104. THE ESTIMATION OF PHOSPHORUS

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Osmond [1887] proposed the estimation of P by causing the formation of phosphomolybdic acid and its subsequent reduction to a blue compound which could be measured colorimetrically. Of the many variants of this procedure, that of Fiske & Subbarow [1925] has perhaps been the most widely adopted.[2] They carried out the reduction in strongly acid solution (0·5*N* H_2SO_4) and used as reducing reagent a solution of 1-amino-2-naphthol-4-sulphonic acid in sulphite-bisulphite mixture. The principal advantages claimed for the method were (1) rapidity of colour development; (2) proportionality between colour density and amount of P over a wide range; (3) relative freedom from interference by such substances as ammonium and iron salts, nitrates, nitrites, silicates and chlorides.

King [1932] recommended the use of perchloric acid (final concentration 0·73*N*) in place of sulphuric acid in the method of Fiske & Subbarow. This facilitates the preliminary destruction of organic material necessary for the determination of total P. The solubility of barium and calcium perchlorates confers added advantages, e.g. in determinations on plant extracts which contain sufficient calcium to cause troublesome precipitates with sulphuric acid and on barium and calcium salts of phosphoric esters.

The method of P estimation originally used by the author was a simple adaptation of King's perchloric acid method for use with an absolute photometer. An aliquot of the solution of orthophosphate[3] under examination was placed in a 25 ml. volumetric flask, and to it were added 2 ml. 60 % perchloric acid, 1 ml. 8·3 % ammonium molybdate and 1 ml. reducing reagent (0·417 g. 1-amino-2-naphthol-4-sulphonic acid, 5 g. crystalline sodium sulphite and 25 g. sodium bisulphite in 250 ml. water). Water was added to 25 ml. and after about 20 min. the extinction coefficient *K* (the extinction or colour density of a layer of solution 1 cm. thick) was measured in the photometer, using a deep red filter (Zeiss S 72) and an absorption cell of appropriate thickness (0·5–3 cm.). The concentration of P present was then read from a standardization (*K* against mg. P) curve established with known amounts of pure primary potassium phosphate.

The relation between *K* and the period of colour development with low, medium and high concentrations of P is shown in Table 1. A comparatively rapid increase in *K* is followed by a long period of slow increase. In order to avoid appreciable errors, it was thus necessary always to determine *K* after a

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[2] The method of Lohmann & Jendrassik [1926], which is frequently cited in the German literature, differs only slightly from that of Fiske & Subbarow.

[3] The procedure for the estimation of total P differed only in that the material under examination was first digested with a slightly greater quantity (2·2 ml.) of perchloric acid (see p. 861).

definite period (20 min.), a matter of considerable inconvenience when a large number of analyses were to be made.[1]

A more serious objection became apparent when it was found that the extinction coefficient at 20 min. varies considerably with the temperature. This was shown in the following experiment.

Table 1. *Relation between time and extinction coefficient in King's method*

P, mg./25 ml. final solution ...	0·010	0·100	0·400
Av. temp. during exp. period ...	17°	14°	19°
Time	*K*	*K*	*K*
2 min.	0·060	0·503	1·540
5 ,,	0·060	0·530	1·960
8 ,,	0·061	0·535	2·044
20 ,,	0·063	0·550	2·145
30 ,,	0·064	0·558	2·180
50 ,,	0·069	0·574	2·200
3 hr.	0·074	0·635	2·370
7 ,,	0·082	0·730	2·600

Table 2. *Influence of temperature on extinction coefficient in King's method*

Av. temp. of solution during colour development (±1°)	*K* (20 min.)
−1·5°	0·370
+3·2°	0·530
8·0°	0·540
12·5°	0·548
16·6°	0·560
20·3°	0·568
24·3°	0·582
26·8°	0·588
34·4°	0·620

5 ml. standard phosphate solution (=0·100 mg. P) were placed in each of nine 25 ml. volumetric flasks and 2 ml. 60% perchloric acid and about 14 ml. water added to each. The flasks were brought to different temperatures, molybdate, sulphonic acid reagent and water to 25 ml. were added and the extinction coefficients determined after 20 min. (Table 2).

