

Phosphorus Assay in Column Chromatography*

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(Received for publication, August 4, 1958)

The phosphorus assay method of Fiske and SubbaRow (1) has been modified to increase its sensitivity and to facilitate multiple total phosphorus analyses on eluates from chromatographic columns.

EXPERIMENTAL

Materials—30 per cent hydrogen peroxide (Baker and Adamson, phosphorus-free) and the ammonium molybdate, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$, were used. The Fiske and SubbaRow (1) reagent was prepared by adding 0.5 gm. of purified (1) 1-amino-2-naphthol-4-sulfonic acid (Eastman Organic Chemicals) with mechanical stirring to 200 ml. of freshly prepared 15 per cent sodium bisulfite (anhydrous), followed by 1.0 gm. of anhydrous sodium sulfite. The solution was filtered, stored in a dark bottle, and freshly prepared weekly.

Procedure for Total Phosphorus Analysis—Up to 2.0 ml. of the sample to be analyzed and 0.5 ml. of 10 N H_2SO_4 were placed in a 12-ml. conical centrifuge tube and heated in a 150–160° oven for at least 3 hours. 2 drops of 30 per cent H_2O_2 were added, and the solution was returned to the oven for at least 1.5 hours more to complete the combustion and to decompose all the peroxide. 4.6 ml. of 0.22 per cent ammonium molybdate (or 4.4 ml. of H_2O plus 0.2 ml. of 5 per cent ammonium molybdate) and 0.2 ml. of the Fiske-SubbaRow reagent were added, mixed thoroughly, and heated for 7 minutes in a boiling water bath, with marbles covering the tubes. The optical density at 830 $\text{m}\mu$ was recorded with the use of a Beckman model B photometer with the red-sensitive phototube. This procedure for color development at 100° is hereafter designated the heating method.

A longer period of digestion at 150–160° did not influence the results. If an aliquot greater than 2.0 ml. was to be analyzed, it was first evaporated to dryness in a mechanical convection oven at about 110°. 1.0 ml. of H_2O and 0.5 ml. of the 10 N H_2SO_4 were then added with rinsing of the walls of the tubes, and the assay was continued as above. When more than 1 mmole of ammonium formate was present, the sample was evaporated to dryness at 110° in order to remove the formate; then H_2O and H_2SO_4 were added and the analysis continued as above.

If the highest accuracy was not required, the following manipulations simplified and speeded multiple total phosphorus determinations on the eluates from column chromatographic separations. Aliquots from successive fractions were transferred to the 12-ml. centrifuge tubes with an automatic syringe,¹ and the various reagents were delivered from elevated storage bottles

through a solenoid dispenser² which was controlled by an electrical timer.³

To assay inorganic orthophosphate by this heating method, the phosphorus-containing solution was made to 4.1 ml. with water and 0.5 ml. of 10 N H_2SO_4 , 0.2 ml. of 5 per cent ammonium molybdate, and 0.2 ml. of the Fiske-SubbaRow reagent were added in succession with mixing. The solution was heated for 7 minutes at 100° and the color read at 830 $\text{m}\mu$.

Standard Curve—The color produced was proportional to the concentration of phosphorus up to 1.5 μM in the reaction mixture. Direct colorimetric assay was convenient to about 0.15 μM (Fig. 1). Solutions with higher color values, however, could, by dilution with water, be brought into the most accurate range for colorimetry without losing proportionality.

Spectrum—Fig. 2 compares the spectrum of the color produced in the phosphorus reaction at 100° with that given by the usual procedure at room temperature. The heating method gave a more intense and different shade of blue, but the greatest increase in light absorption was beyond the range of visual perception as shown by the relatively sharp spectral peak with a maximum at 830 $\text{m}\mu$. The optical density at the maximum (830 $\text{m}\mu$) in the heating method was 0.520 for 0.1 μmole of phosphorus in a total volume of 5.0 ml. (1-cm. light path), a value 7.2 times higher than the conventional method (measured at 660 $\text{m}\mu$).

Influence of Reagent Concentration and Heating Time; Stability of Color—The reliability of the method was reinforced by three factors: insensitivity to variations in the concentration of the reagents and in the heating time, and stability of the final color. The amount of molybdate or Fiske-SubbaRow reagent could be decreased or increased by one-third without influence on the color reaction. At sulfuric acid concentrations below 0.6 N, molybdate was directly reduced to a blue color and at sulfuric acid concentrations above 1.8 N, phosphorus color development was inhibited; between these extremes a constant color value was produced (Fig. 3). The color reached a maximum in 4 to 5 minutes after the solution approached 100° and remained constant for several minutes of heating thereafter (Fig. 4). The final color was stable at room temperature for at least 24 hours. In contrast, the customary Fiske and SubbaRow method at room temperature ((1), see below) is more dependent on the concentrations of the molybdate and Fiske-SubbaRow reagent, has a narrower range of permissible H_2SO_4 concentration (Fig. 3), and produces an unstable color.

