Optimization of Conditions for Accurate Phosphonate and Total Phosphorus Assay on Lipid Samples, in Conjunction with Thin-Layer Chromatography

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The range of inorganic acid normalities for maximum color formation of the phosphomolybdenum-blue complex (under heating) increases by elevating the ammonium molybdate concentration, and at a ratio of molybdate molarity/acid normality equal to 10, there is maximum color development at any acid normality in the range 1–4 N with either $HClO_4$ or H_2SO_4 (or their mixtures).

On the basis of these features a revised method is described for the accurate determination of phosphonate-P percent of total-P, on lipid extracts and on TLC bands. The color at the final step, in both cases, is developed under the same conditions of molybdate, $HClO_4$ and H_2SO_4 concentrations, thus avoiding possible errors produced by the use of two separate calibration curves.

Introduction

During the last two decades it was progressively established that aminoalkylphosphonic acids, as inherent components lipid and protein molecules, are much more widespread in nature than previously recognized. In addition, accumulating evidence reveals a correlation between their occurrence and biological significance [1].

So far, the qualitative and quantitative determination of phosphono compounds in the presence of phospho-derivatives is based on the chemical stability of the C-P bond, which cannot be broken down by prolonged heating with mineral acids, *e.g.* treatment at 160 °C with concentrated sulphuric acid in conjunction with H_2O_2, according to Bartlett [2]. Therefore, the phosphonate content may be calculated [3] as the difference between total phosphorus determined by perchloric acid digestion and orthophosphate-phosphorus liberated according to Bartlett.

However, the phosphonates vary in stability to H_2SO_4, especially when H_2O_2 is added [3]. Furthermore, in a continuing research program on the naturally occurring phospho- and phosphono-lipids we have encountered several additional problems, *e.g.* incomplete decolorization of the lipid samples within the heating time of the Bartlett method [2, 3], nonsuitability of the method for analysis after TLC separation, etc.

With the aim to overcome such problems by simple techniques and to minimize any possible alterations of final conditions (*e.g.* acidity) that might even slightly influence the reproducibility and accuracy of the assay we have undertaken a systematic study of the effects of changing several experimental parameters on the optical density of the final blue color.

Methods and Results

Reagents

Deionized distilled water was used throughout to bring solutions to volume.

All glassware was cleaned with dichromate cleansing solution or with nitric acid to eliminate contaminating phosphates.

Anhydrous analytical grade KH_2PO_4 was used to prepare a stock phosphate standard with a phosphorus content of 40 μg P/ml; portions of this solution were diluted to give working standards as indicated in the following sections.

Analytical grade AEP*(Sigma) was used to prepare a stock phosphonate-P standard (40 μg P/ml).

Ammonium paramolybdate, $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$ was used to prepare ammonium molybdate reagents 0.4%, 1.6% and 4.0%; appropriate volumes of these solutions were used to give final concentrations of ammonium molybdate in the range 0.05–2.0% (see below).

Abbreviations: AEP, aminoethylphosphonate; ANSA, aminonaphtolsulfonic acid.

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The ANSA stock solution was prepared by dissolving 0.5 g of purified 1-amino-2-naphthol-4-sulfonic acid in 250 ml of 12% $NaHSO_3$ plus 2.4% Na_2SO_3. The solution was filtered into a dark bottle and stored in the refrigerator. A portion of this solution diluted *ad hoc* with 1.5 vol distilled water, was used as the working ANSA reagent.

Egg phospholipids and phospho- plus phosphonolipids from *Tetrahymena pyriformis* were prepared by extraction according to Bligh and Dyer (4) and removal of the neutral lipids according to Galanos and Kapoulas (5).

