Molecular Devices

MAXline™
Microplate Readers

Application Note
24

Microdetermination of phosphorus using the SPECTRAmax® *PLUS* microplate spectrophotometer: choice of microplate, cuvette or test tube assay formats

INTRODUCTION

Most spectrophotometric methods for inorganic phosphorus (Pi) analysis are based on the color formed upon the reduction of a phosphomolybdate complex. The classic (and still widely-used) method is that of Fiske and Subbarow[1], in which the reduction is carried out with sulfite and aminonaphtholsulfonic acid. Over the years, modifications have been offered with various reducing agents and differing concentrations of reactants. One of the most sensitive and well-characterized procedures is that of Chen, Toribara and Warner[2] in which the reducing agent is ascorbic acid.

This application note details two methods for measuring inorganic phosphorus using Molecular Devices' SPECTRAmax *PLUS* microplate spectrophotometer. The first method uses a commercial kit obtained from Sigma Diagnostics, based on the original Fiske-Subbarow chemistry, and offers the convenience of ready-made reagents. The second method[2] is approximately 10 times more sensitive, and thus is useful for samples containing 0.1 to 5.0 μg Pi/mL . Whichever method is used, the SPECTRAmax *PLUS* microplate spectrophotometer offers the versatility of making the absorbance measurements in microplates, in standard 1 cm cuvettes, or in 12 x 75 mm test tubes.

SIGMA KIT MATERIALS

1. SPECTRAmax *PLUS* microplate spectrophotometer system
2. Test tubes (12 x 75 mm, glass), *or* 1 cm cuvettes, *or* transparent microplates (e.g., polystyrene)
3. Trichloroacetic acid, (20% w/v) if samples need deproteinization
4. Pipettor and tips
5. Inorganic Phosphorus kit (Sigma Chemical Co., catalog # 670-C)

SIGMA KIT METHODS

Setting up the instrument and the software

Step 1 Using SOFTmax® PRO, open either a Cuvette Set (if using test tubes/ cuvettes) or a Plate Section (if using microplates). If necessary, create a new Cuvette Set or Plate Section by selecting New Cuvette or New Plate from the Experiment menu.

Step 2 Set up the Instrument Settings dialog box as shown in Figure 1 for a cuvette (upper) or for a plate (lower). Select to perform an end-point read at 820 nm. DO NOT SELECT PATHLENGTH CORRECTION (i.e., leave the PathCheck checkbox unchecked). The phosphomolybdate absorption band extends into the near IR and interferes with the measurements needed for pathlength calculation.

Note: The Sigma kit package insert recommends 660 + 40 nm. However, the broad absorbance band of the reduced phosphomolybdate complex is actually maximal near 820 nm, and the latter wavelength gives approximately 25% higher sensitivity.

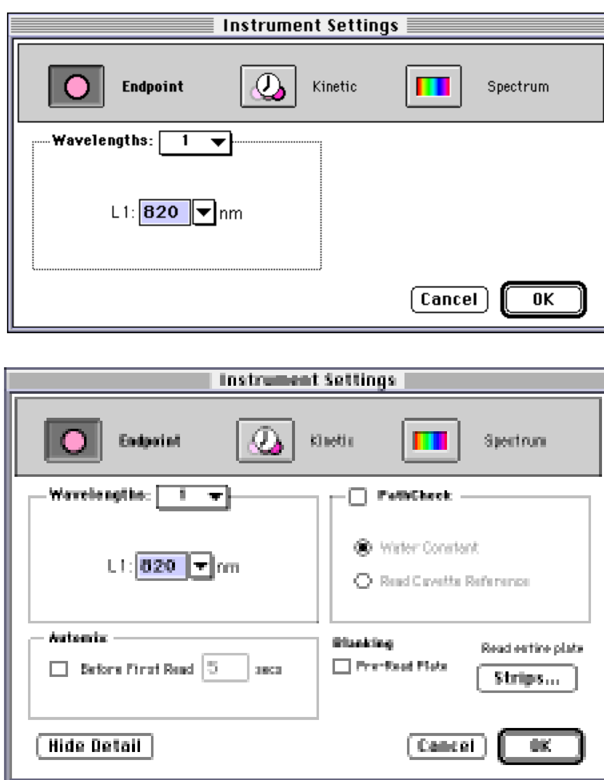

Figure 1: The Instrument Settings dialog box set up for a cuvette (upper) or plate (lower)

Step 3 Create a template showing where standards and unknowns will be located in the Cuvette Set or the Plate Section.

