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Flow injection programmed to function in batch mode is used to determine molar absorptivity and to investigate the phosphomolybdenum blue method

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ABSTRACT

The ultimate goal of flow-based analytical techniques is to automate serial assays of a target analyte. However, when developing any reagent-based assay, the underlying chemistry has to be investigated and understood a step, which is almost always the most challenging component of the optimization effort. The difficulty lies in that almost all reagent-based assays were initially developed and optimized in a batch mode, with the aim to perform assays manually, within a time frame of up to 15 min, while flow injection techniques are designed to monitor concentration gradients at times prior to reaching chemical equilibria and while performing up to two assays per minute. This work resolves this discrepancy by using programming Flow Injection (pFI) that operates in a batch mode within a time frame of 1 min or less, with the aim of optimizing an assay under the same conditions and using the same instrument in which the assay will be performed. This novel concept is verified by determining a molar absorptivity of Fe(II) ferrozine complex and by comparing it with literature data. Next, the pFI-batch technique was used to investigate and optimize the phosphate assay, based on formation of phosphomolybdenum blue, with the aim of maximizing sensitivity and improving the limit of detection of this widely used method.

"Always run a blank!"
Gary D. Christian

1. Introduction

There are two objectives of this work. The first one is to show how Flow Injection, programmed to function in a batch mode, can be used to determine molar absorptivities and to investigate reagent-based assay by monitoring reaction rates and spectra of ongoing chemical reactions.

The second aim is to optimize the phosphate assay based on the formation of phosphomolybdenum blue (PMoB) – a widely used, yet still mostly unexplored method, the complexity of which was recently compared to a "black box" [1].

Before the advent of flow analysis, all measurements in chemical analysis were performed on homogenous solutions, and, with the exception of kinetic assays, at a time when chemical equilibria had been reached. This batch approach, led to the development of reagent-based techniques that are the basis of the majority of wet chemical assays performed today. In a manually performed batch technique, the composition of the homogenously mixed reactants is well defined and the measurement is carried out under equilibrium conditions. Manual protocols for a large variety of reagent-based assays are collected in comprehensive monographs [2,3], which also list spectra and molar absorptivities that serve as a guide for the optimization of spectrophotometric assays. This is also why Skeggs [4] designed the Auto-Analyzer to operate in a batch mode, by air segmenting the carrier stream of sample and the reagent, because segmentation produced a homogenous mixture that could be incubated along its route to the detector until equilibrium was reached.

In contrast to batch mode assays, Flow Injection techniques rely on data obtained by monitoring concentration gradients at times and conditions that fall short of reaching chemical equilibria. This approach has, besides high sampling frequency, numerous advantages that have led to the wide acceptance of Flow Injection techniques. But the disparity between flow and batch mode, and especially the difference in the duration of incubation periods, makes it difficult to optimize flow-based protocols using information obtained from manual batch methods.

This obstacle can now be overcome, using programmable Flow Injection (pFI) [5,6], which combines the advantages of Flow Injection and Sequential Injection [7] by programming them to function in batch mode. Thus, for the first time, the same instrument can be used to

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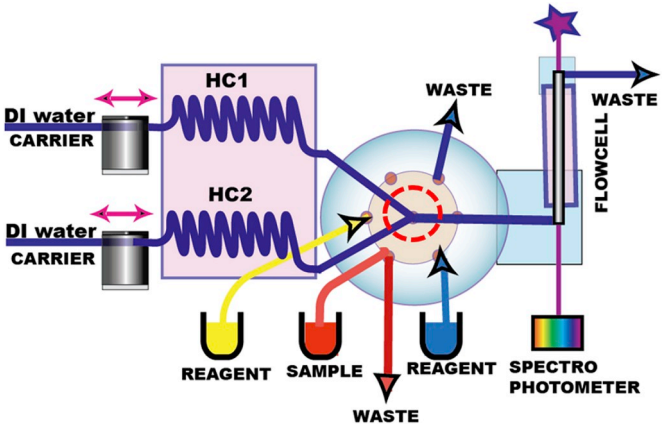


Fig. 1. Programmable flow injection in lab-on-valve format. Red circle indicates confluence point. HC1 and HC2 are temperature-controlled holding coils. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

explore the essential features of the chemical reactions as well as to optimize them and it then can be used to perform the serial assays. The platform for this dual purpose is an instrument that is comprised a lab–on-valve (LOV) manifold equipped with a confluence point (red circle Fig. 1), [8]. In this setup described in detail in the next section, the microfluidic manipulations are programmed in such a way that:

- Sample is mixed with reagents in a known, well-defined ratio.
- The flow cell is entirely filled with a homogenous mixture of these reactants.
- The mixture of reactants is held within the flow cell and spectrally monitored until chemical equilibrium is reached.