These features are less important when the solution under examination is always compared with a standard solution prepared simultaneously, but become serious when the analysis depends on the measurement of the absolute colour density. In the method of P determination described below these disadvantages are eliminated. The necessary period of colour development is also much reduced; not only is a considerable saving in time effected thereby, but the extent of hydrolysis of labile organic P compounds (e.g. glucose-1-phosphate) is proportionately reduced.

The modified method

Several references to the possibility of using amidol (2:4-diaminophenol hydrochloride) as the reducing agent in the colorimetric estimation of P are to be found in the literature. Fiske & Subbarow [1925] described amidol as superior to quinol for this purpose but (for reasons unstated) "not entirely satisfactory

[1] Brose & Jones [1936] have shown that in the case of the original Fiske & Subbarow [1925] method the colour density continues to increase for a number of hours.

from every standpoint," whilst Tschopp & Tschopp [1932] included it in a group of substances which were found satisfactory but rejected on economic grounds. Finally Müller [1935] found that if a solution of amidol in sodium sulphite is substituted for the sulphonic acid reagent in Fiske & Subbarow's method, the blue colour produced with amounts of P between 0·05 and 0·10 mg. reaches a maximum density within 5 min. and remains constant for a further 25 min. It has been found, using Müller's reagent in the presence of perchloric acid, that the colour produced tends to be purple and that the intensity fails to reach a steady maximum with amounts of P much above 0·1 mg. Further, Müller's reagent proved to be inconveniently unstable. These difficulties are eliminated in the procedure now described.

Solutions

Ammonium molybdate solution. An 8·3 % solution of the A.R. salt in distilled water. A small quantity of conc. ammonium hydroxide may be added to facilitate solution.

Perchloric acid. 60 % solution (specific gravity 1·54) A.R.

Amidol reagent. 2 g. amidol and 40 g. pure sodium bisulphite are dissolved in glass-distilled water and diluted to 200 ml. The reagent is best kept in a well-stoppered bottle painted black on the outside. It is advisable to discard the solution after about 10 days.

Hydrogen peroxide. 30 % ("100 volumes") solution, free from P. The "M.A.R." grade sold by Messrs British Drug Houses, Ltd. is suitable, but bottles in which the paraffin lining is broken should be rejected.

Standard phosphate solutions. A stock solution containing 1 mg. P per ml. is prepared by dissolving 1·0967 g. A.R. KH_2PO_4 (dried in an air oven) in distilled water and diluting to 250 ml. This solution remains unaltered for at least 2 years if stored over chloroform at 0°. Suitable standard solutions for the calibration of the photometer are obtained by dilution of the stock solution.

Estimation of orthophosphate

An aliquot ($\geq$ 20 ml.) of the solution under examination, containing up to 0·4 mg. P, is placed in a 25 ml. volumetric flask and to it are added 2 ml. perchloric acid, 2 ml. amidol reagent and 1 ml. ammonium molybdate in that order. Water to 25 ml. is added, the solution mixed and the extinction coefficient is determined after an interval of 5–30 min. The amount of P present is then read from a calibration curve (K against mg. P) obtained by applying the same procedure to aliquots of standard phosphate solutions containing from 0·01 to 0·4 mg. P.

Table 3 gives the calibration data for the deep red filter (Zeiss S 72) used in the present investigation.

Table 3. *Calibration data for the modified method, using a deep red filter (Zeiss S 72)*

P, mg./25 ml. final solution	K	mg. P/K
0·010	0·055	0·180
0·020	0·110	0·182
0·050	0·270	0·185
0·100	0·532	0·188
0·150	0·798	0·190
0·200	1·036	0·193
0·300	1·564	0·192
0·400	2·070	0·193

Estimation of total P

An appropriate amount of the material (solid or solution) under examination is placed in a micro-Kjeldahl flask (hard glass) and 2·2 ml.[1] perchloric acid and a small piece of porous pot (to prevent bumping) are added. The flask is heated over a micro-burner until the contents have become colourless [King, 1932]. The addition of a few drops of H_2O_2 may be required to complete the combustion if much organic material is present. After cooling the flask, its contents are thoroughly rinsed into a 25 ml. volumetric flask, 2 ml. amidol, 1 ml. molybdate and water to 25 ml. are added, and the extinction coefficient is determined between 5 and 30 min. later.

