Quantitative Content of Total Polar Lipids in Soybean Seeds

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ABSTRACT

The exhaustive stepwise extraction of soybean seeds was performed in a specially designed apparatus by a solvent system containing chloroform, methanol, water, mineral acid, and antioxidant in an inert atmosphere. The yield of total lipids was as high as 99.95%. The possible artifact content in the extracted lipids was < 0.5%. Separation of polar lipids and determination of constituent groups of these compounds (fatty acyl groups and phosphate) has shown that 36.7 μmole (±4.6%) of polar lipids may be contained in 1 g dry wt of seeds, 25.9 μmole (±1%) of this amount being phospholipids.

INTRODUCTION

According to current views, lipoprotein membrane of a plant cell is a set of structural units (unit membrane), consisting of individual proteins and individual polar lipids at a definite molar ratio (1). Within the framework of our studies the term "polar lipids" refers to substances containing higher fatty acyls linked to the polar radicals of the molecule by ester bonds, and being more polar than triglycerides under given conditions of adsorption chromatography. For this reason, a quantitative study of polar lipids may serve as an approach to elucidation of the structure of biological membranes.

To now, studies of this kind were confined to determination of percent composition of individual polar lipid classes (2,3). Yet, for understanding of membrane structure, it is essential to obtain reliable data on total molar content of these substances in plant tissues. Investigation of lipid extractability also is revelant to the understanding of lipid-protein molecular interactions in the plant cell membranes (4).

Testing of the currently known lipid extraction procedures (5-9) proved them to be unsuitable for plant tissue such as soybean seeds. Some tests did not yield quantitative recovery of lipids, while others led to considerable breakdown of covalent bonds of lipids during extraction. Methods of quantitative determination of total polar lipids in crude lipid extracts were not available until quite recently (2,10,11).

The purpose of the present communication is to estimate the quantitative content of total polar lipids, and particularly phospholipids, in soybean seeds. The investigation is based on the determination of most characteristic polar lipid functional groups, namely ester and phosphate (12,13). The results obtained show that quantitative extraction of polar and nonpolar lipid in the native state is quite feasible. Fractionation of extract components and subsequent determination of functional groups makes it possible to estimate total polar lipid content of soybean seeds, which contain, along with phospholipids, polar lipids devoid of phosphorus.

EXPERIMENTAL PROCEDURES

Reagents and materials. Aluminium oxide was treated with H_2SO_4, ethyl acetate, and water. As a result, the oxide assumed the activity grade V and an acid surface. The purity of heptadecenoic acid methyl ester preparation was 98.6% (13). Quartz sand (fraction 0.6-0.8 mm) and kieselguhr (0.12-0.16 mm) were washed with acid and silanized (13). Soybean seeds were ground for 2 min on an electrical mill immediately before extraction. Flour sample (particle size ⩽ 0.25 mm) of 2 g air dry wt was taken.

Lipid extraction. Plant material was extracted in the apparatus shown in Figure 1. Operation of the apparatus was described previously (14). For the extraction, settling down of the suspension, and filtration of the supernatant liquid, the 3-way stopcock was placed in positions I, II, and III, respectively. The extraction was performed at room temperature, in an argon atmosphere and in the presence of antioxidant butylated hydroxytoluene (BHT). The flour first was treated 3 times with a mixture of $CHCl_3$:CH_3OH:water (3:2:0.17, v/v; 40, 3, and 3 min) and then 6 times with a mixture of $CHCl_3$:CH_3OH:HNO_3

