipid fractions, however, were used for qualitative lipid analysis and fatty acid analysis.

The larger amount of lipids extracted by any procedure from soy protein products was found in Fraction I, the neutral lipids (Table II). The least amount of lipid was the pure lecithin in Fraction III.

Thin layer chromatograms of lipids extracted from the soy products by Procedures 3 and 4 and separated into three fractions by silicic acid column chromatography are shown in Figs. 1, 2 and 3. Figure 1 (Fraction I lipids) shows Procedure 4 removed fewer lipids from each soy product than Procedure 3. The lipids extracted from soy products by Procedure 3 (chloroform/methanol; A, B, C, D, E, F, G, H, I, J, K and L) but not by Procedure 4 (hexane/ethanol) had the following reactions to the detection tests: F, G, I and J of the meal, H of the concentrate, and G and L of the isolate were positive for phosphorous-containing lipids; A,

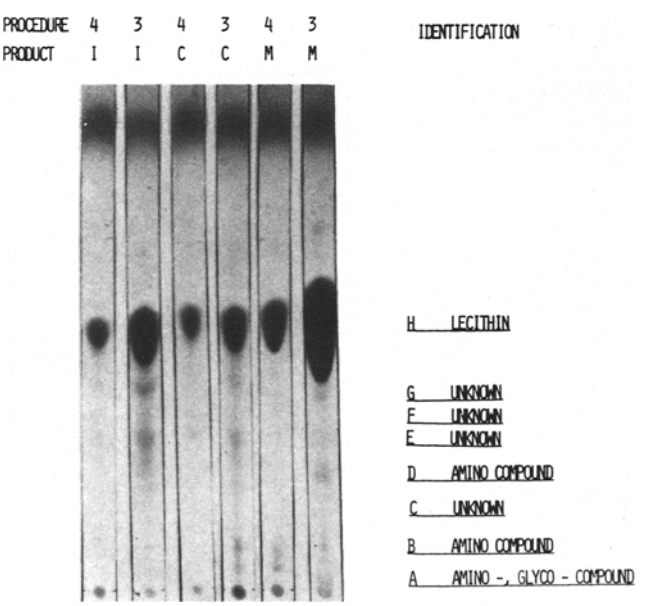

FIG. 3. Fraction III lipids extracted from soy products by chloroform-methanol (Procedure 3) and by hexane-ethanol (Procedure 4). Product I = isolate, C = concentrate and M = defatted meal.

TABLE III

Fatty Acid Composition of Lipid Fractions in Soybean Protein Products

Fatty acid	Soy product[a]	Lipid fraction[b] I	II	III
		Relative percent[c]		
C16:0	M	14.73	21.69	16.96
	C	14.28	21.82	21.36
	I	28.78	27.74	23.29
C18:0	M	3.43	3.53	4.09
	C	4.40	4.96	5.91
	I	12.48	6.25	4.82
C18:1	M	16.38[d]	10.40[d]	8.89[d]
	C	25.91[e]	18.00[e]	15.26[d]
	I	12.86[d]	11.58[d]	12.53[d]
C18:2	M	57.56	57.64	63.84
	C	47.97	49.13	48.02
	I	42.56	50.00	56.61
C18:3	M	7.90	6.41	6.21
	C	7.41	6.07	9.44
	I	3.31	4.40	2.73

[a]M = Defatted meal; C = Concentrate; I = Isolate.
[b]Fraction I = neutral lipids, Fraction II = polar lipids with exception of lecithin, and Fraction III = lecithin.
[c]Each value is the mean of four determinations.
[de]Values in a column for C18:1 bearing unlike superscript letters are significantly different at the $P<0.05$ level.

B, D, E, G, I, J and K of the meal and E, I, J and K of the isolate reacted positively to the diphenylamine test; and F and I of the meal and L of the isolate reacted positively to the antimony chloride test.

Lipids designated as M and N of all soy products for both procedures reacted positively with the antimony trichloride test, and lipid M in the isolate and meal extracted by Procedure 3 also reacted positively with diphenylamine. These results indicate that Fraction I lipids extracted by the chloroform-methanol in contrast to Fraction I lipids extracted by the hexane/ethanol are composed of a number of glycolipids including a sterol glycoside (K, in meal and isolate, Fig. 1), plus other unidentified lipids. Both procedures removed steroids and mono-, di- and triglycerides which are present in M and N in Fig. 1. Development of the TLC plate with ethyl ether/petroleum ether/acetic acid, 70:20:4, v/v/v showed the presence of the glycerides.