Step 4 Click the Reduction button in the Cuvette Set or the Plate Section toolbar to display the Reduction dialog box. Set the wavelength combination to L1 and the data mode to Absorbance as shown in Figure 2.

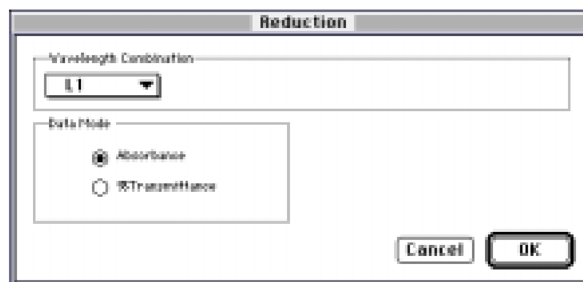

Figure 2: The Reduction dialog box

Prepare the reagents and samples

Step 1 Dissolve 1.0 g Fiske & Subbarow Reducer in 6.3 mL deionized water. The Reducer solution can be stored for 1 month at room temperature, stored in a tightly-capped amber bottle. The acid molybdate reagent is supplied at a ready-to-use concentration in the kit.

Step 2 Prepare the standards by diluting the stock as specified in the package insert, *except* prepare only 2.5 mL (not 5.0 mL) of each dilution and use 12 x 75 mm glass tubes to hold the dilutions. The inorganic standard supplied with the Sigma kit contains potassium phosphate, equivalent to 20 µg Pi/mL. Because the kit is intended to be used with serum samples, a one to ten dilution of the sample is factored in and the standard curve is expressed in mg/dL of the original undiluted sample.

Step 3 Prepare the samples as recommended in the package insert (e.g. diluting urine samples with water, or trichloroacetate precipitation of serum samples to remove protein). The diluted samples should contain 1 - 25 µg Pi/mL. Pipet 2.5 mL of each into 12 x 75 mm glass tubes.

Step 4 Add 0.5 mL acid molybdate solution and 0.125 mL Fiske & Subbarow solution to each sample and standard. Mix briefly with a vortex mixer. If using the microplate method, accurately transfer 250 µL of each reaction mixture into the preassigned wells of the microplate.

Step 5 Incubate 10 minutes at room temperature. If using test tubes, read the absorbance of each tube in the cuvette port of the SPECTRAmax *PLUS* microplate spectrophotometer. For the reference reading, use the "0" standard. If desired, the samples can be transferred into cuvettes before reading. If using a microplate, place it in the drawer of the SPECTRAmax *PLUS*, then read the plate.

SIGMA KIT RESULTS

Figure 3 compares standard curves obtained using the same solutions, but read in 12 x 75 glass tubes and in a microplate. Both curves have excellent linearity from 1 mg/dL to 25 mg/dL based on the concentration of the original samples before dilution. (The actual concentration range of the diluted standards before addition

of reagent is 1–25 µg/mL.) The microplate standard curve is approximately 30% lower than the tube curve, reflecting the correspondingly shorter pathlength through the 250 µL samples in the wells.