* Supported in part by Grant No. H-1071, National Institutes of Health, United States Public Health Service.

¹ Becton, Dickinson and Company, Rutherford, New Jersey.

² Liquid Dispenser No. 665, Microchemical Specialties Company, Berkeley, California.

³ Model P-4R, 15 seconds, Industrial Timer Corporation, Newark, New Jersey.

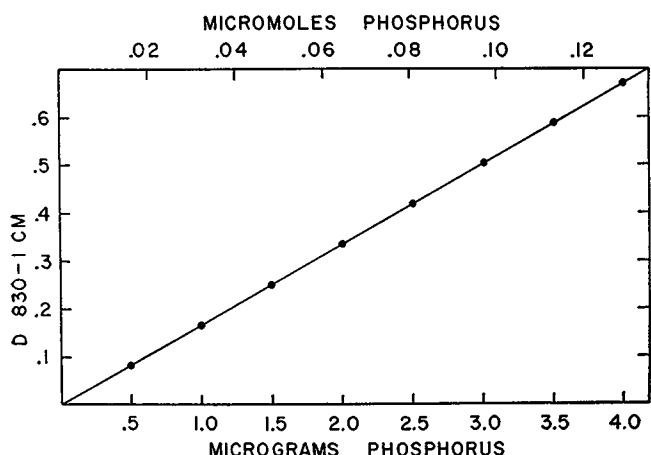


FIG. 1. Phosphorus standard curve with the heating method, optical density (D) measured at $830\text{ m}\mu$ with a 1-cm. light path.

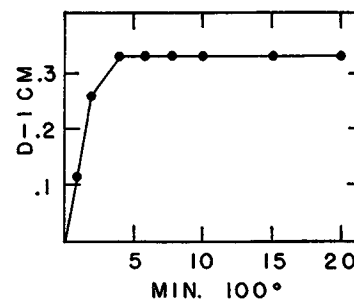


FIG. 4. Effect of the time of heating on color development in the heating method for phosphorus assay; optical density (D) measured at $830\text{ m}\mu$ with $2\text{ }\mu\text{g.}$ of phosphorus.

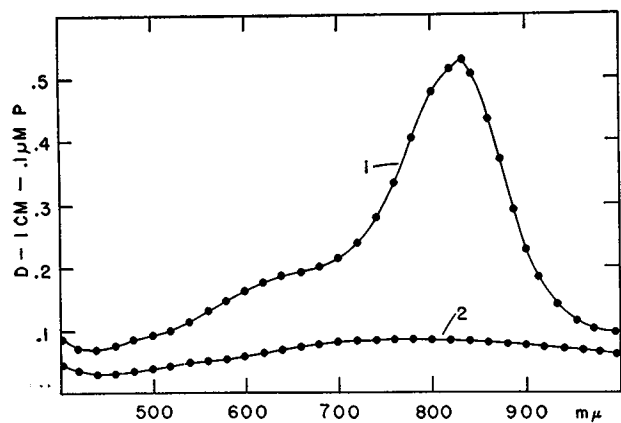


FIG. 2. Spectra of colors given by phosphorus assay procedures. 1. Heating method. 2. Room temperature method.

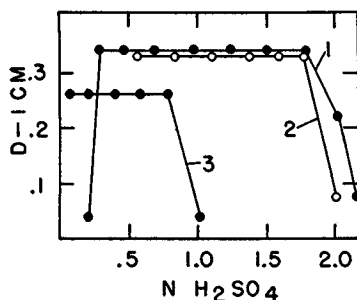


FIG. 3. Effect of sulfuric acid concentration on phosphorus assay procedures. 1. Heating method with the addition of 0.2 ml. of 15 per cent EDTA, optical density (D) measured at $830\text{ m}\mu$ with $2\text{ }\mu\text{g.}$ of phosphorus. 2. Heating method, D , $830\text{ m}\mu$, $2\text{ }\mu\text{g.}$ of phosphorus. 3. Room temperature method, D , $660\text{ m}\mu$, $10\text{ }\mu\text{g.}$ phosphorus. At sulfuric acid concentrations below the lowest points indicated Curves 2 and 3 gave blank colors due to the direct reduction of molybdate, and Curve 1 gave no color with or without phosphorus.

