An Improved Phosphorus Assay for Oils Without Carcinogenic Hydrazine Sulfate

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ABSTRACT: An improved spectrophotometric method for phosphorus determination in oils is proposed. The proposed new method has made significant improvements in safety, sensitivity, and efficiency in comparison with the current American Oil Chemists' Society (AOCS) Official Method (Ca 12-55, corrected 1992). The AOCS method employs hydrazine sulfate as the reducing agent to generate molybdenum blue. Hydrazine sulfate is a known carcinogen in laboratory animals and a suspected human carcinogen. The chemical also irritates skin and mucous membranes. In the improved method ascorbic acid is used as the reducing agent. Ascorbic acid is equally effective as hydrazine sulfate for the color reaction. The improved method is approximately 75 times more sensitive than the AOCS method. The sensitivity is improved by measuring absorbance at the absorption peak, 825 nm, and reducing the final volume of reaction mixture for color reaction. The AOCS method determines phosphorus by measuring absorbance at 650 nm, which is about 40% of that at the absorption peak. The efficiency of this improved method is also significantly increased by reducing sample size and the volumes of sample preparation. Therefore, the improved method is more cost-effective than the AOCS method because less chemical reagents and smaller glassware are used, and the hazardous chemical waste disposal cost is eliminated. The improved method also avoids concentrated HCl and 50% KOH for sample preparation.
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Taylor and Miller (1) first reported that phosphorus in biological samples could be determined by light absorption of molybdenum blue, the reduction product of phosphomolybdate. Fiske and Subbarow (2) later found that 1-amino-2-naphthal-4-sulfonic acid (ANSA) was an effective reducing agent for phosphomolybdate and developed a reliable and widely used method in which the absorbance was measured at 660 nm. More than three decades later, Bartlett (3) reported that adding heat treatment to the ANSA-reduced reaction produced more reproducible and intense blue color with an absorption peak at 830 nm.

The use of ascorbic acid to reduce phosphomolybdate was first reported by Ammon and Hinsberg (4). Several other laboratories reported various requirements (5–7). All the reports recommended heat treatment for the color reaction and absorbance measurements at 820 nm.

The current AOCS Official Method Ca 12-55 (8) is also a spectrophotometric method for the determination of phosphorus in oil samples. This method requires hydrazine sulfate as the reducing agent and heat treatment to generate molybdenum blue. The absorbance is measured at 650 nm. Hydrazine sulfate is a known carcinogen in laboratory animals and a suspected human carcinogen. The chemical irritates eye, skin, and mucous membrane, and, if ingested, it can damage liver and kidney (8). The AOCS Official Method also employs rather large sample sizes, large sample preparation volumes, and bulky glassware.

Daun *et al.* (9) conducted a thorough study on the methodologies of phosphorus determination in canola oil samples. A digestion-spectrophotometric method with ascorbic acid for phosphomolybdate reduction and absorbance measurements at 650 or 750 nm was included in their study. However, the results of this method correlated poorly with the AOCS Official Method and were inconsistent.

The objectives of this study are to identify the absorption peak wavelength of the color product, to investigate the feasibility of substituting hydrazine sulfate with ascorbic acid, and to increase the safety, sensitivity, and efficiency of the spectrophotometric phosphorus assay. A new method has been developed with increased safety, sensitivity, and efficiency.

MATERIALS AND METHODS

Materials. L-Ascorbic acid was purchased from Sigma Chemical Company (St. Louis, MO). Hydrazine sulfate was obtained from Aldrich Chemical Company (Milwaukee, WI). Sodium molybdate was substituted by ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (ACS certified; Fisher Scientific, Fair Lawn, NJ). Anhydrous monobasic potassium phosphate, KH_2PO_4 (analytical reagent; Mallinckrodt, Paris, KY) was used as the standard. All the other chemicals used in this study are of reagent grade or better. Five commercially processed canola oil samples were used to verify the improved phosphorus assay.

