Quantitative determination of phosphorus in soybean lipids

A. V. Zhukov and A. G. Vereshchagin

Lipid Biochemistry Research Unit, Institute of Plant Physiology, Academy of Sciences, Moscow, USSR

SUMMARY Black and Hammond's method for determining lipid phosphorus has been modified for use with oils containing small amounts of phosphorus. The minimum amount of P that can be determined is 3 nmoles, with a coefficient of variation SD of 2%.

SUPPLEMENTARY KEY WORDS perchloric acid · acid molybdate reagent · stannous chloride · phospholipids

Of the many published methods for determining lipid phosphorus (1–8), many are unsuitable for this determination in soybean lipids, which contain a small quantity of phospholipids and about 10 times as much triglyceride. Prolonged heating during the digestion of the necessarily large amounts of oil leads to loss of acid and of reproducibility in the colorimetry (7). We have therefore modified the method of Black and Hammond (5), the main characteristic of which is preliminary oxidation of the lipids by nitric acid to facilitate the subsequent digestion by perchloric acid. The modifications include reducing the scale, use of a modified Kjeldahl flask, and neutralization of excess perchloric acid with ammonia instead of evaporation.

Materials. Reagent grade KH_2PO_4 and $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ were recrystallized. Technical grade nitric acid (d = 1.407) and perchloric acid (d = 1.655) were distilled to remove impurities. Other chemicals were reagent grade. Volumetric glassware was calibrated by standard methods. All calibrations and specific gravity determinations were performed at 20 ± 0.02°C. Solutions were prepared in twice-distilled water. The molybdate reagent, a 1:3 (v/v) mixture of 10% ammonium molybdate and 10 N H_2SO_4 (d = 1.39), remained stable

for several months. The $SnCl_2$ solution was prepared in a 10 ml volumetric cylinder by the solution of 190 mg of $SnCl_2 \cdot 2H_2O$ in 0.15 N H_2SO_4; after the addition of two drops of 4% $CuSO_4 \cdot 5H_2O$, the solution was brought to volume with 0.15 N H_2SO_4. The lipid solution was prepared by the extraction of freshly-ground soybean seeds first with butanol and then with chloroform–methanol 2:1.

Procedure. For the construction of the calibration curve, 2–8 ml aliquots of standard KH_2PO_4 solution (1 μg P/ml, freshly diluted from a stock solution containing 100 μg P/ml) were pipetted into 10-ml glass-stoppered volumetric flasks, followed by addition of water to a volume of about 8 ml and 0.2 ml of the molybdate reagent. After the solution had been shaken, it was brought to volume with water and thoroughly mixed. A drop of the freshly prepared $SnCl_2$ solution was added to each flask at 3-min intervals, and after exactly 45 min the absorbance A of each solution was read at 720 nm (9) against a water reference in a cell of 3.5 ml capacity with a light path of 0.998 cm. The values were corrected for a reagent blank, and P concentrations, corrected for the glassware calibration, were used to plot a standard curve.

The digestion of lipid phosphorus (5) was carried out with single and double volumes of solution, every test being conducted in replicate. An amount of lipid extract containing 10–20 mg of lipid was pipetted into a modified Kjeldahl flask (Fig. 1). The sample size could be reduced to about 1 mg, in which case the necessary dilutions were modified accordingly. The flask was stoppered with the modified stopper (Fig. 1), and the solvents were evaporated in boiling water for 5–10 min under a stream of air. Two drops of nitric acid were added, and the flask was heated on the thermostatted block heater, care being taken to avoid charring (the temperature inside the flask was 175°C). The heating was continued until the vigorous brown foaming had subsided and a homogeneous dark liquid was formed. The flask was cooled, 0.3 ml of perchloric acid was added, and the flask was heated for 2–3 min to 175°C with continuous stirring. After the appearance of heavy white fumes, the flask was heated at 200°C for a further 15–30 min until the mixture was completely decolorized. The flask was cooled, and 0.5 ml of aqueous ammonia (d = 0.914) was added. A rubber stopper was inserted at position *5* (Fig. 1), and the flask was heated at 100°C under reduced pressure (water pump) until the liquid had evaporated off and no odor of ammonia remained. 25 ml of water was added to the flask, and 2 ml of the resultant solution (in replicate) was pipetted into a 10 ml volumetric flask. The phosphorus content was estimated as described above. Solvents used in the procedure were sufficiently free of phosphorus to give blank values of zero. The accuracy of the method was evaluated by the recovery of inorganic phosphorus

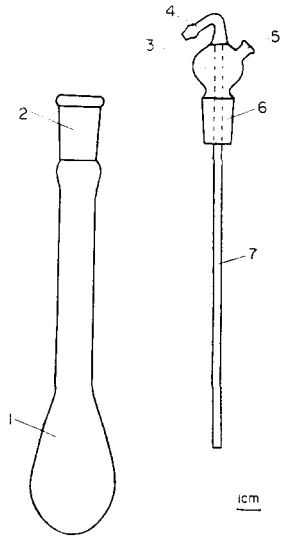

FIG. 1. Modified Kjeldahl flask for digestion of lipids without loss of phosphate. *1*, Kjeldahl flask (volume, 30 ml); *2*, female standard joint No. 14.5; *3*, stopper, pierced by glass tube; *4*, outlet tube for connection with the water pump; *5*, hole for rubber stopper; *6*, male standard joint No. 14.5; *7*, vapor exit tube.

