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SPECTROPHOTOMETRIC DETERMINATION OF PARTS PER BILLION LEVEL PHOSPHATE BY "STOPPED-FLOW TIME DIFFERENCE ANALYSIS" USING MALACHITE GREEN AND MOLYBDATE

Ken-ichiro KANAYA* and Keitaro HIROMI†

Japan Food Research Laboratories, Osaka Branch,
Toyotsu-cho 3-1, Suita, Osaka 564
†Department of Food Science and Technology, Faculty of Agriculture,
Kyoto University, Sakyo-ku, Kyoto 606

The "stopped-flow time difference analysis" method [K. Hiromi, C. Kuwamoto, and M. Ohnishi, *Anal. Biochem.*, 101, 421 (1980)] was applied to the rapid and simple determination of trace amounts of phosphate, using Malachite Green and molybdate. The specific determination of phosphate down to the ppb level was successfully carried out even in the presence of 20 fold-excess (w/w) of AsO_4^{3-}. The detection limit was as low as 2 ppb.

Sensitive and simple analytical methods for phosphate determination have been sought in the pollution control of natural water and in the micro-analysis of medical and biological samples.

The spectrophotometric method using the complex of 12-molybdophosphate (abbreviated to 12MPA) with Malachite Green is more sensitive (the molar absorptivity at 650 nm is as high as about 1×10^5 dm^3 mol^{-1} cm^{-1}) than the methods based on the formation of 12MPA or its reduction product (so-called "molybdenum blue").[1,2] Motomizu *et al.* improved the former method by using a flow injection technique.[3]

Hiromi *et al.* developed the so-called "stopped-flow time difference analysis" (abbreviated to SFTDA) method for rapid and sensitive spectrophotometric determinations.[4] This method, which belongs essentially to the category of end-point assay, is based on the analysis of progress curves of reactions observed (from

about 5 ms to several minutes) by the stopped-flow method. From the signal amplitude, *e.g.*, the magnitude of the absorbance change, quantitative determination is made in high sensitivity. The rate constant that can be obtained from the progress curves as well may be used for qualitative analysis.[4] The reasons for the high sensitivity of this method are as follows: (a) Since the cell and the solution are both immobile during the measurement with the stopped-flow apparatus, any possible error from the replacement and/or the movement of the solution can completely be eliminated. (b) Since the magnitude of the absorbance change (the signal amplitude) is determined in steady situation (after the reaction has terminated in most cases), the sensitivity and precision are much more enhanced compared with usual kinetic (rate assay) method. (c) Small change as a function of time (even on large background) can easily be picked up and recorded in an expanded scale by the apparatus: for example, the absorbance change as low as 0.002 O.D. on the background of 1.0 O.D. can be measured in good precision. Moreover, owing to the small dead time (about 2 ms) of the apparatus, fast reactions accompanying absorbance change can be observed in short period of time, which is useful to exclude possible slow parallel or consecutive reactions accompanied with the main reaction. The first application of the SFTDA method was successfully done for the determination of ascorbic acid (down to 50 ng cm^{-3}, or 3×10^{-7} mol dm^{-3}) with large excess of 2,6-dichlorophenolindophenol, even in the presence of triose reductone.[4]

In this paper, the SFTDA method with Malachite Green and molybdate is proposed for the rapid and simple analysis of the ppb level phosphate.

The color-developing reagent (which consists of 0.00025 mol dm^{-3} Malachite Green, 0.05 mol dm^{-3} ammonium molybdate and 1.1 mol dm^{-3} H_2SO_4) and the sample solution (which contains 0.05 mol dm^{-3} ammonium molybdate and 1.1 mol dm^{-3} H_2SO_4 besides phosphate to be measured) were rapidly mixed in 1:1 ratio with the stopped-flow spectrophotometer (Union Giken SF-70) equipped with a data accumulation and averaging device (Union Giken RA-450). For fast and reproducible measurements, the sample solution was allowed to stand for about 15 min to form 12MPA before the mixing. The absorbance change at 650 nm was recorded as a function of time. The time required for one measurement was about 1 min.

A typical example of the progress (reaction) curves obtained is shown in Fig. 1. Although the kinetic feature of the curve is not simple and no mechanistic study will be undertaken here, the reaction curve can be conveniently divided into

two main phases; the first sigmoidal phase that completes within about 20 s after mixing, and the second much slower phase that proceeds linearly for at least several tens of minutes. After confirming that the slope of the linear part in the second phase of all progress curves is invariant irrespective of the phosphate concentration, we adopted the following procedure as a convenient and reliable one for the determination of phosphate concentration: (1) Let ΔA and ΔA_0 be the magnitudes of the absorbance changes determined at a certain definite time in the linear region of the second phase in the progress curves of a sample solution and the reagent blank, respectively (see Fig. 1). (2) Then the difference between ΔA and ΔA_0, $(\Delta A - \Delta A_0)$, which is proportional to the phosphate concentration, is used as the reliable measure. The standard deviation (SD) of ΔA obtained by this procedure was less than 0.0003, which is about 1/10 of that by conventional spectrophotometric methods. A typical example of the calibration curves is shown in Fig. 2. It was recognized that straight line was obtained for phosphate concentration from 5 to 1000 ppb.