If the highest accuracy is not essential, considerable time may be saved by developing the blue colour in the digestion flask. In order to do this 20 ml. distilled water, followed by 2 ml. amidol and 1 ml. molybdate, are added to the cooled colourless digest and the extinction coefficient is determined as before. This abbreviated procedure will involve no important error as long as the volume of perchloric acid at the end of the digestion does not differ appreciably from 2 ml. It has been employed successfully for some time with trichloroacetic acid extracts of apples and potatoes; the following experiment (Table 4) shows that even in the presence of large amounts of organic material (and NaCl) a good recovery of added P is obtained.

Table 4

0·100 mg. P (as KH_2PO_4) was added to each of 10 digestion flasks together with 2·2 ml. perchloric acid, a small piece of porous pot and 0·5 g. of the substance indicated. The digestion and colour development were then carried out by the abbreviated method.

No.	Substance added	mg. P recovered
1	Glucose	0·100
2	Glucose	0·100
3	Glycine	0·100
4	Glycine	0·100
5	Oxalic acid	0·100
6	Oxalic acid	0·101
7	Citric acid	0·100
8	Ammonium acetate	0·101
9	Sodium chloride	0·103
10	Sodium chloride	0·103

The only substance so far encountered which is not readily digested by the King perchloric acid technique is urea. If a considerable amount of this substance is present the contents of the digestion flask become solid, and if the heating is prolonged most of the perchloric acid is volatilized. According to Rae & Eastcott [1939] this difficulty may readily be overcome by a preliminary treatment of the urea solution with HNO_3.

Relation between extinction coefficient and time

The extinction coefficients with 0·010, 0·100 and 0·400 mg. P observed at different times after addition of the usual reagents are shown in Table 5. It will be seen that during the interval between 3 and 30 min. the colour density in each case was virtually constant. Later a slow increase is detectable.

[1] This slightly larger amount of perchloric acid is used in order to compensate for the loss (0·2 ml.) during digestion [King, 1932].

Table 5. *Relation between time and extinction coefficient in the modified method*

P, mg./25 ml. final solution ...	0·010	0·100	0·400
Time after addition of molybdate		*K*	
1 min.	0·049	0·498	—
1·5 ,,	0·055	—	—
2 ,,	—	0·525	—
3 ,,	—	0·531	2·060
4 ,,	—	—	2·070
15 ,,	0·055	0·531	2·070
30 ,,	0·055	0·531	2·070
40 ,,	0·056	0·532	2·100
60 ,,	0·057	0·535	2·130
2 hr.	0·059	0·548	2·220
3·7 ,,	0·066	0·590	—
6·5 ,,	0·076	0·635	—

Influence of temperature on extinction coefficient

The results in Table 6 were obtained in an experiment analogous to that summarized in Table 2, but using the modified method of P estimation. The extinction coefficient was measured after 5 and after 30 min. colour development at the temperatures indicated.

Table 6. *Influence of temperature on extinction coefficient in the modified method*

Av. temp. during the period 0–30 min. (±1°)	P, mg./25 ml. final solution	*K* (5 min.)	*K* (30 min.)
8·2	0·010	0·055	0·055
10·4	0·010	0·055	0·055
17·1	0·010	0·055	0·055
26·0	0·010	0·055	0·055
27·8	0·010	0·055	0·057
32·8	0·010	0·068	—
12·0	0·100	0·532	0·532
16·0	0·100	0·531	0·532
19·6	0·100	0·532	0·532
24·5	0·100	0·532	0·533
8·0	0·400	1·980	2·070
8·2	0·400	2·070	—
27·9	0·400	2·070	2·080
28·4	0·400	2·070	2·110
33·5	0·400	2·070	2·200

It will be observed that variations in temperature between approximately 8° and 26° do not affect significantly the extinction coefficient during the 5–30 min. interval.