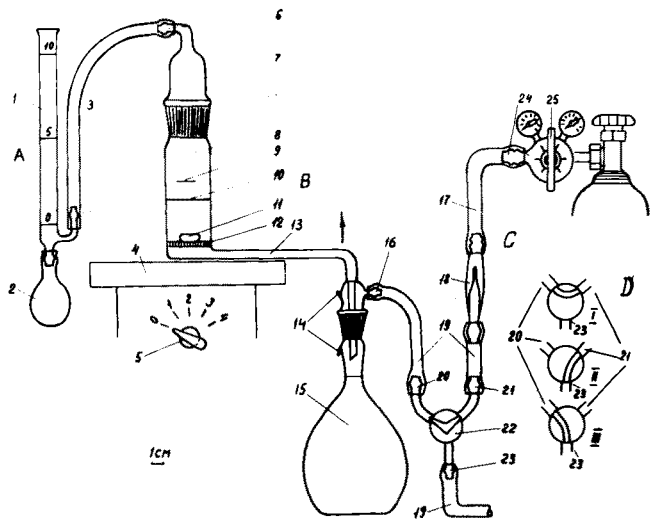

FIG. 1. Apparatus for lipid extraction. (A) gas flowmeter; (B) extractor with a head and receiving flask; (C) tube array with an inert gas source; (D) various positions of 3-way stopcock 22; (1) burette of the flowmeter with 0, 5, and 10 ml marks; (2) rubber bulb for soap solution; (3) rubber tubing of the flowmeter; (4) plate of a magnetic stirrer; (5) stirrer speed switch; (6) outlet tube of the extractor head; (7) head of the extractor, standard joint No. 29; (8) extractor chamber, standard joint No. 29; (9) lower level of the funnel tube when soybean flour sample is introduced; (10) initial level of solvent I in the extractor, 13 ml; (11) magnetic bar in a glass ampule; (12) glass sintered filter No. 3; (13) tube for drainage of liquid from the extractor chamber; (14) glass studs for fastening the flask by a rubber ring; (15) a 150-ml receiving measuring flask, standard joint No. 14.5; (16) extractor outlet tube for connection with tube array B; (17) rubber sleeve connection of capillary 18 with outlet tube of the cylinder gauge; (18) capillary throttling gas flow [14]; (19) vacuum rubber sleeves; (20) outlet tube of 3-way stopcock 22 for connection with the extractor; (21) outlet tube of the 3-way stopcock for connection with the capillary outlet tube; (22) 3-way stopcock; (23) outlet tube of the 3-way stopcock for connection with a water jet pump, not shown at the figure; (24) outlet tube of the cylinder gauge; (25) gauge grip. The arrow shows direction of the extractor tilting.

TABLE I

Lipid Content in Plant Material After Extraction

Experiment Number	Extraction conditions	M_1 (μmoles)[a] $\bar{x}$	S_{rel}(%)[c]	n
1	Standard conditions[b]	0.3	15.6	5
2	Standard conditions without BHT[c]	0.2	14.2	6
3	Standard conditions with methanol replaced by isopropanol	0.6		1
4	$CHCl_3$-CH_3OH(3:2) 3x(20 ml, 60, 30, and 30 min) and 5x(8 ml, 3 min)	5.4		1
5	Water saturated n-butanol-1 (20 ml, 60 min); $CHCl_3$-CH_3OH(3:2) 5x (5 ml,3 min)	17.1		2
6	Acetone (20 ml, 45 min) and 3x(10ml, 3 min); water saturated n-butanol-1 (20 ml, 60 min); $CHCl_3$-CH_3OH(3:2) 3x (8 ml, 3 min)	0.7		2

[a] M_1 = number of μmoles of polar lipids in plant material upon extraction of 2 g air-dry wt of soybean flour. $\bar{x}$ = arithmetic mean of separate determinations of M_1. S_{rel} = relative standard deviation (12); n = number of determinations.

[b] See Section on *Lipid extraction.*

[c] BHT = butylated hydroxytoluene.

(2:1:0.01 , v/v). After extraction, HNO_3 was neutralized by an excess of aqueous ammonia.

For the determination of fatty acid methyl esters (FAME) content in the extract, pure chloroform, without methanol, was used occasionally instead of the above solvents.

Determination of residual lipid content in plant material after extraction. To the flour residue in the apparatus chamber p_s = 10.4 μg of heptadecenoic acid methyl ester used as an internal standard (13) was added; the mixture was saponified for an hr by 0.1% Na solution in ethanol and fatty acids were converted into FAME (13). The esters were separated by gas liquid chromatography (GLC), and areas of separate peaks of the sample (s_i) and peaks of the standard (s_s) were measured (13). Wt content of FAME ($p^1{}_o$) found by internal standard procedure from GLC data was calculated as follows:

$$p^1{}_o = 100 \times p_s \times \Sigma\, s_i/\bar{k} \times s_s, \quad \text{(I)}$$

where calibration coefficient $\bar{k}$ = 115.8% (13). Molar content of lipids left in the flour after extraction (M_1, μmole), was evaluated from the FAME wt ($p^1{}_o$) and the molecular weights of these esters (m_i) using the equation:

$$M_1 = 50 \times p_s \times s_s^{-1} \times \bar{k}^{-1} (s_1 \times m_1^{-1} + s_2 \times m_2^{-1} + \ldots s_N \times m_N^{-1}), \quad \text{(II)}$$

where N = number of peaks in the sample (14).

Determination of FAME content in the extract. Internal standard (p_s = 26.0 μg), 2 g kieselguhr, and 3 g sand were added to the freshly prepared extract, solvent was evaporated, and the mixture was transferred on the starting zone of a plate covered with a loose 1 mm Al_2O_3 layer (second activity grade). The plate was developed twice with hexane. Aluminium oxide between solvent front and clearly seen triglyceride spots was collected, extracted with hexane, and lipids recovered were subjected directly to GLC (14).

Estimation of free fatty acids (FFA) in the extract. Freshly prepared extract was evaporated, a small quantity of water was added to the residue, and the mixture was extracted by hexane. Lipid solution in hexane was extracted with a buffer solution of equal volumes of 0.025 M Na_2CO_3 and $NaHCO_3$ solutions, pH 10 (15), buffer extract was acidified with an excess of H_2SO_4 and regenerated FFA were extracted with hexane. The recovered FFA were purified by loose layer chromatography on kieselguhr, 2 cm band layer in the middle of the plate being impregnated with alkali. After separation with acetone as a solvent, this band was extracted with buffer solution, and FFA were recovered again (16). FFA were separated by paper chromatography; the paper was impregnated with a 5% benzene solution of dodecane, v/v (17).