General Procedure

The influence of reagent concentration and heating time on maximum color formation was investigated on samples of inorganic phosphate standard solutions as follows: One ml of the standard phosphate solution (2 or 4 μg P/ml) was pipetted into each Pyrex test tube (15 × 180 nm) and mixed with 1 ml of ammonium molybdate reagent appropriately diluted in order to give the desired final concentration of ammonium molybdate. Then, 0.2–3.0 ml of 12 N $HClO_4$ or 24N H_2SO_4 (or their mixture, see below) were added and the volume was adjusted to 5.0 ml with distilled water. After mixing, 0.5 ml of the working ANSA reagent was added, the contents were mixed well (cyclo-mixer) and all the test tubes were placed in a boiling water bath for 20 min. After cooling, the absorbances were measured at 820 nm against distilled water. (Reagent blanks were run in all conditions and their absorbances at 820 nm (A_{820}) against distilled water were practically constant, *i.e.* in the range 0.030–0.040).

Influence of molybdate and inorganic acid concentrations

Preliminary tests had indicated that by increasing the final ammonium molybdate concentration the permissible highest concentration of $HClO_4$ or H_2SO_4 for maximum color development was also increased, this being more pronounced with $HClO_4$ that with H_2SO_4.

These effects were then investigated in a systematic series of tests involving measurements in the full range of 0.05–6.55 N $HClO_4$ and H_2SO_4 (alone and in 1:1 mixture) for each ammonium molybdate concentration in the range 0.2–1.0%. The results are depicted in Figs. 1 and 2, from which the following general features may be drawn:

1. The range of acid normality for maximum color development depends on both, the concentration of molybdate and the nature of the acid. However, in all the above conditions the blue color reaches the same maximum extinction (A_{820} = 0.94 ± 0.02 /μg P/ml = 0.680 ± 0.015 /4 μg P/5.5 ml).

2. With $HClO_4$ the plateau of maximum extinction (observed in the curves of A_{820} VS. acid normality) is much larger than with H_2SO_4, while with mixtures of $HClO_4$–H_2SO_4 (of equal normalities) the results are intermediate, closer to those with H_2SO_4 alone.

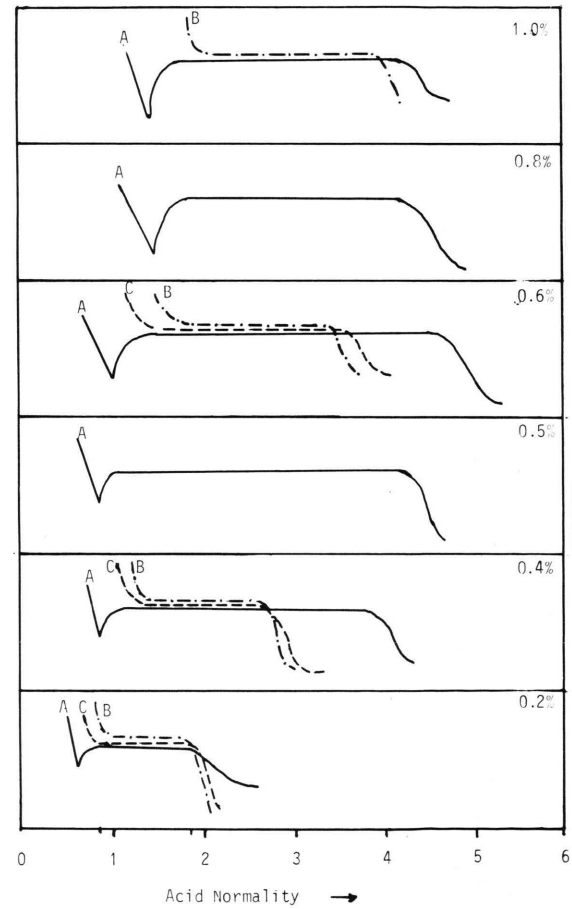

Fig. 1. Ranges of acid normalities allowing maximum color formation at different ammonium molybdate concentrations (as indicated in the upper right parts of each section). Curves A correspond to $HClO_4$ alone, curves B to H_2SO_4 alone, and curves C to $HClO_4$–H_2SO_4, 1:1. Maximum color of horizontal parts (plateaus) of all curves was the same (A_{820} = 0.680 ± 0.010).