Modified AOCS Official Method. The AOCS Official Method Ca 12-55 (8) was modified by reducing the sample size and the amount of reagents employed. However, the

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reagent preparations and their proportion to sample size were adjusted in accordance with the AOCS Official Method, except that sodium molybdate was substituted by ammonium molybdate. One gram of oil was accurately weighed at 0.0001 g into a 20-mL fused-quartz crucible (Fisher, Pittsburgh, PA). The oil was charred by a Bunsen burner after the addition of 20 mg of ZnO. The oil was then ashed in a muffle furnace at 550–600°C for 2 h. The ash was dissolved in 1.0 mL of 6 N HCl (or 0.5 mL of concentrated HCl and 0.5 mL of H_2O) by heating on a hot plate. The washing was filtered into a 10-mL volumetric flask through Whatman No. 1 filter paper. Two consecutive washes with 2 mL hot water were carried out, and the washes were filtered and combined with the first wash. After the solution was cooled to room temperature, 50% KOH was added dropwise to neutralize the wash. Concentrated HCl was then added dropwise to dissolve the ZnO precipitate. One additional drop of HCl was added to the solution. Deionized water was added to bring the volume to 10 mL. An aliquot of 0.5 mL of the solution was transferred to a 13 × 100 mm test tube with a PTFE-lined screw cap in duplicate. The following reagents were added in the order given: 0.4 mL hydrazine sulfate solution, 0.1 mL ammonium molybdate solution, and 1.5 mL deionized water. The reaction mixture was vortexed and then heated in a boiling water bath for 10 min with screw cap on loosely. After the reaction mixture was cooled to room temperature, the absorbance at 820 nm was determined in a Hitachi U-2000 spectrophotometer (Danbury, CT). The blank was prepared in duplicate with the same treatments and reagents, except that the oil was omitted.

The standard stock and working solutions were prepared according to the AOCS Official Method (8). Depending on the level of phosphorus in the sample, a standard curve was established from either the series with 0, 0.25, 0.50, 0.75, and 1.00 μg, or 0, 0.50, 1.00, 1.50, 2.00, 2.50, and 3.00 μg phosphorus. The reaction mixtures for standard curves were prepared in the same way as that described for sample solution.

Improved phosphorus assay. The improved method was directly developed from the method described by Ames and Dubin (7). Oil sample was charred and ashed as described previously. The ash was dissolved in 2 mL of 0.5 N HCl by heating on a hot plate. The solution was then filtered into a 10-mL volumetric flask. The crucible was washed two more times with 2 mL of hot 0.5 N HCl, and the filter paper was washed with 1 mL of 0.5 N HCl. The washes were filtered and combined with the first wash. After the solution cooled to room temperature, the volume was brought to 10 mL by adding 0.5 N HCl. A portion of 0.5 mL of the sample solution was transferred to a 13 × 100 mm test tube with a PTFE-lined screw cap in duplicate. The sample solution was mixed with 1.0 mL of coloring reagent, consisting of 1 part of 10% ascorbic acid solution and 6 parts of 0.42% ammonium molybdate solution in 1 N H_2SO_4. Coloring reagent needs to be prepared fresh immediately before use. The reaction mixture was incubated in a water bath at 45°C for 20 min. The absorbance was determined at 820 nm. The blank was prepared in the same way without oil as described above.

The standard stock and working solutions were prepared in 0.5 N HCl. A standard curve of either 0–1 μg or 0–3 μg phosphorus was prepared depending on the level of phosphorus in the sample. The reaction mixtures of standards were prepared in the same manner as the sample solution in duplicate.

Wavelength scan. The color solutions of the standard of 3 μg phosphorus, prepared from both the modified AOCS method and the improved phosphorus assay, were scanned from 400 to 1,000 nm with a Hitachi U-2000 spectrophotometer.

Recovery of inorganic phosphorus. Both the modified AOCS and the improved methods were tested for the recovery of inorganic phosphorus. Proper amounts of a KH_2PO_4 standard solution were placed in a crucible and then dried to give the following amounts of phosphorus: 0.5, 1.0, 5.0, 10.0, 25.0, and 50.0 μg. Because refined, bleached, and deodorized (RBD) oil usually contains only trace amount of phosphorus, 1 g of RBD oil was added to each crucible. The recovery of inorganic phosphorus at each level was investigated in triplicate.