This concept is verified in this work by determination of molar absorptivity of Fe(II)ferrozine complex (Section 2.3.) and applied to study and optimization of phosphomolybdenum blue method (Section 2.4.).

2. Experimental

2.1. Instrumentation

The instrument, miniSIA-2 (GlobalFIA, Fox Island, WA, USA), fitted with a 20 cm long thermostatted flow cell of internal volume 100 µL, comprises two high precision, synchronously refilling milliGAT pumps, two temperature-controlled holding coils, and a 6-port lab-on-valve module. All tubing connections, downstream from the milliGAT pumps including the holding coils (volume 1000 µL), were made with 0.8 mm I.D. PEEK tubing. The conduits between the carrier stream reservoirs and the milliGAT pump were made from 1.6 mm I.D. PTFE tubing in order to minimize degassing under reduced pressure at higher aspiration flowrates. Spectrophotometer (USB4000, Ocean Optics, Dunedin, FL) and a tungsten light source were connected to the flow cell by optical fibers. Because the light intensity of light source mounted within miniSIA-2 instrument was not strong enough, the external tungsten Halogen light source SL-1 (StellarNet, Tampa, FL) was used in all experiments. Assay protocols were computer controlled using FloZF software by Global FIA.

2.2. Reagents and materials

Deionized (DI) water, was generated by Barnstead Water Purification System, Nano Pure Diamond (Thermo Fisher Scientific, www.thermofisher.com). In order to eliminated air bubbles formed by exsolving dissolved air, DI was either stored over night before use, or it was de-aerated by stirring under a mild vacuum.

2.2.1. Fe(II)-ferrozine method

0.01 M Ferrozine solution was prepared by dissolving 0.492 g FerroZine TM iron reagent (ACROS Organics, www.acros.com) in 100 mL 0.2 M ammonium acetate buffer. The solution was prepared weekly, and stored refrigerated in the dark. 0.2 M Ammonium acetate buffer (pH 4.6) was prepared from isothermal distilled acetic acid Certified ACS (Fisher Scientific, www.fishersci.com) and ammonium hydroxide, Certified ACS PLUS (Fisher Scientific, www.fishersci.com) in deionized water. Hydrochloric acid, Certified ACS PLUS (Fisher Scientific, www.fishersci.com) was purified by isothermal distillation. Hydroxylamine hydrochloride, Certified ACS (Fisher Scientific, www.fishersci.com). A 2.00 ppm stock standard iron solution was prepared by diluting a 200 ppm commercial iron standard (LabChem, Zelienople, PA) in 0.01 M HCl and 0.5% hydroxylamine hydrochloride. The stock solution was further diluted to obtain working standards in 0.01 M HCl. The carrier solution was 0.2 M ammonium acetate buffer without any surfactant.

2.2.2. Phosphomolybdenum blue method

DI water without any surfactant was used as carrier in all experiments. Input concentrations and resulting reagent concentrations in the flow cell are summarized in Table 1. Ascorbic acid combined with surfactant was prepared daily. Stock solution of ammonium molybdate was prepared by dissolving ammonium molybdate tetrahydrate in DI water. Stock solutions of sulfuric, hydrochloric and nitric acids were prepared by dilution of analytical grade concentrated acids. Their concentration was adjusted to 4.00 N and confirmed by titration with standard solution of sodium hydroxide. Input concentrations of molybdate reagent were selected to yield 0.50, 1.0 and 1.5 mM concentrations of molybdate in the flow cell, while normalities of strong acids were varied stepwise between 0.05 N and 0.7 N Concentration of potassium antimony tartarate that resulted on 0.03 mM KSb tartarate in the in the flow cell was kept unchanged in all experiments. Input concentration of orthophosphate standard solutions were prepared by

Table 1
Input reagent concentrations and of reactants within in the flow cell.

