Lipids of the Cottonseed. III. Perchloric Acid Ashing of Lipids for the Determination of Phosphorus

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EVIDENCE has been presented (1, 2) that in the extraction of cottonseed with several organic solvents the composition of the extract obtained with a given solvent will vary from seed specimen to seed specimen, and the composition of the extracts obtained with a given seed specimen is dependent upon the nature of the solvent used. The quantity of material extracted with a given specimen is also dependent on the nature of the solvent(1).

An interest has developed in the phosphorus content of the various extracts obtained from cottonseed, and the data presented in this communication indicate that the phosphorus contents of the extracts obtained on extraction with several solvents is likewise a function of the solvent.

Oxidation of Organic Matter

The phosphorus content of the several extracts from cottonseed is too small to permit gravimetric methods to be used to good advantage, and the method which suggests itself as being the most practical is that of Fisk and Subbarow (3). It is not feasible to carry out the oxidation of the organic matter with sulfuric acid and hydrogen peroxide or sulfuric acid and nitric acid, as is ordinarily used with biological material in the determination of phosphorus where the Fisk and Subbarow method is employed. The difficulty arises because the acid content of the digests, where sufficient sulfuric acid has been added to completely clarify the digest, is too great to permit the development of the molybdenum blue.

The difficulties arising with the sulfuric acid oxidation procedure may be circumvented, as has been done by King (4), by carrying out the oxidation with perchloric acid. In his procedure for the determination of phosphorus in several biological materials King did not add sulfuric acid to the ammonium molybdate, as directed by Fisk and Subbarow, but attempted to produce the correct acidity in the solutions by adding a small amount of perchloric acid to the digests after oxidation had been completed. Reiser (5) also has used perchloric acid for the oxidation of lipid material. Since the quantities of perchloric acid distilled off during the digestion varied greatly, the final acidity of his solutions was controlled by neutralizing the perchloric acid in the digest and then adding a fixed amount of sulfuric acid to the neutralized solution.

Although the quantity of acid present in the reducing mixture is important in the development of the molybdenum blue, the quantity which may be present is not as critical as would be implied in the papers by King, by Reiser, and by Fisk and Subbarow. The dependence of the development of the maximum blue color on the acidity of the solution is indicated in Figure 1. These data were obtained on the perchloric acid digestion of a petroleum ether extract of a specimen of Rowden-Harper's type cottonseed. The directions of Fisk and Subbarow were followed in the development of the molybdenum blue, and the

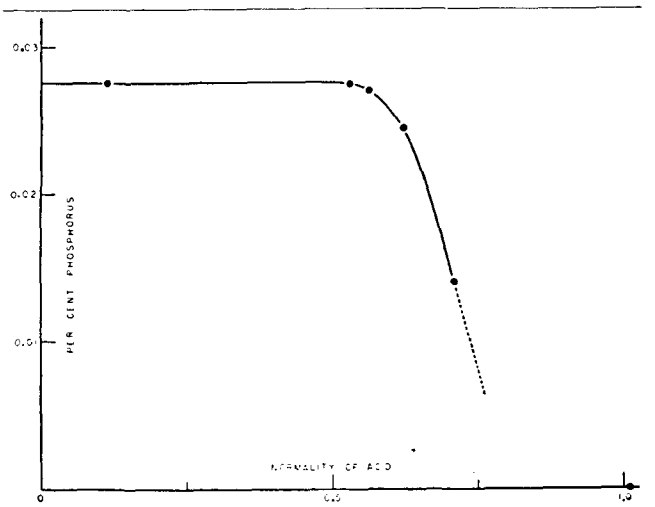

FIG. 1. The dependence of the reduction of phosphomolybdic acid on the acid content of the medium.

Beckman spectrophotometer with 1-cm. cells was used to determine the optical density at 800 millimicrons (6). The stipulated quantities of perchloric acid were added to aliquots of the digest. It will be noted that the calculated quantity of phosphorus, as reflected in the intensity of the blue color, remains constant until an acid concentration of about 0.5 normal is reached, after which it decreases. The initial experimental point in this curve corresponds approximately to the concentration of acid in the reducing medium when the digestions have been carried out in the manner presented herewith.