Recovery of phosphorus from phospholipid. Because most of the phosphorus in oil samples comes from phospholipids, the recovery of phosphorus from phospholipid was investigated. L-α-Phosphatidylcholine (P-3556, Type XVI-E, 99%; Sigma Chemical Company) was added to RBD oil to prepare oil samples with 0.5 to 25.0 ppm phosphorus, based on an average molecular weight of 750. The phosphorus in the specific lot purchased (Lot No. 83H8371) was 3.9%, so the actual concentrations of phosphorus used in this study were 0.47, 0.94, 4.7, 9.4, and 23.6 ppm. The amount of sample used for the study was as follows: 1.0 g of 0.47 and 0.94 ppm; 0.5 g of 4.7 ppm; 0.1 g of 9.4 and 23.6 ppm. The final weight of each sample was made up to 1.0 g by adding RBD oil. The blank contained ZnO and 1.0 g of RBD oil. The recovery was tested in triplicate by using the improved method.

Verification of the improved phosphorus assay. Canola oil samples, containing various levels of phosphorus, were analyzed by both the improved method and the ICP–AES method, and the results were compared. Sample Nos. 1 through 3 were washed oils; No. 4 was degummed oil; and No. 5 was crude oil.

RESULTS AND DISCUSSION

Wavelength of absorption peak. The color solutions prepared from 3 μg phosphorus by both the improved and the modified AOCS methods were scanned from 400 to 1,000 nm (Fig. 1). Both profiles had an absorption peak at 825 nm, and a mild shoulder around 650 nm. The absorbance at 650 nm is about 40% of that at the absorption peak.

The improved method, reported in this study, was developed from the procedures reported by Chen *et al.* (6) and Ames and Dubin (7). Both procedures required ascorbic acid as the reducing agent, heat treatment to generate the color product, and molybdenum blue absorbance measurement at

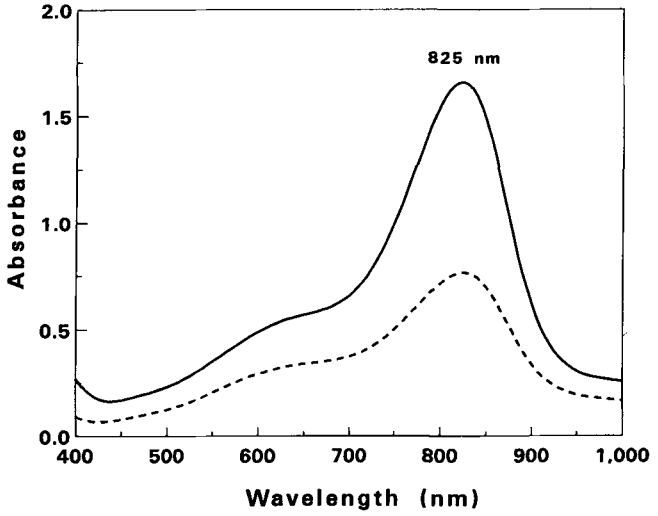

FIG. 1. Wavelength scans of the color product generated by either ascorbic acid (—) or hydrazine sulfate (----) as reducing agent.

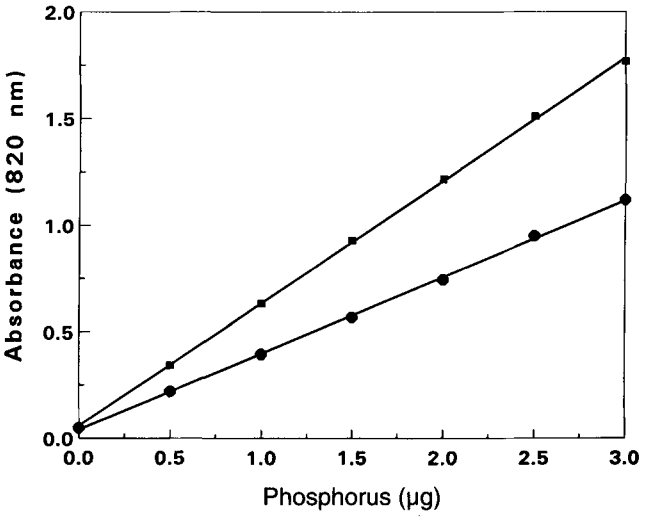

FIG. 2. Standard curves of phosphorus assay with either ascorbic acid (■) or hydrazine sulfate (●) as reducing agent.

820 nm. The wavelength scans (Fig. 1) confirmed the use of a wavelength between 820 and 830 nm for phosphorus quantitation.