(0.5 ml of the stock phosphate solution being added to occasional samples before digestion).

Results. The calibration curve for the lipid phosphorus assay was constructed from the data in Table 1. The color of the reduced phosphomolybdate obeyed the Lambert–Beer law in the range of 2–8 μg of P. The SD was calculated by the method of Gänshirt and Polderman (10). The relative SD (average coefficient of variation) for successive estimations was 0.56%. The sensitivity of the quantitative method was evaluated using the molar extinction coefficient at 720 nm (11). As derived from the data of Table 1, ϵ_{720} = 25,200. This value was used to calculate the minimum amount of phosphorus (M) that can be determined by the spectrophotometric procedure described. Calculation by the method referred to in

TABLE 1 CALCULATION OF STANDARD DEVIATION IN THE SPECTROPHOTOMETRIC ASSAY OF INORGANIC PHOSPHORUS

	P, μg/10-ml*						
	2	3	4	5	6	7	8
	$A \times 10^3$						
x	167	243	323	400	478	554	632
	166	243	325	399	478	561	634
	164	247	325	400	478	558	633
$\bar{x}$	165	244	324	400	478	558	633

* Actually in μg × 1.017, the correction factor for the glassware calibration.

Designations: x, single measurements of absorbance obtained successively by a single experimenter; $\bar{x}$, arithmetic mean. If m is number of P solutions of different concentrations (=7) and S = pooled standard deviation (ref. 10) = 0.0018, relative SD (average coefficient of variation) = $10^5 S/m\Sigma\bar{x}$ = 0.56%

TABLE 2 CALCULATION OF STANDARD DEVIATION IN ASSAY OF PHOSPHORUS IN SOYBEAN LIPIDS*

Test No.	A†	B†	x_i
1	3.62	3.61	99.7
2	3.46	3.55	102.6
3	3.27	3.31	101.2
4	3.26	3.29	100.9
5	3.57	3.48	97.5
6	3.26	3.38	103.7
7	3.32	3.37	101.5
8	3.03	2.98	98.3
9	3.01	2.97	98.6
10	2.96	2.95	99.7
11	3.09	3.01	97.4
12	1.89	1.93	102.1

* The data were obtained by two experimenters at different times.

† Arithmetic mean of four measurements.

Designations: A, μg P in 1 volume of the lipid extract; B, μg P in 2 volumes of the same extract divided by 2; $x_i = 100\ B/A$; $n = 12$; $x = \Sigma x_i/n = 100.3$; $S = [\Sigma(x_i - \bar{x})^2/(n-1)]^{1/2} = 2.06$. Relative SD (coefficient of variation) $= 100\ S/\bar{x} = 2.05\%$.

Gorchakov's manual (11) gives M = 2.77 × 10^{-} moles of P. The recovery of inorganic phosphorus after the lipid digestion was 102%.

In order to evaluate the accuracy and precision of the method, single and double volumes of the soybean lipid solutions were wet ashed and the P content in the inorganic residues was measured (Table 2, columns 2 and 3). The estimated absolute values of the phosphorus concentrations varied from test to test since the content of phospholipids in the extracts obtained under different conditions differed slightly and since various volumes of extracts were used for digestion in successive experiments. Therefore, absolute (11) and relative (10) standard deviations were calculated using relative x_i values obtained as shown in Table 2. It was found that the coefficient of variation in the assay of lipid phosphorus after digestion (2.05%) was somewhat higher than that in the estimation of inorganic phosphorus (0.56%). In our samples of soybean lipids the phosphorus content was 0.6–0.7%.

Discussion. The method of Black and Hammond (5) has been modified as follows: the sample volume was reduced 10-fold, and the amount of oxidants (HNO_3 and $HClO_4$) was decreased from 4- to 5-fold. This allowed digestion without glass beads that have been used (5) to achieve less vigorous boiling. Use of a powerful oxidant (perchloric acid) and high temperature (over 200°C) at the terminal stage of digestion made it possible to carry out reliable digestion of lipid material for up to 30 min without any loss of orthophosphate.

Stannous chloride is a powerful reducing agent (5), but reproducible results with it can be obtained only if the pH of the solution is accurately maintained and if the time interval between the addition of stannous chloride and measurement of the absorbance is strictly observed With other reducing agents (1–4, 6–8, 12, 13) the acidity of the solution is usually created by the acid used for the digestion. If perchloric acid serves as the oxidizing agent, it cannot be used for accurate acidification of the medium because of the inevitable losses of acid during lipid digestion. Black and Hammond (5) attempted to solve this problem by evaporating perchloric acid in a current of compressed air that passed through the Kjeldahl flask as it was heated. In our experiments we failed to achieve complete removal of the acid when a stream of inert gas was passed through the hot flask. Connection of the heated flask (175°C) with the water pump (7) resulted in a better evaporation of perchloric acid, but this method proved to be unsuitable because of a significant loss of phosphate. We therefore removed excess perchloric acid from the reaction mixture by neutralization with aqueous ammonia followed by the vacuum distillation of water and excess ammonia at a moderate temperature (100°C). The acidity necessary for color development was obtained by an addition of a precise amount of the molybdate reagent. As shown in Table 2, this method assures complete retention of phosphorus in the Kjeldahl flask, and the ammonium salt formed after neutralization does not affect the colorimetric determination. The sensitivity of the proposed method compares favorably with other modern methods of phosphorus assay (2, 4, 12, 13), and the method can be used for the determination of microamounts of phospholipids after their analytical separation by TLC.

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