Reagents	Input reagent concentration		Reactant concentration in FC.	
	R+S = 1+1	R+S = 1+3	R+S = 1+1	R+S = 1+3
	(4-fold dilution)	(8-fold dilution)	(4-fold dilution)	(8-fold dilution)
MO.				
Molybdate	4 mM	8 mM	1 mM	1 mM
Acid	1.6 N	3.2 N	0.4 N	0.4 N
KSb	0.12 mM	0.24 mM	0.03 mM	0.03 mM
ASC.				
Ascorbic acid	3%	6%	0.75%	0.75%
SDS	3%	3%	0.75%	0.38%

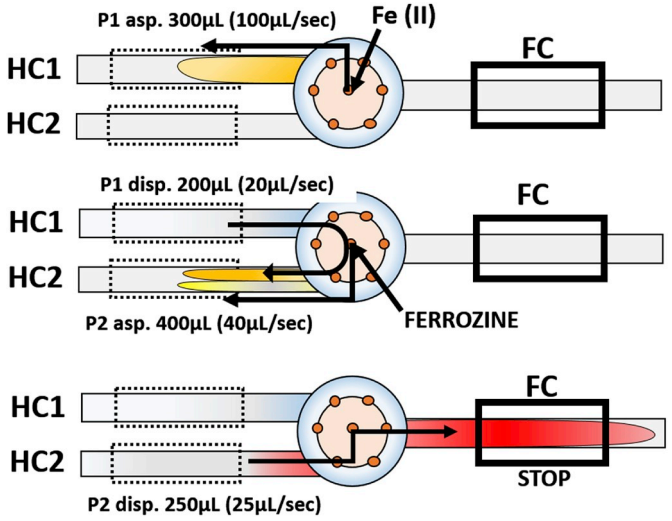


Fig. 2. Flow programming for single reagent batch type determination of Fe(II)-ferrozine molar absorptivity. HC1 and HC2 are temperature-controlled holding coils. P1 and P2 are milliGAT bi-directional pumps, FC is the flow cell. Carrier is deionized water.

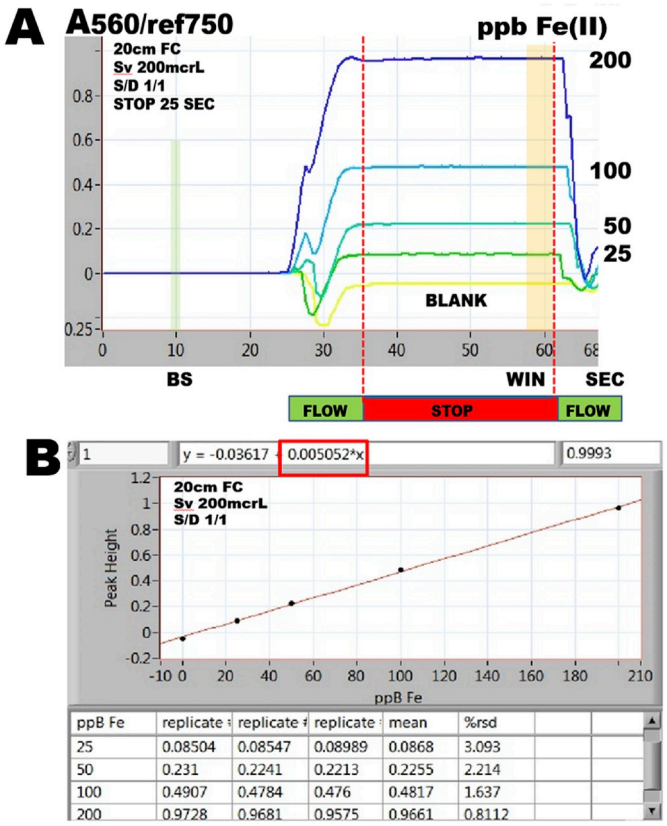
serial dilution of 1000 ppm P commercially available standard by DI. They are, in this work, referred to in ppb units in order to distinguish them from P concentrations in flow cell.