The modified AOCS method employs a different reducing agent (hydrazine sulfate) and a different heat treatment (boiling for 10 min) to produce the color (8). The wavelength of the maximal absorption also appeared to be 825 nm.

Bartlett (3) used ANSA as the reducing agent and boiling for 7 min for the color reaction. The color product also had a similar absorption profile with an absorption peak at 830 nm. Without heating, the color developed by ANSA at room temperature had no such peak and was not stable (3,6).

The absorption peak of the blue reduction product of phosphomolybdate is independent of both the reducing agent and the heat treatment. The absorbance at 825 nm should be used for phosphorus determination instead of 650 nm. Most spectrophotometers allow the measurement of absorbance between 750 and 1,000 nm.

Standard curves. When the standard curves were prepared in the range from 0.5 to 3 µg with either the modified AOCS or the improved methods, near-perfect linearity (r = 1.000) was observed in both curves (Fig. 2). A near-perfect linear response was also demonstrated by the standard curves in the lower range from 0.25 to 1.0 µg phosphorus (data not shown).

A noteworthy characteristic of the phosphorus standard curve generated by ascorbic acid is its linearity over a wide range of absorbance from 0 to 1.7. The near-perfect response may result from absorption of a nonvisible wavelength (825 nm) and allows for quantitation of a wide range of phosphorus with the same standard curve.

Molar extinction coefficient. Based on the absorbance, the amount of phosphorus, and the final volume of the reaction mixtures, the molar extinction coefficient (ε) was calculated for the color product prepared from both methods (Table 1). The ε of the color products, prepared by other methods published in the literature, were also calculated from the reported data.

According to the calculated ε values, the color products prepared by the methods involving heat treatments had the same value, regardless of the type of reducing agents used and the type of heating conditions. The ε of the color products obtained by using heating methods were seven to eight times higher than those from the Fiske and Subbarow method (2), which did not heat the color reaction mixture.

These results suggest that hydrazine sulfate, ascorbic acid, and ANSA have the same capacity to reduce phosphomolybdate as long as the heat treatment is adequate. Ascorbic acid can replace hydrazine sulfate as the reducing agent for phosphorus determination and increase the safety of this procedure without compromising sensitivity or accuracy.

Recovery of inorganic phosphorus. Both the modified AOCS method and the improved method quantitatively recovered inorganic phosphorus in the range of 0.5 to 10 µg. However, the recovery rate of the improved method slightly decreased above the level of 25 µg, and the recovery rate of the modified AOCS method was significantly reduced above the level of 10 µg (Table 2). Although other factors could

TABLE 1
The Molar Extinction Coefficient (ε) of the Color Product and Reaction Conditions of Various Phosphorus Assays

Reducing agent	ε ($M^{-1}cm^{-1}$)	Reactions conditions	Reference
ANSA[a]	3.6×10^3	room temp., 7–10 min	3
ANSA[a]	2.6×10^4	100°C, 7 min	3
Ascorbic acid	2.6×10^4	37°C, 90 min	6
Ascorbic acid	2.4×10^4	45°C, 20 min	7
Ascorbic acid	2.6×10^4	45°C, 20 min	This study
Hydrazine sulfate	2.6×10^4	100°C, 10 min	This study

[a]ANSA = 1-amino-2-naphthal-4-sulfonic acid.

TABLE 2
The Recovery Rate of Inorganic Phosphorus by the Improved Method and the Modified AOCS Method

Phosphorus (μg)	Improved method		Modified AOCS method	
	Recovered (μg)	Recovery rate (%)	Recovered (μg)	Recovery rate (%)
0.50	0.51 ± 0.03	102	0.48 ± 0.07	96
1.00	0.98[a]	98	1.06 ± 0.21	106
5.0	5.3 ± 0.1	106	5.0 ± 0.2	100
10.0	10.7 ± 0.3	107	10.5[a]	105
25.0	24.0 ± 0.5	96	21.9 ± 1.0	88
50.0	46.9 ± 0.6	94	43.3 ± 0.4	87

[a]The average of two determinations. The rest are mean ± SD.

have caused the recovery rate decrease at high phosphorus levels, personal error could not be eliminated. Recovery rates that exceed 100% at low levels of phosphorus suggest possible contamination of exogenous phosphorus.