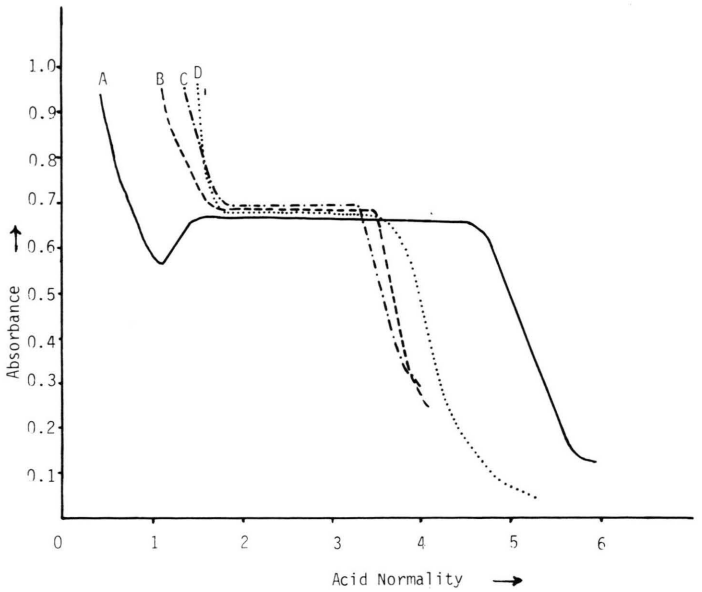

Fig. 2. Complete curves of absorbance versus acid normalities at 0.6% ammonium molybdate. Curve A corresponds to $HClO_4$ alone, B to $HClO_4$–H_2SO_4, 1:1, C to H_2SO_4 alone and D to $HClO_4$–H_2SO_4, 1:2. All four plateaus correspond to $A_{820} = 0.680 \pm 0.010$.

3. The highest permissible normality of $HClO_4$ for maximum extinction increases continuously as the ammonium molybdate concentration increases up to 0.5% and then it remains at approx. 4.5 N. With H_2SO_4 this increase is slower but it continues up to 0.8% (40 mM) molybdate and reaches the value of approx. 4 N.

As a consequence, when the ratio of molybdate molarity/acid normality is equal to 10 there is maximum color development at any final acid normality in the range 1–4 N with either $HClO_4$ or H_2SO_4 or any mixture of them.

4. The decrease of the non-specific molybdenum blue color formed at low acid normalities is steeper in the case of $HClO_4$ and this results in the appearance of an absorbance minimum to the left of the plateau. With H_2SO_4 this latter effect is almost hidden by the non-specific absorbance extending to higher acid normalities.

The features mentioned above are very useful for minimizing the possibilities of experimental error, especially in the differential determination of phosphonate-P where the normally accepted limits of experimental error may lead to highly erroneous values of phosphonate-P. Namely, the final normality 3 N of H_2SO_4-$HClO_4$ mixture in conjunction with 0.6% (30 mM) ammonium molybdate was chosen as offering the following advantages.

The amount of concentrated H_2SO_4 per unit mass of sample digested, according to Bartlett [2, 3] or to Kapoulas *et al.* [6], for liberation of inorganic phosphate from phosphate esters may be increased. The digestion for total-P determination may be carried out in a H_2SO_4–$HClO_4$ mixture that is a stronger oxidizing agent (than $HClO_4$ alone), ensuring the stoichiometric production of inorganic phosphate from phosphonate derivatives [6–9]. Furthermore, the color at the final step of the procedure may be developed under the same conditions of molybdate concentration and acidity, thus avoiding the possible errors from the use of two separate calibration curves while, at the same time, eventual losses of $HClO_4$ up to 50% during digestion for total-P do not affect the development of maximum absorptivity.