Effect of variation of the quantities of perchloric acid, amidol reagent and ammonium molybdate used in the estimation

From Table 7 it will be seen that the amount of perchloric acid per 25 ml. of final solution may vary between 1·0 and 2·4 ml. without sensibly affecting the extinction coefficient of the solution; the amount of amidol reagent may vary

between 1·7 and 2·2 ml., and that of molybdate between 0·9 and 1·5 ml. (1·1 ml. in the case of very low amounts of P).[1]

Table 7. *Effect of variation of the quantities of reagents used*

Perchloric acid			Amidol reagent			Ammonium molybdate		
mg. P	Amount added ml.	*K* (5–30 min.)	mg. P	Amount added ml.	*K* (5–30 min.)	mg. P	Amount added ml.	*K* (5–30 min.)
0·100	1·0	0·532	0·010	1·8	0·055	0·010	0·9	0·055
0·100	1·5	0·533	0·010	**2·0**	**0·055**	**0·010**	**1·0**	**0·055**
0·100	1·9	0·531	0·010	2·2	0·055	0·010	1·1	0·055
0·100	**2·0**	**0·532**				0·010	1·2	0·060
0·100	2·2	0·532	0·400	1·7	2·065	0·010	1·3	0·064
0·100	2·3	0·532	0·400	1·8	2·065			
0·100	2·4	0·532	0·400	**2·0**	**2·070**	0·400	0·7	2·000
0·100	2·5	0·500	0·400	2·2	2·080	0·400	0·8	2·050
0·100	3·0	0·461	0·400	2·3	2·096	0·400	0·9	2·070
						0·400	**1·0**	**2·070**
						0·400	1·3	2·080
						0·400	1·5	2·076

Effect of interfering substances

In Table 8 are given the approximate maximum concentrations of a number of substances which may be present in the final solution in the estimation of P without interfering with the analysis. The first 8 substances when present in concentrations higher than those indicated cause a decrease in colour density, the last 2 an increase.

Table 8. *Maximum permissible concentrations of interfering substances*

No.	Substance	Permissible concentration in presence of 0·400 mg. P *M*	Permissible concentration in presence of 0·010 mg. P *M*	Remarks
1	$(NH_4)_2SO_4$	0·4	0·75	—
2	NaCl	0·5	0·6	—
3	$NaNO_3$	0·5	0·8	—
4	$CCl_3.COOH$	0·25	0·5	—
5	NaF	0·01	0·02	—
6	$K_2C_2O_4, H_2O$	0·002	0·004	—
7	$FeCl_3$	0·0013	0·0013	—
8	Na_2SiO_3	0·0043*	0·00011*	Ratio Si : P = 7·5
9	C_2H_5OH	1·8	1·8	Equivalent to 2·5 ml. 95 % alcohol per 25 ml. final solution

* The sensitivity to silicate is considerably greater if the molybdate is added to the test solution before the amidol reagent [cf. Tschopp & Tschopp, 1932].

The effects of most of the above substances on the determination of P by the method of Fiske & Subbarow [1925] were examined by these authors and by Vársárhelyi [1930]; King [1932] made a shorter but similar study in connexion with his modified method. Although the maximum permissible concentrations of these substances were not clearly defined in these investigations, it can be stated that the present method exhibits at least as high a degree of tolerance as the older methods.

[1] It is, of course, not suggested that the reagents be added so carelessly as to give rise to variations of this magnitude. In the presence of interfering substances the tolerances to variations in the amounts of reagents used may be considerably less than these indicated here.

A method for the determination of phosphorus in turbid or highly coloured solutions

The determination of orthophosphate in higher plant extracts is frequently complicated by the fact that these exhibit appreciable turbidity or colour; this is the case, for example, with trichloroacetic acid extracts of potatoes, apples, bananas and onions. The turbidity, due apparently to the presence of polysaccharide material, increases in some cases after the addition of perchloric acid and molybdate. An approximate correction for these effects may be applied if a separate measurement is made of the extinction value exhibited by a blank determination from which the reducing reagent is omitted; this correction cannot be made, however, when the extract contains strong reducing substances, e.g. ascorbic acid.