Separation of polar lipids from triglycerides. The extract was transferred as above to the starting zone of a plate covered with aluminium oxide (activity grade V). The plate was developed overnight in a horizontal chamber (16) containing hexane:water saturated benzene (3:2) mixture, then treated 3 times in a chamber with anhydrous acetone until only the starting zone was impregnated by the solvent and finally redeveloped for 2 more hr in the hexane:benzene mixture. Then, the upper portion of the layer, the 3 cm band, containing neutral lipids was discarded, and remaining adsorbent together with polar lipids was transferred onto a glass filter (18).

For the estimation of residual triglycerides in polar lipids adsorbed on Al_2O_3, the adsorbent was washed by anhydrous acetone, and recovered lipids were separated by paper chromatography (14). Linseed oil triglycerides were used as a standard.

Quantification of polar lipids by the fatty acid content. After separation of triglycerides, the adsorbent was transferred to a glass filter and polar lipids were eluted with 5 x 12 ml $CHCl_3$:isopropanol:water (20:10:1) and 5 x 12 ml water saturated butanol. Then, firmly bound lipids were continuously extracted overnight by hot water saturated butanol. Internal standard was added to an aliquot of the obtained eluate; the lipid mixture was converted into FAME and analyzed by GLC. The content of polar lipids in the eluate (M_2,μmole) was calculated as described above. The content of polar lipids not extracted from aluminium oxide (M_3,μmole) was determined using the same procedure and equation as for M_1 (18).

Quantification of phospholipids by the phosphorus content. The extract was quantitatively transferred to the starting zone of a plate covered with silanized kieselguhr. The plate was developed overnight with a $CHCl_3$:isopropanol:aqueous NH_3 mixture (12:12:1) in a horizontal chamber, then put into a chamber with hot water saturated butanol ca. 80 C for 30 min. The band of kieselghur layer, 3 cm wide from the upper portion of the plate, was transferred on the filter, and lipids were eluted by the same solvents (18). Mineralization and quantification of lipid phosphorus was carried out as described previously (12).

Calculation of quantitative content of total polar lipids (M, μmole) and total phospholipids (M_{ph}, μmole) in the seeds was carried out using the equations

$$M = (\bar{x}_{M_I} + \bar{x}_{M_2} + \bar{x}_{M_3}) \pm (S_{rel})_M \quad \text{(III)}$$

and

$$M_{ph} = (\bar{x}_{M_I} + \bar{x}_{M_4} + \bar{x}_{M_5}) \pm (S_{rel})_{M_{ph}} \quad \text{(IV)}$$

where $\bar{x}$ and S_{rel} are arithmetic mean and relative standard deviation for 5 parallel determinations. Calculation of M and M_{ph} was based on the following assumptions: 1) all polar lipids contain 2 acyl residues; 2) all phosphorus compounds eluted from kieselguhr are phospholipids; and 3) polar lipids not extracted from the flour and kieselghur are phospholipids (18).

RESULTS

Quantitative extraction of lipids and evaluation of their native state. Table I shows the results of the quantitative determinations of lipids not extracted from plant material. After extraction under standard conditions (experiment 1) ca. 0.3 μmole polar lipids remain in the material. Assuming

that soybean seeds contain 24% lipids (18), and the residue lipids are phosphatidyl choline (mol wt ca. 780), it may be calculated that in experiment 1 99.95% of total lipids was extracted from the seeds.

The results of experiment 2 (Table I) show that absence of antioxidant BHT (1) caused a marked decrease of the remaining lipid concentration. The difference between the arithmetic means ($\bar{x}$) in experiments 1 and 2 calculated by t_q criterion = 3.86 for 9 degrees of freedom (18) is significant with the probability of $99\% < P < 99.9\%$. The presence or absence of 0.001% of neutral antioxidant in the solvents does not affect their polarity, and a lower content of the residue lipids seems to be due to their partial oxidation during the extraction without BHT. Hence, an antioxidant is absolutely required for quantitative isolation of intact lipids from plant tissues.

When anhydrous neutral chloroform:methanol (3:2) mixture, water saturated butanol, or both (7) were used after preliminary removal of water and part of lipids with acetone (experiments 4-6), lipids were not as completely extracted as by the usual procedure (experiment 1). In addition, butanol extraction frequently led to the clogging of the filter as a result of swelling of plant material.

Thus, for a sufficiently complete extraction of lipids, it is necessary to use organic extractants of high polarity; this usually is achieved by the addition of some alcohol. For lipid extraction in experiment 1, we used solvents containing methanol, a primary alcohol with the most active hydroxyl group. In addition, slight acidification was applied at the second stage of extraction.