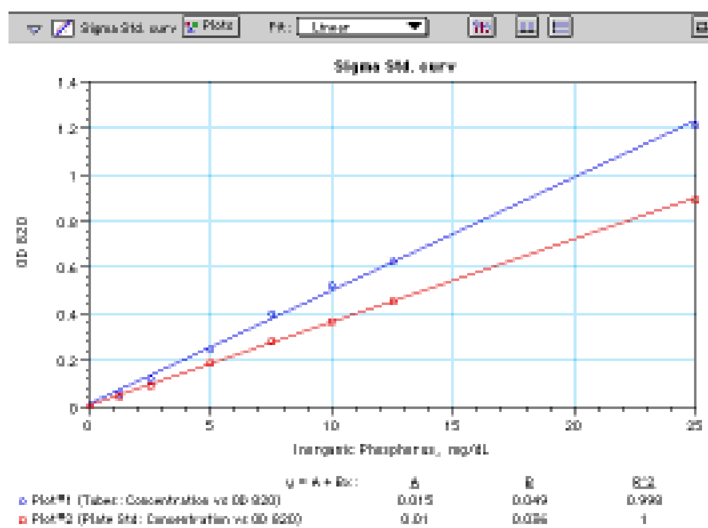

Figure 3: Phosphorus standard curve prepared using the Sigma kit and read i n 12 x 75 test tubes (Plot #1) and in a microplate (Plot #2)

CHEN et al MATERIALS

1. SPECTRAmax *PLUS* microplate spectrophotometer system
2. Test tubes (12 x 75 mm)
3. Transparent microplates (e.g. Polystyrene) with lids
4. 1 cm cuvettes (if desired)
5. Samples (200-300 mL each, containing 0.1 to 5.0 µg Pi/mL)
6. Pipettor and tips
7. 37° C incubator (for test tube/cuvette method)
8. Sulfuric acid, reagent grade
9. Ammonium molybdate, ACS reagent grade
10. Ascorbic acid, ACS reagent grade

CHEN et al METHOD

Setting up the instrument and the software

Step 1 Using SOFTmax PRO, select a Cuvette Set (test tube/cuvette method) or a Plate Section (microplate method). If necessary, create a new Cuvette Set or Plate Section by selecting New Cuvette or New Plate from the Experiment menu.

Step 2 Set up the Instrument Settings dialog box as shown in Figure 4 for a cuvette (upper) or for a plate (lower). Select to perform an end-point read at 820 nm. DO NOT SELECT PATHLENGTH CORRECTION (i.e., leave the PathCheck checkbox unchecked). The phosphomolybdate absorption band extends into the near IR and interferes with the measurements needed for pathlength calculation. It is not necessary to pre-

read the microplate because polystyrene contributes negligible absorbance at 820 nm.

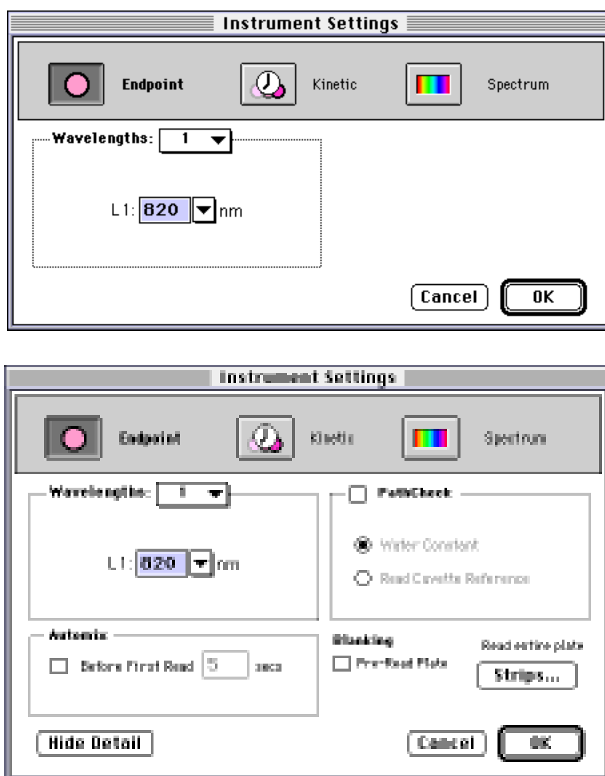

Figure 4: The Instrument Settings dialog box set up for a cuvette (upper) or plate (lower)

Step 3 Create a template showing where standards and unknowns will be located in the Cuvette Set or the Plate Section.

Step 4 Click the Reduction button in the Cuvette Set or the Plate Section toolbar to display the Reduction dialog box. Set the wavelength combination to L1 and the data mode to Absorbance as shown in Figure 5.