Effect of ANSA concentration and age; heating time

Test solutions were prepared by mixing in a beaker 10 ml of standard solution (4 μg P/ml) with the appropriate tenfold amounts of acid and ammonium molybdate reagent to a final volume of 50 ml. Then, 5 ml aliquots were transferred to separate test tubes and mixed with 0.5 ml of working ANSA reagent diluted up to 1:5. The measurements of A_{820} have shown that maximum color development occurs even at the lowest ANSA concentration used, correspond-

ing to 1/5 of its normal concentration. The same results were obtained by using ANSA solutions kept in the refrigerator for several periods up to 4 months.

Test solutions prepared in bulk as described above and including the appropriate amount of ANSA reagent were also used to study the rate of color formation during the final heating step of the procedure. The test tubes with the same contents were placed in a beaker containing vigorously boiling water (on the heater) and each test tube was withdrawn and cooled at different times up to 30 min. This type of experiment was repeated several times at different dates. In all cases it was found that extending the heating time up to 30 min has no effect on the maximum extinction, which remains constant. However, the results concerning the rate of color development were poorly reproducible. Namely, on one occasion maximum A_{820} was reached at 10 min in all samples, acidified either with $HClO_4$ or with H_2SO_4. On another two occasions this happened at 6 min with $HClO_4$ and 9–10 min with H_2SO_4. In other cases maximum color development occured after 10 min with $HClO_4$ and after 12–15 min with H_2SO_4 or $HClO_4$–H_2SO_4 mixtures. There is no record of the age of ANSA reagent corresponding to each of the above cases but it is reasonable to postulate that this may be a source of the poor reproducibility in the rate of color development mentioned above.

Since this source of possible error is eliminated by the prolonged heating time of 20 min, it is wise to adopt it in routine analysis.

Color extraction and spectra

Extraction of the blue color by ethyl acetate is very convenient to overcome problems of incomplete decolorization of samples during phosphonate-P determination [5], as well as the problem of turbidity caused by fine particles of silica in the phosphorus assays after TLC separation. Furthermore, extraction with smaller volumes of ethyl acetate may increase the sensitivity of the method (by concentrating the color). By using the data depicted in Fig. 3 it may be calculated that by extracting 5.5 ml of aqueous layer with 4.8 or 3.3 or 2.0 ml, respectively, and taking into consideration that the A_{780} of the extracted color is higher than the A_{820} of the aqueous color by approx. 20%, that the overall sensitivity is increased in the above cases by 37%, 100% or 230% respectively.

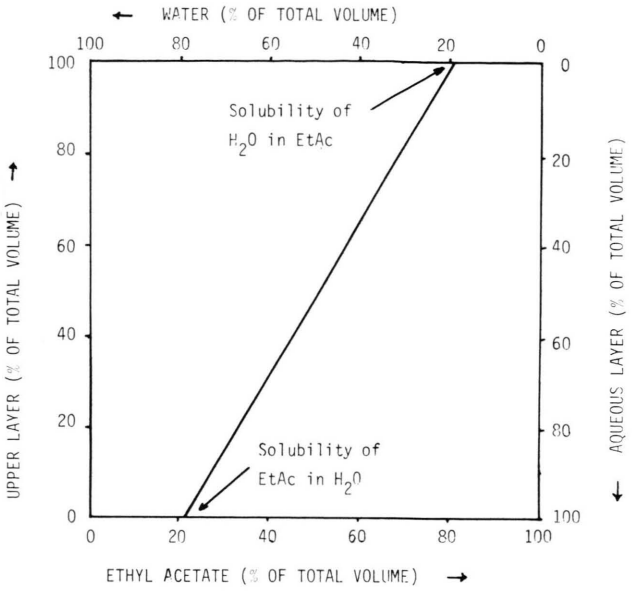

Fig. 3. Relative volumes of upper and lower layers formed in binary systems of water-ethyl acetate.