The extraction condition described above presumably stimulated transesterification of acyl residues in the extracted lipids with methanol (19); therefore, it was necessary to quantitate FAME in the freshly prepared extract (Table II, series 1). FAME were isolated by adsorption chromatography in a loose Al_2O_3 layer. For a quantitative transfer of total lipids on the plate a special procedure was employed. Nonadsorbing particles were coated with a thin film of the extracted substances, the support was separated from the flask walls under the action of quartz sand, and the resulted mixture was put on the starting zone. It has been shown that ester concentration in the extract is 1.2-1.9 mg per 2 g of air dry wt.

Some of the FAME found in the extract could have been natural products, because some plant tissues were found to contain FAME of biosynthetic origin (20). It was shown by the estimation of ester concentration in the extracts obtained under the conditions excluding transesterification (Table II, series 2), that 2 g of seeds contain 0.1-0.4 mg of "native" methyl esters. Hence, during extraction of this sample about 1.1-1.5 mg FAME was formed as a result of lipid methanolysis.

It also might be expected that during further storage of chloroform:methanol lipid solutions, transesterification would continue. In fact, long storage of these solutions, even at lower temperature, increased initial FAME concentration 3 to 5-fold (Table II, series 3). Upon replacement of methanol by isopropanol, whose hydroxyl group is less reactive, the content of residual lipid was found to be twice as great (Table I, experiment 3).

The results of experiment 4 (Table I) suggest that upon extraction of the plant material with anhydrous neutral solvents, much lipid is retained in the meal. Therefore, for the extraction under the standard conditions (Table I), it was imperative to use solvents containing a marked amount of water. Moreover, the original air dry seeds included ca. 7% water. Because of the presence of water in the extractants, the possibility of hydrolysis of the extracted lipids yielding FFA could not be excluded.

For a semiquantitative estimation of trace amounts of FFA in the extract containing a lot of triglycerides, phospholipids and proteins, a special technique was developed. The method ensures satisfactory recovery of 0.2 mg FFA added to the extract (Figure 2, A and B). The visual comparison of chromatograms A and B suggests that FFA concentration in the extract from 1 g of seeds is ca. 0.4 mg.

TABLE II

Content of Fatty Acid Methyl Esters in Freshly Prepared and Stored Extracts of Soybean Seeds

Number of series of experiment	Conditions of extraction and storage[a]	Methyl ester content (mg/2g of air-dry wt)[b]
1	Extract recovered under standard conditions without storage	1.24 1.92 1.43
2	Solvents 1 and 2 replaced by $CHCl_3$; no storage of extract.	0.45 0.22 0.12
3	Extract recovered under standard conditions with addition of excess of ammonia; stored 48 hr at 5 C	5.11 7.08

[a] See Section on *Lipid extraction.*

[b] Results of several experiments of a given series.

Separation of polar lipids from triglycerides. It can be shown by visual comparison (Figure 3, st and 40) that under standard conditions of separation on Al_2O_3, a polar lipid sample applied on a chromatogram contained less than 40 μg triglycerides. Spot C (9) of experiment 40 (Fig. 3) as well as experiments 35 and 30 correspond to nonglyceride components, because no triglyceride with 9 double bonds (trilinolenin) has been found in soybean seeds (21).

A small amount of residual triglycerides in the polar lipid fraction has a negligible effect on the results of quantitative estimation, and on the other hand, ensures that no other more polar components would be present as an impurity in the triglyceride fraction. In fact, after the eluting power of the solvent was increased by raising benzene concentration to 45-50%, components A-C (Fig. 3) were no longer visible on liquid-liquid chromatograms of the polar lipid fraction. Therefore, it was not worthwhile to increase polarity of the solvent mixture used for preparative isolation of polar lipids. A decrease of polarity by reduction with hexane of benzene concentration from 40 to 35% brought about an increase of residual triglycerides; this increase became more pronounced with further decrease of polarity with 30% benzene (Fig. 3).

Thus, a hexane:benzene (60:40) mixture employed here provided optimal conditions for separation of neutral lipids. Under these conditions, the polar lipid preparation contained 200 μg triglycerides; one fifth of the preparation was applied on chromatogram 40. This quantity corresponds to ca. 0.7 μmole aliphatic acids in residual triglycerides, because the average mol wt of fatty acids is 290.

Quantitative content of polar lipids and phospholipids. Satisfactory separation of triglycerides from polar lipids permitted quantitative determination of the latter by the molar content of fatty acyl groups. It can be seen (Table III, column 5) that 2 g of air dry soybean seed flour contain 68.6 μmole (±4.6%) of polar lipids.

It might be expected that these lipids consist, for the most part, of phospholipids (2). A determination of phospholipids by lipid phosphorus showed that the quantity of phospholipids in the flour sample was 48.4 μmole (±1.1%) (Table III, column 8), ca. 71% of total polar lipids.