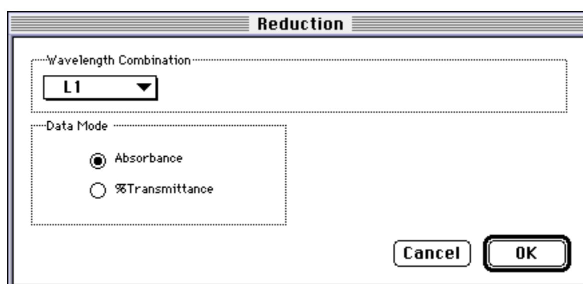

Figure 5: The Reduction dialog box

Step 5 If using the microplate method, you have the option of performing the incubation step in the microplate reader or in an external incubator. If the plate is to be incubated in the plate reader, set the incubator to 37 °C (using either the front panel of the instrument, or SOFTmax PRO's incubator (thermometer) button). If using tubes (or a microplate, if you don't want to use the instrument to incubate it), check that a 37 °C incubator is available for them.

Reagent and sample preparation

Step 1 Prepare the following stock solutions:

6 N sulfuric acid (dilute 16.7 mL concentrated acid to 100 mL.)

2.5% ammonium molybdate (store at room temperature).

10% ascorbic acid (store for up to 7 weeks at 0–5 °C).

Step 2 Prepare the stock standard, equivalent to 5.0 µg Pi/mL (0.161 mM). Weigh accurately 22.2 mg sodium phosphate, monobasic, anhydrous (MW 120) into a 100 mL volumetric flask. Bring to volume with 0.05 N HCl.

Dilute 10 mL of the stock standard to 100 mL with 0.05 N HCl (final concentration: 5.0 µg Pi/mL.

Step 3 The samples must be aqueous, protein-free and contain 0.1 to 5 µg Pi/mL. If they have been acid-treated (to deproteinize or to ash them), either dilute or neutralize, so that the acid concentration is less than 1 N (See reference 2).

Step 4 Prepare the standard curve by making a serial, 1 to 2 dilution of the stock standard in deionized water to obtain the following concentrations of Pi: 5.0, 2.5, 1.25, 0.625, 0.3125, 0.156 and 0.078 µg/mL. For the "0" standard, use deionized water.

Step 5 Prepare the working reagent by mixing 1 volume 6 N sulfuric acid, 1 volume 10% ammonium molybdate, 1 volume 10% ascorbic and 2 volumes deionized water. The working reagent should not be stored for more than 24 hours.

Step 6 *Test tube/cuvette method:* Pipet 1.0 mL aliquots of each sample and standard into 12 x 75 mm test tubes. For the "0" standard, use deionized water. Add 1.0 mL reagent and vortex briefly to mix. Cover each tube (e.g. with Parafilm®) and place in 37 °C incubator for at least one hour.

Microplate method: Pipet 150 µL aliquots of each sample, standard and blank into the designated wells of the microplate. Add 150 µL reagent to each well. Place a lid on the microplate and put the plate in the drawer of the reader and mix for approximately 5 seconds. Incubate the plate at 37 °C for approximately one hour.

Step 7 After incubation, read each tube at 820 nm in the SPECTRAmax *PLUS* cuvette port. For the reference reading, use the "0" standard. Alternatively, transfer each standard and sample to a clean glass or quartz cuvette and read 820 nm. If using a microplate, read it in the SPECTRAmax *PLUS*.

Color development proceeds gradually until reaching a maximum at approximately 90 minutes (Figure 6). The reaction is approximately 85%, 91% and 96% complete at 30, 45 and 60 minutes, respectively. The plate could be read as early as 30 minutes, though maximum sensitivity is obtained after 90 minutes incubation, as specified in the original procedure.