To overcome a somewhat similar difficulty encountered with extracts of animal tissue Delory [1938] has recommended the preliminary precipitation of orthophosphate by the addition of $CaCl_2$, NH_4OH and "light" magnesium carbonate; the precipitate is separated on the centrifuge, and, after washing, the determination is carried out on this material. When this process is applied to extracts of higher plants, however, only a small proportion of the inorganic phosphate present is precipitated. Thus with trichloroacetic acid extracts of the banana, only a trace of inorganic phosphate is precipitated, and even if a double excess of the precipitating reagents is employed and the time allowed for precipitation is increased up to 4 hr., only about a quarter of the phosphate present is thrown down. The Delory procedure is therefore not generally applicable to extracts of the higher plants.

It was hoped that the difficulty might be eliminated by the use of the Berenblum & Chain [1938] modification of the Kuttner & Cohen [1927] method. In this, H_2SO_4 and molybdate are added to the solution of orthophosphate to be analysed and the yellow molybdic acid is extracted from the aqueous phase with *iso*butyl alcohol. The alcohol layer is removed and washed, shaken with stannous chloride solution for a standard time and the blue colour, which remains in the *iso*butyl alcohol, measured in a photometer. When the method is applied to trichloroacetic acid extracts of bananas, however, two defects become apparent: (1) added inorganic phosphate can only be recovered to the extent of 70–80 %; (2) when *iso*butyl alcohol is shaken with a trichloroacetic acid extract without previous addition of molybdate, some material passes into the alcohol layer which gives a distinct blue colour with the subsequent reagent.

While the method of Berenblum & Chain [1938] is thus not applicable to extracts from certain higher plants at least, the following procedure, based on extraction of the reduced phosphomolybdic acid in *iso*butyl alcohol, has been used successfully.

A peculiar defect observed in the first trials of the method was that the density of the blue colour increased at an appreciable rate after its extraction into *iso*butyl alcohol. This effect, which was apparently due to reduction of molybdic acid, has been eliminated by the addition to the aqueous solution, before the extraction, of enough oxalic acid to combine with the excess molybdate. (Molybdic and oxalic acids combine to form a very stable oxalomolybdic acid [see Davies & Davies, 1932].) The extracted blue colour is thereby rendered stable for at least 24 hr.

Solutions. Besides those described on p. 860, ethyl alcohol (95 %), *iso*butyl alcohol (redistilled) and 10 % (saturated) oxalic acid solution are required.

Procedure. An aliquot ($\geq$ 20 ml.) of the solution of orthophosphate under examination, containing up to 0·4 mg. P, is placed in a 25 ml. volumetric flask

and treated exactly as described under "Estimation of orthophosphate" on p. 860. After the blue colour has developed for from 5 to 30 min., 1 ml. 10% oxalic acid is added. After mixing thoroughly, the contents of the flask are transferred quantitatively to a separating funnel, the flask being rinsed out with a little *iso*butyl alcohol. About 10 ml. *iso*butyl alcohol are then added to the contents of the funnel, the mixture is swirled for a few moments, the phases are allowed to separate and the aqueous (lower) layer is run off into a clean beaker. The blue *iso*butyl alcohol layer is run into a 25 ml. flask and the sides of the funnel are rinsed into the same flask with a little ethyl alcohol. The aqueous phase is then returned to the funnel and the extraction repeated with a further 5 ml. of *iso*butyl alcohol. The combined *iso*butyl alcohol extracts are then diluted to 25 ml. with ethyl alcohol, and the extinction coefficient is measured within 24 hr. The amount of P present is read from a calibration curve (K against mg. P) obtained by applying the same procedure to a series of standard phosphate solutions.

The calibration data for the deep red filter used in the present investigation are given in Table 9.

Table 9. *Calibration data for the* iso*butyl alcohol extraction method, using a deep red filter (Zeiss S 72)*

mg. P	K	mg. P/K
0·010	0·064	0·156
0·050	0·319	0·157
0·080	0·505	0·158
0·100	0·631	0·158
0·125	0·760	0·164
0·150	0·920	0·163
0·200	1·220	0·164
0·300	1·820	0·165
0·400	2·440	0·164

SUMMARY

Certain defects in the King-Fiske & Subbarow method of P estimation, which become serious when the method of analysis depends on the determination of absolute colour density, are described. A method of P estimation is proposed in which these adverse features are eliminated.

A procedure for the determination of P in cloudy or highly coloured solutions is described.

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