Lipids in Fraction II (Fig. 2) extracted from the soy products by chloroform-methanol are more numerous than lipids extracted by Procedure 4. Positive identification was made for lipid M (phosphatidyl ethanolamine), and tentative identification for lipids K (phosphatidyl inositol), H (phosphatidic acid) and N (sterol glycoside). The major differences in types of lipids extracted by the two procedures were the lipids C through G, I and J which reacted positively to the diphenylamine test and C, D, and E which were positive to the antimony trichloride test. Spots A and B were also positive for the diphenylamine test.

Incomlete separation of the glycolipids and sterol glycolipids were obtained by the fractionation of lipids as described. However, the fraction eluted with absolute methanol contained primarily lecithin (Fig. 3) and some contaminants which were probably sugars and amino acids. The contaminants were not present in the hexane extract to the same extent that they were in the chloroform (Procedure 3, Fig. 3). For researchers interested in investigation of this one lipid in soy products, this elution pattern from silicic acid columns should be of interest.

The relative percentages of fatty acids in lipids extracted from all soy products and fractionated by column chromatography are shown in Table III. The most abundant fatty acid in any fraction of the lipid extracted from any soy

TABLE IV

Palmitic and Oleic Acids in Lipid Fractions of Soy Protein Products Extracted by Different Procedures

Fatty acid	Procedure[b]	Lipid fraction[a] I	II	III
		Relative percent[c]		
C16:0	3	20.79	29.33	21.10
	4	17.73	18.17	19.96
C18:1	3	15.02[d]	7.58[d]	8.91[d]
	4	21.75[e]	19.08[e]	15.54[e]

[a]Fraction I = neutral lipids, Fraction II = polar lipids except lecithin and Fraction III = lecithin.
[b]3 = modified chloroform/methanol method (8) and 4 = modified hexane/ethanol method (5).
[c]Each value is the mean of 6 determinations.
[de]Values in a column for C18:1 bearing unlike superscript letters are significantly different at the $P<0.05$ level.

product was linoleic (C18:2, Table III). The next most abundant fatty acid palmitic (C16:0) and the least abundant fatty acid was either stearic (C18:0) or linolenic acid (C18:3).

Significant differences ($P<0.05$) among products were found for palmitic, oleic, linoleic, and linolenic acids when the percentages of each fatty acid were averaged across lipid fractions and extraction procedures. Significant differences ($P<0.05$) among lipid fractions were found for palmitic, oleic and linolenic acids when the percentage of each acid was averaged across soy product and extraction procedures. However, a significant ($P<0.05$) product X lipid fraction interaction also was found for each fatty acid. This made it necessary to look at each fatty acid on a product-lipid fraction subgroup basis (Table III). The only significant difference found on this subgroup basis was for oleic acid (C18:1, Table III). The concentrate lipids in Fractions I and II had significantly greater quantities of oleic acid than the meal or isolate lipids in the same fractions.

For each product the other fatty acids varied in different ways among the lipid fractions (Table III). In lipid Fraction I, the isolate had almost two times the quantity of C16:0 than in either the concentrate or defatted meal. The isolate in lipid Fraction I also had the greatest quantity of C18:0 and the least amount of C18:3 of the three products. The meal Fraction I lipids contained the largest quantity of C18:2 and the least amount of C18:0 of the three products. For any one product a larger quantity of lipids was found in Fraction I (Table II). These observations tend to confirm the report (5) that refining soybean meal to an isolate removes unsaturated fatty acids.

Extraction procedure had a significant effect on C16:0 and C18:1 content in lipids when percentages of these fatty acids were averaged across soy product and lipid fraction. However, a significant ($P<0.05$) extraction procedure X lipid fraction interaction also was found for these same acids. This made it necessary to look at these fatty acids on a procedure-lipid fraction subgroup basis as shown in Table IV. There was significantly more ($P<0.05$) C18:1 in each lipid fraction extracted by Procedure 4 than in the same lipid fraction extracted by Procedure 3. The difference in C18:1 content between the two procedures was greater in Fraction II than in Fractions I and III. No significant differences in palmitic acid content between the two procedures were found (Table IV). Procedure 3 tended to extract lipids containing more palmitic acid than Procedure 4; the difference in palmitic acid content between the two procedures was greater in Fraction II than in Fraction I or III.

A limitation of this paper is the comparison with the modified procedure of Maga and Johnson (5). A better

method for comparison probably would have been the method of Honig et al. (4) as it was reported or one of the procedures reported for extraction of lipid from soybeans (2, 3). The modified method of Ostrander and Dugan (8) as reported in this paper has been shown to extract as much lipid from soy concentrate and isolate as much more stringent extraction procedures. From present data it is impossible to state whether this method removes all of the acidic phospholipids, especially phosphatidyl inositol. More research is needed to answer this question.

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