Taking into account lipid losses owing to incomplete extraction from flour (Table III, column 2), aluminium oxide (Table III, column 4), and kieselguhr (Table III, column 7), the fraction of polar lipids and phospholipids actually isolated from seeds was 99.6 and 99.4%, respectively, whereas, the fraction of corresponding lipids recovered for further purification and preparative isolation was

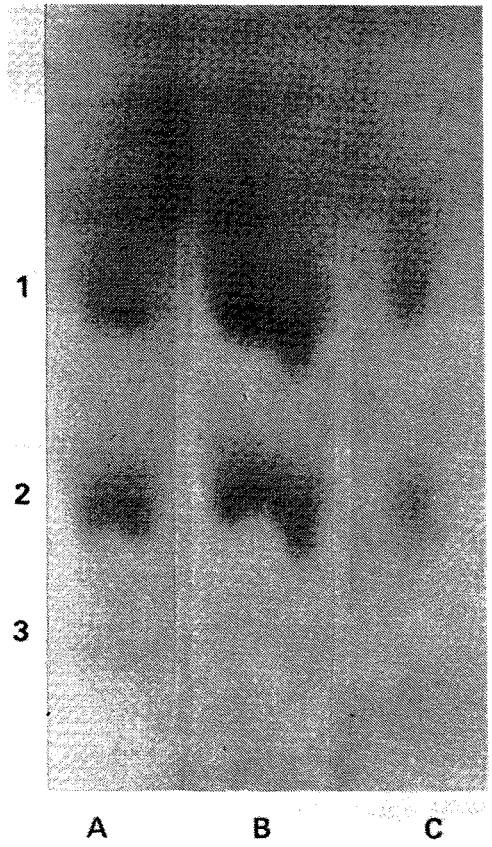

FIG. 2. Reversed phase paper chromatograms of free fatty acids (FFA). (A) FFA isolated from 1 g seed extract; (B) FFA isolated from 1 g seed extract in which 0.2 mg FFA was added prior to isolation; (C) 0.2 mg standard FFA obtained by saponification of soybean oil; (1) linoleic; (2) oleic plus palmitic; (3) stearic acid.

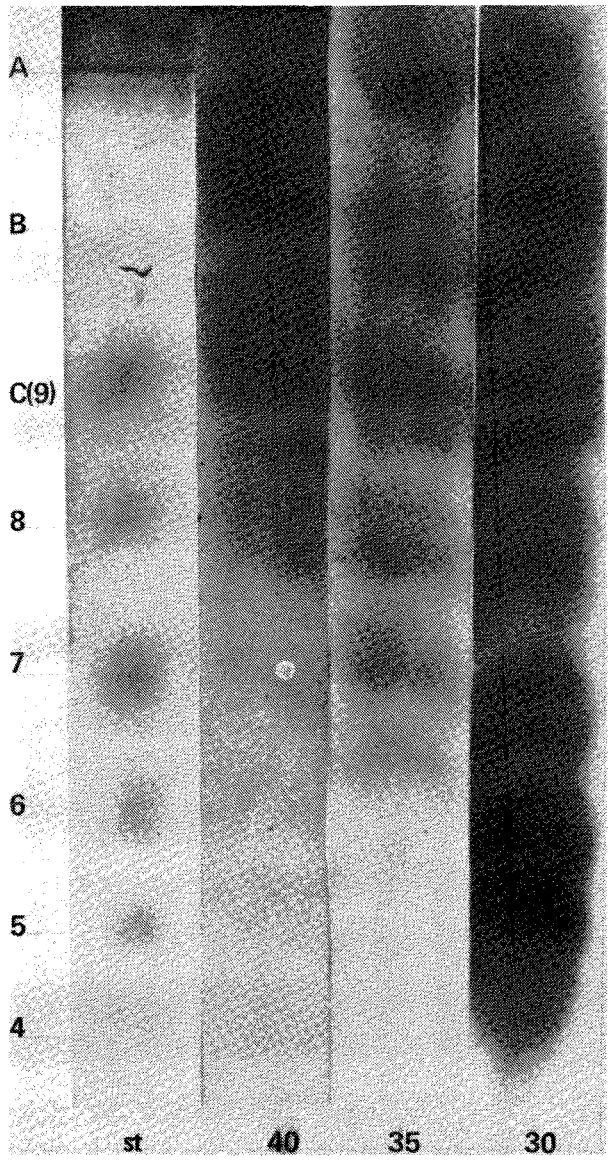

FIG. 3. Paper chromatograms of triglycerides. (st) = standard 40 μg linseed oil triglycerides; 40, 35, 30 = residual triglycerides of polar lipid fraction after separation of triglycerides with n-hexane:benzene (60:40, 65:35, and 70:30, v/v, respectively; A, B, C = nonglyceride components of polar lipid fraction on chromatograms 40, 35, 30, respectively; 4-9 individual triglyceride groups having 4-9 double bonds in a glyceride molecule. Group 9 occurred only in the standard triglycerides.

