Megazyme

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PHOSPHATE

ASSAY PROCEDURE

K-PHOS 09/19

(100 Manual Assays per Kit) or
(400 Auto-Analyser Assays per Kit)

INTRODUCTION:

Inorganic sources of phosphate are often added to foods to extend shelf life, improve taste and colour. As a result, phosphate is commonly found in highly processed, convenience food items, such as frozen meats, processed meats, processed cheeses, colas, cereals, and bakery items. Phosphate is also a key parameter used in water quality assessment. Excess levels of phosphate can lead to excessive growth of algae, a process termed eutrophication.

Traditionally, inorganic phosphate has been detected using the "molybdenum blue reaction" through the resulting UV absorbance change associated with complexation to molybdenum-based reagents.[1] This reaction has recently been described as a "black box" owing to the myriad of potential UV active products formed. The limitations of this method include the long reaction time, the use of heavy metals, and most notably, the requirement to run a phosphate calibration curve with each set of assays. In 1992 Webb published a far superior method for the measurement of phosphate,[2] which Megazyme have now further optimised to address all of the limitations of the molybdenum blue based assay methodology.

PRINCIPLE:

The phosphate quantification assay is based on the purine nucleoside phosphorylase (PNPase) mediated, conversion of 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to 2-amino-6-mercapto-7-methylpurine and ribose-1-phosphate in the presence of free inorganic phosphate. This reaction occurs with a shift in the UV absorbance maximum from 330 nm to 360 nm. The assay principle is depicted in Figure 1 (page 9).

$$P_i + \text{MESG} \xrightarrow{\text{(PNPase)}} \text{ribose 1-phosphate} + \text{2-amino-6-mercapto-7-methylpurine}$$

SENSITIVITY, LINEARITY AND PRECISION:

The smallest differentiating absorbance for the assay is 0.03 absorbance units, this corresponds to 0.49 mg/L of sample solution at the maximum sample volume of 0.7 mL or 3.43 mg/L with a sample volume of 0.1 mL. The detection limit is 0.16 mg/L which is derived from an absorbance difference of 0.01 and the maximum sample volume of 0.7 mL. The assay is linear over the range of 0.1 to 10 µg of phosphate per assay, this is shown in Figure 2 (page 9).

In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 0.7 mL, this corresponds to a phosphate concentration of approx. 0.08 mg/L to 0.16 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multipliedby the dilution factor, F.

INTERFERENCE:

If the conversion of phosphate has been completed within the time specified in the assay (approx. 20 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding phosphate (approx. 5 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding phosphate to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 100 assays in manual format (or 400 assays in auto-analyser format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1:	Buffer (20 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.
Bottle 2: (x2)	MESG. Freeze dried powder. Stable for > 5 years below -10°C.
Bottle 3:	PNPase suspension (1 mL). Stable for > 2 years at 4°C.
Bottle 4:	Phosphate standard solution (5 mL, 0.05 mg/mL) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years; store sealed at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of one of bottle 2 in 5 mL of distilled water. Use immediately or divide into 1 mL aliquots and store in polypropylene tubes below -10°C. Do not re-freeze once thawed. Stable for > 2 years below -10°C. Do not dissolve the contents of the second bottle until required.

3. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottle to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position.
Stable for > 2 years at 4°C.

4. Use the contents of bottle 4 as supplied.
Stable for > 2 years; store sealed at 4°C.

NOTE: The phosphate standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of phosphate is determined directly from the extinction coefficient of MESG.

EQUIPMENT (RECOMMENDED):

1. Polypropylene tubes (5 mL capacity).
2. Disposable plastic cuvettes (1 cm light path, 1.5 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.2 mL aliquots of buffer (bottle 1) and 0.1 mL aliquots of MESG solution].
   - with 25 mL Combitip® [to dispense 5 mL aliquots of distilled water used for dissolving MESG powder].
5. Analytical balance.
6. Spectrophotometer set at 360 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 360 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: 22°C
Final volume: 1.01 mL
Sample solution: 0.1-10 µg of phosphate per cuvette (in 0.1-0.7 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 22°C)	0.70 mL	0.60 mL
sample	-	0.10 mL
solution 1 (buffer)	0.20 mL	0.20 mL
solution 2 (MESG)	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reaction by addition of:		
suspension 3 (PNPase)	0.01 mL	0.01 mL
Mix* and read the absorbance of the solutions (A_2) at the end of the reaction (~ 20 min).		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining ΔA_{phos} .

In order to achieve sufficiently accurate results the value of ΔA_{phos} should as a rule be at least 0.1 absorbance units, and not more than 1.0 absorbance units.