Effect of EDTA—First trials with the heating method, involving a final concentration of $0.5\text{ N H}_2\text{SO}_4$, disclosed that the addition of 0.2 ml. of 15 per cent of sodium EDTA prevented

⁴ The abbreviation used is: EDTA, ethylenediaminetetraacetate.

blank color development. Subsequently, it was found that increasing the sulfuric acid concentration made this addition unnecessary. However, it is of interest in respect to the mechanism of molybdate blue formation and it may prove experimentally useful in some circumstances that EDTA could extend the quantitative phosphomolybdate blue reaction to a lower range of acid concentration (Fig. 3). EDTA completely blocked the direct reduction of molybdate to a blue color at any pH but also prevented reduction of phosphomolybdate at higher pH values.

Effect of Arsenate and Silicate—Arsenate and silicate form molybdate compounds reducible to blue colors. At the high acidity used in this test silicate gave no reaction. Arsenate produced 60 per cent of the $830\text{ m}\mu$ absorption of an equimolar quantity of phosphate.

Ultramicro Modification of Total Phosphorus Method—Because of the constant low volume resulting from the wet ashing with sulfuric acid and hydrogen peroxide, the final volume of the phosphorus assay mixture could be reduced, with appropriate adjustment of the reagent concentrations, to increase the sensitivity of the method. 1.0 ml. proved a workable final volume, obviating resort to highly specialized microtechniques. The sample was combusted as before except that the amount of $10\text{ N H}_2\text{SO}_4$ was reduced to 0.3 ml. After combustion, 0.65 ml. of H_2O , 0.2 ml. of 5 per cent ammonium molybdate, and 0.05 ml. of the Fiske-SubbaRow reagent were added, and the solution was heated for 7 minutes at 100° . The optical density was read at $830\text{ m}\mu$ with the use of a $40 \times 10 \times 3\text{-mm.}$ cuvette (Pyrocell Manufacturing Company). The color value obtained was, as expected, 5 times that given by the procedure involving a 5-ml. volume.

Assay of Inorganic Orthophosphate in Presence of Phosphate Esters—The phosphorus heating method could not be used to assay inorganic orthophosphate in the presence of a number of phosphate esters which hydrolyzed under these conditions. The original Fiske and SubbaRow method (1) was found most reliable for measuring inorganic phosphate when mixed with phosphate esters, and the following conditions were used. 4.4 ml. of an approximately neutral orthophosphate-containing solution was mixed with 0.2 ml. of $10\text{ N H}_2\text{SO}_4$, 0.2 ml. of 5 per cent ammonium molybdate, and 0.2 ml. of the Fiske-SubbaRow reagent. The color was recorded at $660\text{ m}\mu$ after standing at room temperature for 7 to 10 minutes. The optical density at $660\text{ m}\mu$ was 0.363 for $0.5\text{ }\mu\text{mole}$ of phosphorus (1-cm. light path). The color gradually increased on further standing at room temperature with a more rapid rise in the $830\text{ m}\mu$ region as the character of the light absorption changed towards that obtained in the heating method.

Very acid-labile phosphates, such as acetyl phosphate and phosphocreatine, were largely converted to inorganic phosphate during this procedure. However, substances of intermediate lability, for example, the adenosine 5'-polyphosphates, glucose 1-phosphate, and the hexose diphosphates, were not appreciably cleaved.

DISCUSSION

Although many contributions have been made to the analysis of phosphorus based on the reduction of phosphomolybdate to blue colors (2), including proposals for augmenting the color at elevated temperature with the Fiske-SubbaRow reagent (3, 4), it is believed that the modifications described here offer significant advantages in ease of manipulation, accuracy, and sensitivity. The heating method for the analysis of total phosphorus

has been an important contributing factor in the development of ion exchange column chromatographic techniques for the separation of small amounts of organic phosphates of interest in intermediate carbohydrate metabolism (5) and for the isolation and identification of phosphate compounds from red blood cells (6, 7).

SUMMARY

A modification of the Fiske and SubbaRow method for the analysis of phosphorus is described which greatly increases the sensitivity and reliability of the method and is of special value for multiple total phosphorus determinations on eluates from chromatographic columns. The method is based on a large increase in the light absorption at 830 m μ which is produced by heating the phosphorus reaction mixture in relatively strong sulfuric acid.

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