The implication from these data is that the presence of perchloric acid does not interfere with the reduction of the phosphomolybdic acid. The data presented in Table 1 substantiate this statement. In these determinations known quantities of phosphate phosphorus were added to Kjeldahl flasks, some of which contained weighed quantities of cottonseed oil. The digestions were then carried out in the manner which will be discussed below. It will be noted that in every case the quantity of phosphorus recovered is identical with that which was added.

TABLE 1

The Effect of Digestion on the Phosphorus Yield

Phosphorus in oil (mg.)	Phosphorus added (mg.)	Phosphorus found (mg.)
No oil	0.0016	0.0016
No oil	0.00032	0.00032
No oil	0.00024	0.00024
No oil	0.00096	0.00096
0.000175	0.00028	0.00046
0.000202	0.00024	0.00045

Method

The procedure for the determination of phosphorus that we have developed on the basis of the observations just reported is as follows: Weigh 0.15

g. of oil into a micro-Kjeldahl flask. Add 2 ml. of concentrated nitric acid and then drive off the acid with gentle heating. This step is repeated twice. On the third addition of nitric acid a few drops of perchloric acid are added, and the mixture is gently warmed. One should be cautioned to avoid the addition of perchloric before the third addition of nitric acid, and then to add only a few drops, for on the addition of perchloric acid in quantities that are too great, or before the nitration has been substantially completed, a violent reaction is likely to occur. When the mass almost ceases to boil, more nitric and perchloric acids are added in the ratio previously used. The sample may then be heated more strongly, and no more nitric acid should be required. If, however, charring occurs, it may be cleared up with the addition of a small quantity of nitric acid. The digestion now is continued until the dense fumes of perchloric acid begin to flow out of the Kjeldahl flask. It may be noted that the digests have a tendency to bump. The digest is diluted to 100 milliliters in a volumetric flask, and the phosphorus is determined with a suitable aliquot, using the method of Fisk and Subbarow.

TABLE 2

The Phosphorus Content of Crude Cottonseed Oil From Harper's-Rowden Type Cottonseed

Source of oil	Sample number	% phosphorus in the oil
Isopropyl alcohol extract	1	0.117
	2	0.117
	3	0.117
Hydraulic press	1	0.0211
	2	0.0215
	3	0.0214
Hydraulic press	1	0.0217
	2	0.0216
	3	0.0214
Petroleum ether extract	1	0.0261
	2	0.0270
	3	0.0269
Benzene extract	1	0.0829
	2	0.0829
	3	0.0829
Diethyl ether extract	1	0.0844
	2	0.0848
	3	0.0846
Acetone extract	1	0.0071
	2	0.0070
	3	0.0072
Chloroform extract	1	0.103
	2	0.102

Results

The phosphorus content of several extracts obtained from Harper's-Rowden type cottonseed are presented in Table 2. Each datum is from a completely independent determination. These several extracts were obtained with the Frampton-Giles low pressure extraction apparatus (7).

It will be noted that the phosphorus content of the acetone-extracted material is low in comparison with the phosphorus contents of the other extracts. A waxy material high in phosphorus was obtained on the diethyl ether (peroxide-free) extraction of a seed specimen that had been exhaustively extracted with acetone. After the material was kneaded in cold acetone (—10°C.), dissolved in ethyl alcohol, and precipitated with acetone six times, the phosphorus content remained constant at 3.7%.

Summary

1. Cottonseed oil is effectively oxidized with a mixture of perchloric and nitric acids in connection with the determination of phosphorus by the Fisk and Subbarow method.

2. The development of the molybdenum blue in the procedure is independent of the acid content in the reducing medium up to 0.5 normal.

3. The presence of perchloric acid does not interfere with the development of the molybdenum blue.

4. The phosphorus content of cottonseed extracts depends on the nature of the solvent. The quantities obtained varied from 0.0071 to 0.117%.

5. A phospholipid containing 3.73% phosphorus was isolated from cottonseed.

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6. The absorption maximum at 800 millimicrons was observed where 1,4-aminonaphthol sulfonic acid was used as the reducing agent. Boltz and Mellon [Anal. Ed., Ind. and Eng. Chem. *19*, 873 (1947)] observed a maximum at 830 millimicrons when they used hydrazine as the reducing agent. The difference in these maxima is probably due to differences in the phosphomolybdenum complex formed.

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