The concentration of phosphate can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{phos} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of phosphate [g/mol]
ε = extinction coefficient of MESG at 360 nm
= 8400 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for phosphate

$$c = \frac{1.01 \times 94.97}{8400 \times 1.0 \times 0.1} \times \Delta A_{phos} \quad [g/L]$$

$$= 0.114 \times \Delta A_{phos} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of phosphate

$$= \frac{c_{phosphate}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for phosphate can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of phosphate **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
distilled water	12.5 mL
solution 1 (buffer)	5.0 mL
solution 2 (MESG)	2.5 mL (after adding 5 mL of H_2O to bottle 2)
Total volume	20 mL

Preparation of R2:

Component	Volume
distilled water	12.5 mL
suspension 3 (PNPase)	5.0 mL
Total volume	17.5 mL

EXAMPLE METHOD:

R1:	0.2 mL
Sample:	0.025 mL
R2:	0.025 mL
Reaction time:	~ 20 min at 22°C
Wavelength:	360 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.1 g/L of phosphate using 0.025 mL sample volume

SAMPLE PREPARATION:

1. Sample dilution.

The amount of phosphate present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.1 and 10 µg. The sample solution must therefore be diluted sufficiently to yield a phosphate concentration between 0.001 g/L and 0.10 g/L.

Dilution Table

Estimated concentration of phosphate (g/L)	Dilution with water	Dilution factor (F)
< 0.1	No dilution required	1
0.1-1.0	1 + 9	10
1.0-10.0	1 + 99	100
> 10.0	1 + 999	1000

If the value of ΔA_{phos} is too low (e.g. < 0.1), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 0.7 mL, making sure that the sum of the sample and distilled water components in the reaction is 1.01 mL and using the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 7.5 using 2 M NaOH.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide should be degassed by increasing the pH to approx. 7.5 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no PNPase may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to room temperature and fill the volumetric flask to the mark with distilled water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein with Carrez reagents.

APPENDIX:

A. Assay Principle - Phosphate detection

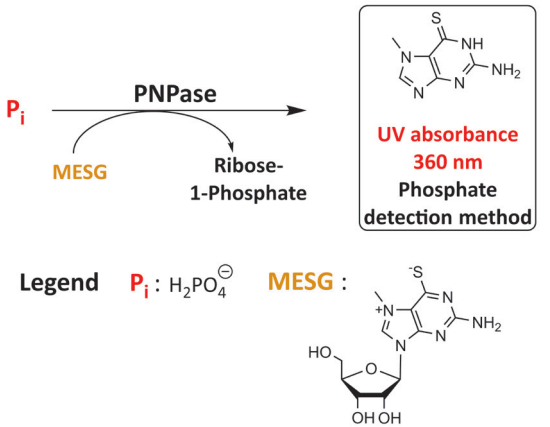

Figure 1. Theoretical basis of the phosphate detection assay

B. Linear range of assay

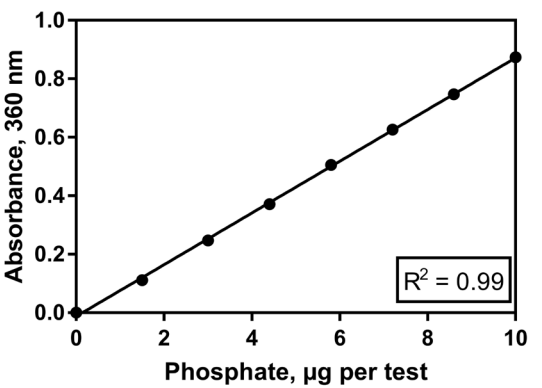

Figure 2. Linearity of the phosphate assay

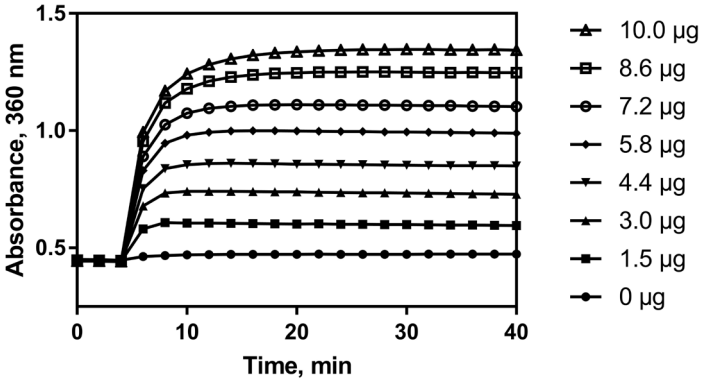

Figure 3. Increase in absorbance at 360 nm on incubation of 0-10 µg of phosphate per test

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1. McKie, V. A. & McCleary, B. V. (2016). A Novel and Rapid Colorimetric Method for Measuring Total Phosphorus and Phytic Acid in Foods and Animal Feeds. *J. AOAC Int.*, **99**, 738-743.

2. Webb, M.R. (1992). A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. *PNAS*, **89**, 4884-7.

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