99.1 and 98.6%, respectively. Polar lipids eluted from Al_2O_3 (68.0 μmole, column 3) may include < 0.5% (0.7 μmole) of residual triglycerides. If calculated on a dry wt basis with 6.6% water content in original flour, the molar concentration of polar lipids and phospholipids was 36.7 and 25.9 μmole/g, and their wt content as phosphatidylcholine was 3 and 2%, respectively. Table III shows that the accuracy of the quantitative determination of total polar lipids is lower than that of phospholipids (S_{rel} = ±4.6 and ±1.1%, respectively). This difference is due to different accuracy of the methods employed for each determination. In the gas chromatographic determination of fatty acid wt with an internal standard, S_{rel} was 3.7% (13), whereas, in spectrophotometry of phosphate this value was < ±0.6% (12).

DISCUSSION

Extraction conditions. Soybean seeds have a higher content of phospholipids compared to other plant seeds (5). The completeness of extraction is known to be directly proportional to the particle size of the extracted material (22). In our experiments, flour grains were ≤0.25 mm; further grinding caused clogging of the filter during extraction. Ground seeds must be placed into the solvent containing methanol as soon as possible to inhibit lipase hydrolysis. Prolonged air storage of the flour and wetting with water before extraction (8,22), are impossible because of lipid hydrolysis.

The apparatus designed by us (Fig. 1) is based on the principle of exhaustive stepwise extraction. The solvent is retained in the extraction chamber by gas pressure on the lower surface of the filter or by hermetizing the space under the filter. At the same time, a liquid phase may be completely removed from the chamber by vacuum filtration. Constant stirring of the suspension ensures more complete extraction of lipids from plant material. This stirring is affected both by means of a magnetic bar and by a continuous stream of argon through the filter. Until now, lipid extraction was carried out with the usual laboratory equipment, i.e., separatory funnels, filters, and centrifuges. Special extractors designed by some authors (8,22) were based on the well known principle of the Soxhlet apparatus. These extractors were unfit for isolation of native lipids, because prolonged stay of the extracted material in boiling alcohol inevitably led to a significant breakdown of covalent bonds, a native state, of lipid molecules (23). By passing inert gas, it was possible to expel air from the extractor. To prevent extracted lipid from contact with oxygen, solvents were saturated with nitrogen before extraction, or the gas phase over the suspension was replaced by nitrogen (9). Commercial argon generally contained less oxygen than nitrogen, and, being heavier than air, effec-

TABLE III

Results of Quantitative Estimation of Total Polar Lipid and Total Phospholipid Content in Soybean Seeds (μmoles/2g of air-dry weight)[a]

Experiment Number	Nonextracted flour lipids, M_1	Polar lipids (μmoles/2g air-dry wt)			Phospholipids μmoles/2g air-dry wt)		
		M_2	M_3	M	M_4	M_5	M_{ph}
1	0.6	63.4	0.3	-	48.1	0.3	-
2	0.3	69.2	0.2	-	47.6	0.5	-
3	0.3	71.1	0.1	-	48.3	0.3	-
4	0.2	70.0	0.3	-	47.7	0.3	-
5	0.3	66.4	0.4	-	46.9	0.2	-
$\bar{x}$	0.3	68.0	0.3	68.6	47.7	0.4	48.4
S_{rel}(%)	23.7	4.6	40.0	4.6	1.1	25.0	1.1

[a]Values of nonextracted flour lipids (M_1, μmoles) are taken from Table I. For designations of M_{ph}, M, M_2-M_5, $\bar{x}$ and S_{rel} and the methods of their calculations see Experimental Procedures. Values of M_1-M_3 and M_5 are calculated from the molar content of acyl residues in respective fractions, that of M_4 from the molar content of phosphoric acid residues.

tively replaced the latter in the extraction medium. To obtain lipids from the tissue in a native state, the isolation conditions were sufficiently mild; however, such conditions did not always ensure quantitative yield of lipids, which required more drastic factors. Therefore, to develop a quantitative method, it was imperative to find some compromise between the extractive and disruptive action of a given technique.

The data in Table I suggest that lipid extraction was closest to quantitative when a solvent system contained chloroform and methanol, as well as small amounts of water, mineral acid, and antioxidant. It was thought that in this system chloroform largely acts on weak intermolecular interactions of a hydrophobic nature, i.e., lipid-lipid or lipid-protein, while methanol and water stimulated disruption of hydrogen bonds between lipid carbonyl, hydroxyl, and amino groups and compounds of the nonextractable residue (5,24). This suggestion may presumably explain the fact that the frequently used (6) equimolar chloroform:methanol (2:1, v/v) mixture ensured more effective lipid extraction than each of these solvents applied alone (24). Increased yield of lipids upon addition of water to the solvent was noted frequently (8,22,24,25). Conceivably, it may have been due to an increase of permeability of the extracted material upon its swelling in the presence of water (25). When swollen, smaller particles of flour were better retained by the filter and at the same time did not cause clogging. However, even after repeated treatment with chloroform:methanol:water the material still contained a considerable quantity of lipids, almost completely extracted with a solvent containing traces of mineral acid. It is known that polyphosphoinositides can be extracted from animal tissues only in the presence of mineral acids (7,16,27,28). Apparently, in a slightly acidic medium, ionic bonds between acidic lipids and some components of the nonextractable residue, possibly alkaline-earth metal ions, are disrupted.