To facilitate the separation and equilibration of phases, 2 ml of 2–5% Na_2SO_4 may substitute for 2 ml H_2O in the test solution. It was found that this addition of Na_2SO_4 to increase the density of the aqueous layer has no effect in maximum color development.

Fig. 4 compares the spectra of the colors extracted by ethyl acetate with those given by the aqueous test solutions (before extraction). Although the absorbance maxima of aqueous colors derived with $HClO_4$, H_2SO_4 alone and in mixture are at 815 nm, 819 nm and 812 nm, respectively, those of all the extracted colors are at 780 nm.

Hydrolysis conditions of phosphate esters

Standard samples of AEP containing 3–10 μg P (total) were subjected to digestion with 0.5 ml 24 N H_2SO_4 for 5 h in a sandbath at 210 °C. It was found that even at this elevated temperature and prolonged heating there is no liberation of inorganic phosphate from AEP by concentrated H_2SO_4 alone.

Revised methods for phosphonate and total P determinations

In the light of the findings described above the recently published methods [6] were revised as follows:

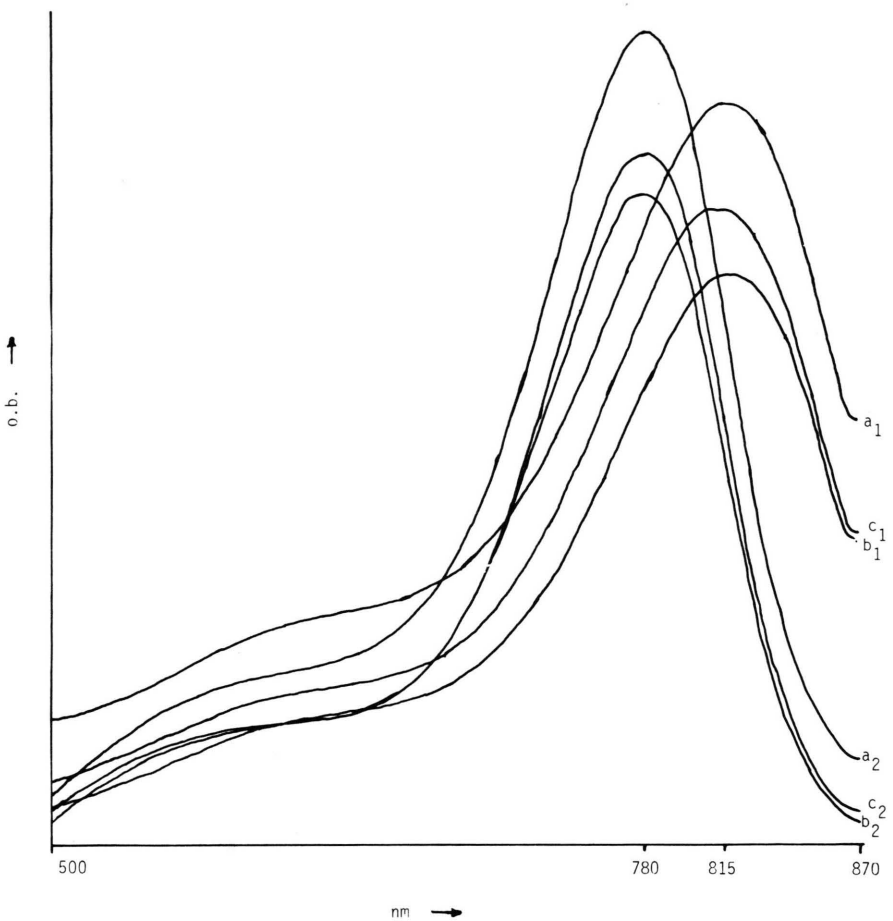

Fig. 4. Spectra of the phosphomolybdenum blue with 4 μg P of stock Phosphonate standard, a_1, b_1, c_1, without extraction and a_2, b_2, c_2 after extraction with 5 ml ethyl acetate, a_1, a_2: 72% $HClO_4$ as the mineral acid, b_1, b_2: 12 N H_2SO_4 as the mineral acid and c_1, c_2: 12 N H_2SO_4/72% $HClO_4$, 1:1 (v:v) as the mineral acid. Spectrophotometer Vis-UV Hitachi 100–80.