Recovery of phosphorus from phospholipids. The recovery of phosphorus from phospholipid by the improved method was close to 100% through the whole range from 0.47 to 23.6 ppm investigated in this study. The recovery rate slightly decreased at high levels of phospholipid (Table 3). Because phosphorus in phospholipid or other organic matters was first converted to inorganic phosphorus, only the improved method was tested.

The overall recovery rate of phosphorus of either inorganic or organic origin by either the modified AOCS method or the improved method was excellent. These results also indicated that both methods could accurately detect inorganic or organic phosphorus at the level of 0.5 ppm in oil. The detectable phosphorus in the reaction mixture was actually 25 ng (1/20 of 0.5 μg) because only 1/20 of the sample preparation was used for the color reaction.

The sensitivity of the improved method was six times as that of the micro-method developed by Chen *et al.* (6), and thirty times greater than the AOCS Official Method. The sensitivity was improved mainly by reducing the final volume of the reaction mixture (from 50 to 1.5 mL) because the ε values of the color products were the same, regardless of the choice of reducing agent or heating condition.

Performance of the improved phosphorus assay. The phosphorus levels determined by either the improved method or the ICP–AES method in five canola oil samples were in good agreement (Table 4). The difference of the phosphorus level of sample No. 5 between these two methods was relatively large, possibly because No. 5 is a crude oil sample. Nevertheless, the overall results clearly indicate that the improved method was able to determine the phosphorus in a wide range of oil samples with high accuracy.

The effect of reagent concentrations, acidity, and heat treatment. Heating time has no effect on the stability of the color product once the phosphomolybdate reduction was completed (3,6). When ascorbic acid was used as reducing agent, different temperatures and times (37°C for 90 min vs. 45°C for 20 min) could be used to complete the color reaction (6,7). Because the results are insensitive to variations in heating temperature and time, the experimental error is minimized.

The determination of phosphorus by reducing phosphomolybdate with heat treatment is also insensitive to the concentration of reagents. The concentration of ascorbic acid did not affect the color development, even when it exceeded many times the minimal requirement for complete reduction of phosphomolybdate (6). Bartlett (3) also reported that an increase or decrease of the amount of molybdate or reducing agent by 1/3 did not affect the color reaction.

The formation of color product was proportional to the amount of phosphorus in the presence of molybdate within a rather broad range of acidity from 0.5 to 1.0 N when ascorbic acid was used as reducing agent (6). The color product of the reduced phosphomolybdate was stable. Chen *et al.* (6) found that the color only increased slightly after 1.5 h. Bartlett (3) reported that the color was stable at room temperature for at least 24 h.

The overall characteristics of the improved phosphorus assay. The improved method is safe because hydrazine sulfate, a carcinogenic reducing agent, is replaced by ascorbic

TABLE 3
The Recovery Rate of Phosphorus in Phosphatidylcholine (PC) by the Improved Method

Phosphorus in PC (μg)	Recovered (μg)	Recovery rate (%)
0.47	0.47 ± 0.03	100
0.94	0.94 ± 0.12	100
4.7	4.6 ± 0.1	98
9.4	9.1 ± 0.6	97
23.6	23.3 ± 0.4	99

TABLE 4
Comparison Between the Improved Method and ICP–AES Method

Sample number	Total phosphorus (ppm)	
	Improved method	ICP–AES method[a]
1	0.83	1.1
2	8.4	8.1
3	24	20
4	52	55
5	270	210

[a]Determined by outside laboratories.

acid, which has the same reducing capacity. By reducing the volume of the final reaction mixture thirty times (from 50 to 1.5 mL) and measuring absorbance at the absorption peak, which is about 2.5 times as high as 650 nm, the sensitivity of the improved method has increased approximately 75-fold over the AOCS Official Method. The improved method is also efficient and cost-effective. Sample size, sample preparation, and reaction mixture volumes have been reduced, as have the glassware size and reagent volume. The replacement of hydrazine sulfate by ascorbic acid also eliminates the carcinogenic waste disposal cost. Finally, the colorimetric phosphorus assay is robust. The formation of molybdenum blue tolerates some variation of reagent concentration, heating conditions, acidity, and type of reducing agent.

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