Finally, antioxidant BHT inhibited the formation of free radicals which arose under the action of oxygen from the air during the suction filtration of a liquid phase, thereby preventing chain reactions of lipid autoxidation (4).

For lipid extraction from cereal grains, water saturated n-butanol is often used (9,29). Yet, in our experiments (Table I), as well as in lipid extraction from wheat flour (24), it proved ineffective.

The results obtained suggest that practically all lipids of soybean seeds are potentially soluble, and, consequently, are not bound covalently to substances of the nonextractable residue. This conclusion is made without account for a small fraction of lipids (0.05%) remaining in the flour, because the reason for their nonextractability is not yet clear.

Saponification of residual lipids of flour, together with an internal standard, and subsequent GLC determination of their molar concentration is the best method of evaluation of completeness of lipid extraction (Table I). Formerly, alkaline hydrolysis of defatted erythrocytes and bacteria was followed by titration or colorimetric analysis of higher fatty acids obtained from the hydrolysate (28,30). Isolation of these acids strongly absorbed by proteins was, however, inevitably accompanied by their loss, and the experimental technique was inferior to GLC in selectivity and sensitivity. The latter procedure was so far employed in experiments of this kind only for analysis of lipid inositol (26). Acid hydrolysis of the extracted tissue and weighing of fatty acids and other hydrolysate lipids (22) were not sufficiently accurate, for the reasons discussed above and also because of a fatlike artifact arising as a carbohydrate breakdown product (22). Less reliable is the conclusion about the complete extraction achieved, based on a decrease of the content of lipids or the disappearance of the latter in the subsequent extracts (6), because the quantity of lipids remaining in the tissue was not determined.

The native state of isolated lipids. Quantitative or semiquantitative analysis of FAME and FFA (Table II; Fig. 2) showed that the sum of these artifacts amounted to $< 0.5\%$ of total lipids. It is not clear whether FFA found in the extract were formed as a result of hydrolysis of lipids during their extraction or were originally present in the flour. Nevertheless, the data (Fig. 2; Table II) suggest that the hydrolysis of ester bonds proceeds much slower than their methanolysis. Concentration of methyl esters formed during extraction was almost twice as high as FFA content in the extract. It seems quite unlikely that these esters arose in the course of extraction by reesterification of FFA with methanol, because the extractant always contained a significant amount of water. Thus, there was almost no cleavage of covalent bonds in the lipid molecules during extraction in the chloroform:methanol:water:mineral acid:BHT system.

To now, the native state of the isolated lipids has been evaluated largely qualitatively by the occurrence of lysoforms of phospholipids, phosphatidic acids, and FAME in the extract (23,27), or by comparing lipids isolated by various methods according to their fatty acid composition (8). Quantitative estimation was attempted only by Gordon, et al., (19) where degradation of lipids during their extraction from rat intestine was 11%.

Finally it should be emphasized that the present work is the only one where quantitative estimation of extraction completeness and of the native state of the recovered lipids was carried out simultaneously on the same material. Our method has some other advantages, such as low solvent comsumption, short duration, no heating, and minimal contact with oxygen.

Determination of constituent groups. In this study, total polar lipids were estimated by means of quantification of

constituent groups most characteristic of these compounds, namely fatty acyl and phosphate groups. Other radicals, such as residues of amino alcohols, inositol, and monosaccharides, were not investigated by us because these groups were confined to individual polar lipid classes.

With our principle of analysis of polar lipid content we had to be aware that higher fatty acyls linked to alcohol radicals by ester bonds also are found in triglycerides and other neutral lipids of seeds, whereas, phosphate residues are also parts of nonlipid compounds such as phytine and carbohydrate phosphates (10,11). Therefore, it was imperative that prior to functional group determination the initial seed extract was fractionated so that the fractions obtained had the groups in question only in polar lipids. At the same time, there could be no loss of polar lipids themselves.

Separation of total polar lipids and their quantitative estimation. Fractionation of the extract was carried out by preparative loose layer chromatography on aluminum oxide or hydrophobic kieselguhr. To prevent lipids from being attacked by free hydroxyl groups on the Al_2O_3 surface, we used adsorbent treated with excess H^+ ions, ethyl acetate to eliminate catalytically active centres (31), and water. Chromatographic solvent also was saturated with water to prevent activation of the plate during fractionation.