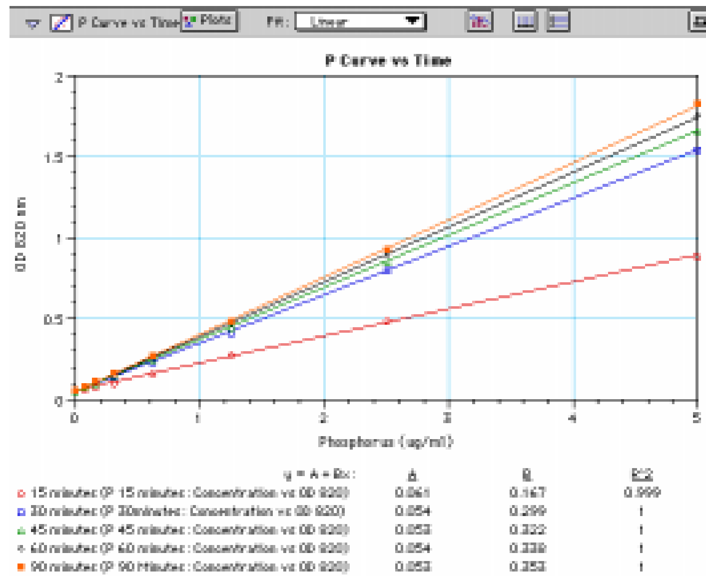

Figure 6: Color development of the phosphorus standard curve prepared according to the Chen *et al.* method and read in a microplate after 15 to 90 minutes incubation

Figure 7 compares standard curves prepared and read in a microplate with standard curves read in tubes or transferred to a standard 1 cm cuvette. All curves have excellent linearity from 0.078 µg/mL to 5 µg/mL. The standards have slightly higher absorbance when read in 12 x 75 tubes than in a cuvette, indicating the tubes are a little more than 1 cm in diameter. The standard curve generated in the microplate is lower, reflecting the shorter pathlengths through the samples in the microplate. Identical results are ultimately obtained, whether the user chooses to perform the analyses in tubes, cuvettes, or a microplate.

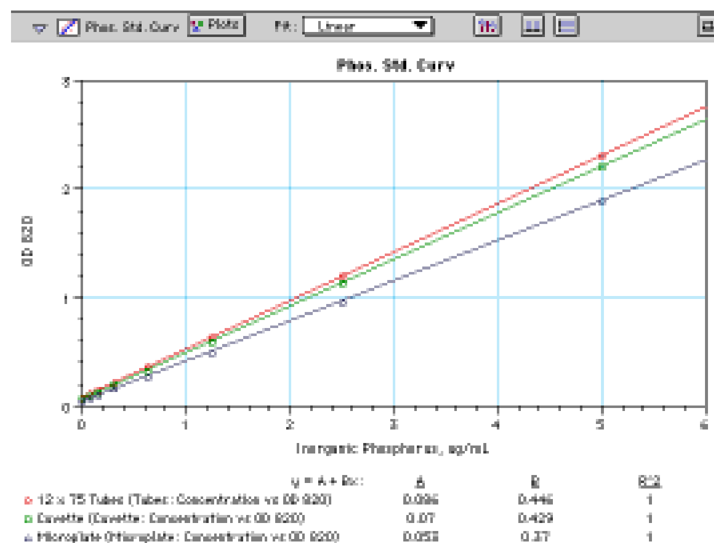

Figure 7: Phosphorus standard curve generated in test tubes, cuvettes and a microplate using the Chen *et al.* method

SUMMARY

Procedures are detailed for measuring inorganic phosphorus using Molecular Devices' SPECTRAmax *PLUS* microplate spectrophotometer. The first method takes advantage of a commercial kit and is suitable for samples which contain fairly high concentrations of inorganic phosphorus, such as serum or urine. The second procedure is much more sensitive and is useful for samples containing as little as 0.1 μg Pi/mL. For either method, Molecular Devices' SPECTRAmax *PLUS* microplate spectrophotometer offers the versatility of making the absorbance measurements in a microplate format, in standard 1 cm cuvettes, or in 12 x 75 mm test tubes.

REFERENCES

1 Fiske, C.H. And Y. Subbarow. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375 (1925).

2 Chen, P.S., T.Y. Toribara and H. Warner. Microdetermination of Phosphorus. *Analytical Chemistry*, **28**:1756-8 (1956)

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