1. Transfer duplicate aliquots A and B (0.4–4.0 μg P) of lipid sample into Pyrex test tubes (15 × 180 nm) and evaporate solvents. Add 0.5 ml 72% $HClO_4$ to sample A and 0.4 ml 24 N H_2SO_4 to both samples. Prepare also a set of standards and blank by adding to the aqueous standard solutions 0.5 ml of both acids (as in sample A).

2. Place all tubes in a sandbath of 175–180 °C for 90 min.

3. Remove from the sand bath the tubes of standards and sample(s) A, raise the temperature to 205 ± 5 °C and continue heating the tube(s) B for another 3 h, then allow them cool to ambient temperature. To accelerate evaporation of water it is helpful to prevent the free circulation of air by protecting the tube walls, with a piece of aluminium foil surrounding all the tubes together.

4. Add to all tubes 2 ml 2% Na_2SO_4 and 2 ml 1.6% ammonium molybdate and mix the contents. Then, add to tube(s) B 0.5 ml 72% $HClO_4$ and mix again.

5. Add to all tubes 0.5 ml of the working ANSA reagent, mix well and place in boiling water for 20 min.

6. Cool, add 5 ml ethyl acetate and mix well by vortexing for 30 sec. Allow 15 min for phase quilibration and measure the A_{780} of the clear upper layers against the extract of the blank (very dense extracts may be diluted with ethyl acetate).

7. The same calibration curve is used to calculate the inorganic phosphorus contents of both samples, A and B. The "phosphonate-P % of total-P" is equal to 100 $(M_A - M_B)/M_B$ where M_A and M_B are either the respective A_{780} values, or the respective P contents calculated from the calibration curve which, obviously, is not necessary for the determination of the percentage of phosphonate-P in the total-P.

Methods in conjunction with TLC

The total-P content of spots after TLC separation may be determined by the above method as applied to samples A and standards, with the following modification:

For the preparation of blanks and standards appropriate areas of the chromatoplate, free of phospholipids are transferred to the respective tubes.

For the determination of phosphonate-P % of total-P in the same TLC spot the following modification was used:

1. Transfer the silica gel area of the spot into a test tube, add 0.5 ml 24 N H_2SO_4 and digest for 3 h at 205 ± 5 °C (sand bath).

2. Cool, add 0.9 ml water, mix well and after cooling, transfer 0.5 ml aliquots into two separate test tubes, A and B. To the tube A add 0.25 ml 72% $HClO_4$ and heat both tubes for 90 min at 180 °C, then cool to ambient temperature.

3. Proceed as in the original procedure by using half of the reagent volumes, *i.e.* 1 ml 2% Na_2SO_4, 1 ml 1.6% ammonium molybdate and 0.25 ml ANSA for all tubes plus 0.25 ml 72% $HClO_4$ for tube(s) B. The color may be extracted with 3 ml (or more) ethyl acetate if this is required by the minimum permissible volume in the spectrophotometer cuvette.

Discussion

In the revised methods described above the liberation of inorganic phosphate from phosphate esters is effected with increased amounts of H_2SO_4 per unit mass of lipid sample and at higher temperatures than in previous methods [6]. The so formed inorganic phosphate and total phosphate are measured under the same final conditions of color formation. In addition, the present methods are not sensitive to eventual losses of $HClO_4$ during digestion. These are important advantages in favor of reproducibility in routine work, especially for the analysis of lipid samples of low phosphonate content (percent of total P).

The present methods were tested on samples of phosphonate-free egg phospholipids, Tetrahymena phospholipids of known phosphonate content, pure AEP and artificial mixtures of them. In all cases the results verified the arguments mentioned above.

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