As a result of a consecutive use of hexane:benzene and acetone, polar lipids adsorbed on aluminium oxide and kieselguhr became practically free from netural lipid contamination. Most of the polar lipids could be eluted from these adsorbents under mild conditions at room temperature. Nevertheless, for complete isolation of a small quantity of highly polar lipids tightly bound to adsorbent, prolonged continuous extraction with hot butanol was found to be necessary. To prevent the possibility of oxidation of unsaturated acyls at heating, extraction was carried out in presence of antioxidant. As a consequence, 99.6% of adsorbed polar lipids were solubilized.

Quantification of acyl groups in the recovered lipids by internal standard gas chromatographic technique (13) permitted accurate determination of the molar content of fatty acid residues bound to alcoholic radicals by ester and also amide bonds; concentration of aliphatic chains of alkenyl and alkyl lipids were not taken into account, as these lipids were not found in soybean seeds so far (2).

The results of a gas chromatographic study were used for the quantitative estimation of possible total polar lipid concentration in seeds (Table III); these calculations were based on an assumption that every lipid molecule had 2 acyl residues. Clearly enough, these results alone were not sufficient to conclusively determine polar lipid content, i.e., number of moles of lipids per unit dry wt. To this end, it was necessary to know molar concentration of individual polar lipid classes or their groups characterized by a definite number of acyl residues in a molecule.

Separation of phospholipids and their quantitative estimation. At the beginning of our study we tried to determine phospholipid concentration in seeds by a usual procedure, consisting of estimation of phosphorus in the residue after digestion of organic matter in the original extract. However, the average phospholipid content in the extract of 2 g seeds (73 μmole) determined by this technique was found to exceed total polar lipid concentration (68.3 μmole); variation of this determination also was quite considerable, ranging from 63 to 83 μmole.

Currently known classes of soybean seed phospholipids contain a single phosphorus atom in the molecule (2,18). Therefore, it was suggested that this highly unlikely result was brought about by solubilization of nonlipid phosphorus compounds (10,11). In fact, after fractionation of the extract on kieselguhr, the molar content of phospholipids in the same sample was as little as 48.4 μmoles. Taking into account that only a negligible quantity of lipids, 0.4 μmole, remained on kieselguhr in a bound state, it should be concluded that the observed decrease of organic phosphate concentration as compared to the original extract was not caused by phospholipid losses during fractionation. On the contrary, the phosphorus concentration found after fractionation must be regarded as reflecting maximal possible phosphlipid content in the analyzed seeds, because nonlipid phosphorus compounds could still solubilize at extraction from kieselguhr. Extraneous phosphorus found by us in the original lipid extract seemingly will impose considerable changes on the present methods of phospholipid quantification in plant tissues.

On the polar lipid content in Leguminosae seeds. Comparison of the estimated wt content of polar lipids and phospholipids with the respective published data is no simple matter. Results reported in the literature are for the most part obtained by imperfect methods, and, therefore, are not accurate enough. At the same time, estimates of the present work are not definitive either.

Weighing of polar lipid preparations after isolation from plant tissues most frequently gave distorted quantitative data, because preparations contained numerous nonlipid contaminants (1,11,32,33). Another important source of error were partial lipid losses during their extraction, purification, and chromatographic separation (10,33). Assays of lipid recovery and balance studies after each of these operations were made only occasionally. Finally, significant inaccuracy of quantitative results was introduced by incomplete separation of polar and neutral lipids by chromatography or dialysis (34,35). The most recent method of lipid quantification, gas chromatography with an internal standard, was used for studies of Leguminosae only once (35).

Taking into account above limitations, we shall discuss total polar lipid content in Leguminosae seeds. For pea, according to the data of dialysis and crystallization from acetone solutions this content is 3.0% (34), while countercurrent distribution of clover lipids suggested that polar content in its seeds was about 2.4% on dry wt basis (36). It can be seen that these concentrations are close to those of the present work (3%).

Leguminous seeds have been studied somewhat better with respect to total phospholipids. The average estimate (1.8%) for soybean seeds is only slightly different from ours (2%), though variations reported by different authors ranged from 1.0 to 3.2% (11,32,37,38). Similar phospholipid content has been found in lupin seeds (1.8% [38] and 2.2% [32]). In other leguminous seeds, the concentration was lower: pea, 1.1% on the average (ranges 0.7-1.7% [32,34,37,38]); beans, 0.6%; clover, 0.5% (37). Comparison of these data with those given in the previous paragraph revealed that pea and clover seeds, like soybean seeds investigated by us, contained much polar lipid devoid of phosphorus.

Plants belonging to other families, namely cereals, cruciferae, sunflower, buckwheat, watermelon, castor seed, and sesame are characterized by a low content of total polar lipids and phospholipids in the seeds, 0.4-1.1% and 0.1-0.9%, respectively (18,32). Rather high concentrations of phospholipids (1.7-2.0%) was found only in cotton seeds and linseed (11,18). Thus, Legiminosae, and particularly soybeans, stand in marked contrast to other higher plants by the highest level of membrane lipids in seeds; therefore, they may serve as a convenient object for investigatiing polar lipid content, composition, and metabolism.

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