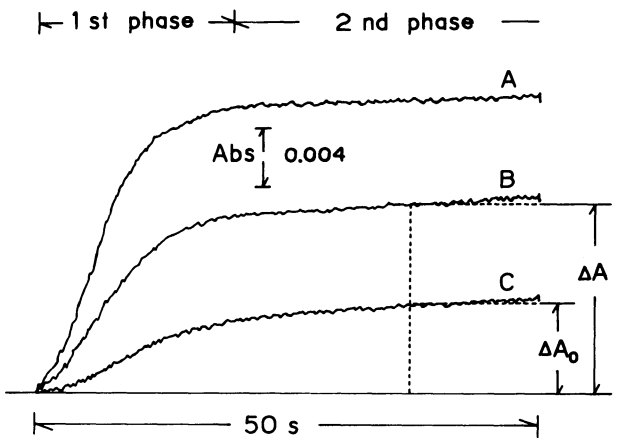

Fig. 1. Typical progress curves.

phosphate concentrations: A, 54 ppb; B, 27 ppb; C, 0 ppb (reagent blank).

Each curve was obtained by accumulating 5 runs.

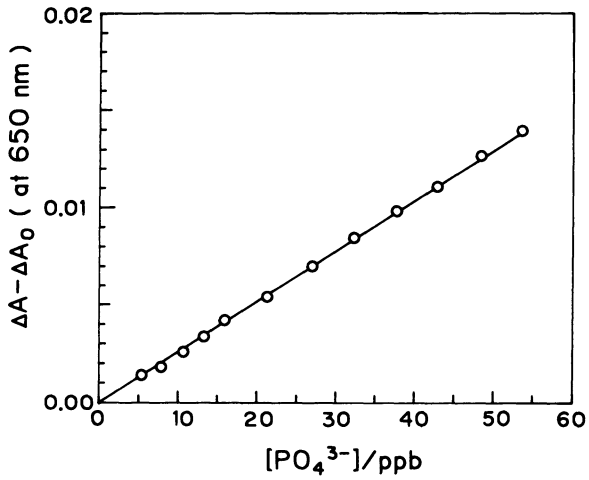

Fig. 2. Calibration curve for phosphate determination.

Each point represents the mean of three determinations.

Slope: 0.00025/ppb, Intercept: 0.00010, Correlation coefficient: 0.998.

Since SD of ΔA_0 obtained by 10 measurements of the reagent blank was 0.0002, which corresponds to about 0.7 ppb of phosphate, the detection limit was estimated to be 2 ppb ($\simeq 3 \times$SD) according to the IUPAC recommendations. This detection limit is about 1/10 of that obtainable by conventional spectrophotometric methods.

The serious interference by AsO_4^{3-} in conventional methods for determination of phosphate was able to be reduced, due to the fact that the reaction of molybdoarsenate with Malachite Green is about 60 times slower than that of 12MPA and these reactions can easily be differentiated. Table 1 shows the effects of the coexisting ions, AsO_4^{3-} and SiO_3^{2-}, on the recovery of phosphate. It is notable that in contrast to other methods[3,5] the effect of AsO_4^{3-} is as low as that of SiO_3^{2-}, and is negligible within 20 fold-excess (w/w) to the amount of phosphate. Thus the present method is considered to be superior in both its higher sensitivity and less interference by AsO_4^{3-}.

Table 1. Recovery of phosphate in the presence of arsenate or silicate[a)]

Ratio (w/w) $\frac{As}{P}$ or $\frac{Si}{P}$	Recovery of P %	
	As present	Si present
0	100.0±4.8 (n=5)	100.1±5.4 (n=4)
18	100.6±5.1 (n=5)	100.6±4.5 (n=4)
36	108.6±4.2 (n=4)	105.4±5.8 (n=5)
54	118.4±5.6 (n=4)	116.0±5.1 (n=5)
72	129.7±6.1 (n=5)	125.6±7.0 (n=5)
90	137.7±5.1 (n=5)	129.7±9.3 (n=4)

a) Determined at 31 ppb of phosphate concentration. Abbreviations: P, PO_4^{3-}; As, AsO_4^{3-}; Si, SiO_3^{2-}.

The phosphorus contents in several food stuffs obtained by the present method were in good agreement with those by molybdovanadophosphoric acid method.[6] These results will be published later.

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