2.3. Molar absorptivity of the Fe(II)-ferrozine complex

Ferrozine®, the sodium salt of pyridyldiphenyltriazine sulfonic acid (m.w. 492.46) forms a water-soluble red chelate with iron (II) which absorbs at 562 nm. The Fe(II)-ferrozine complex is formed rapidly at room temperature in acetate buffer at pH = 4.5 in the presence of hydroxylamine which serves as a reducing agent. The flow programming for determination of its molar absorptivity (Fig. 2.) comprises three steps:

1. The sample containing iron (II) is aspirated into the holding coil 1 (HC1).
2. the valve is switched to the ferrozine reagent and a total volume of 400 µL is aspirated into holding coil 2 (HC2), composed of 200 µL of reagent and 200 µL of sample solution which is delivered from HC1. In this way, the sample is homogenously mixed with the ferrozine reagent in ratio 1 + 1 at the confluence point.
3. The valve is then switched to the flow cell and 250 µL of the reactant mixture is transported from HC2 into the flow cell, where it is held for 25 s, while the absorbance of reaction mixture is being monitored. Because the internal volume of the 20 cm long flow cell is 100 µL, the flow cell is entirely filled with the homogenous mixture of the reactants of a known concentration [10].

Standard solutions of Fe(II) in the range of 0–200 ppb Fe, were analyzed and the absorbance values were recorded during the 25-s long stop flow period. The response curves show that the absorbance initially increases as the flow cell is filled with reactants, but then remains constant during the stop flow period because the reaction equilibrium has already been reached (Fig. 3.). Therefore the absorbance values, collected during window (WIN, shown in orange in Fig. 3A.), represent the complete formation of the Fe(II)-ferrozine complex. The slope of the calibration line (Fig. 3B.) is 5.05 mAU/1 ppb Fe(II), measured in a 20 cm long light path, on a solution of Fe(II), diluted in ratio 1 + 1. This yields a molar absorptivity (ε) for Fe (a.w. 55.9) at 562 nm: ε = 28,300, while the value listed in the literature is ε = 28,600 [2, p. 326.]. Close agreement of the tabulated value of molar absorptivity with the value obtained in this experiment confirms that programmable Flown



ppB Fe	replicate	replicate	replicate	mean	%rsd
25	0.08504	0.08547	0.08989	0.0868	3.093
50	0.231	0.2241	0.2213	0.2255	2.214
100	0.4907	0.4784	0.476	0.4817	1.637
200	0.9728	0.9681	0.9575	0.9661	0.8112

Fig. 3. A. Stop flow response curves obtained by analyzing Fe(II) standards in the range 0–200 ppb Fe. BS - baseline absorbance measurement, WIN - calibration data collection window where absorbance was measured. **B.** Calibration graph obtained from data collected in A.

Injection, configured to function in batch mode, yields the same results as the classical manual batch technique, and is therefore suitable for exploration of chemistry of phosphomolybdenum blue.

2.4. Investigation of the phosphomolybdenum blue method

2.4.1. Phosphomolybdenum blue method

Discovered almost 200 years ago, the phosphomolybdenum blue (PMoB) method has undergone countless optimizations and modifications, and is reported in over 2500 publications. Yet the findings in these works are incomplete and sometimes contradictory, a state-of-art that needs to be addressed, because the determination of phosphate by the molybdenum blue method is one of the most frequently performed reagent-based assays.

The PMoB is produced in two steps:

1. the first one results in formation of a polymolybdate structure around the phosphate anion.
2. during the second step, the yellow phosphomolybdate is reduced to a compound that *appears* to be blue.

$$PO_4^{3-} + 12MoO_4^{2-} + 27H^+ \rightarrow H_3PO_4(MoO_3)_{12} + 12H_2O \quad (1)$$

$$H_3PMo(VI)_{12}O_{40} + \text{Reductant} \rightarrow [H_4PMo(VI)_8Mo(V)_4O_{40}]^{3-} \quad (2)$$

It is widely recognized that both reactions are best performed in strongly acid solution (pH 0 to 1) in order to prevent interference from molybdenum blue (MoB) as well as from Silicomolybdic blue (SiMoB) which is formed in presence of silicic acid.

A wide variety of reducing agents has been used in the search for the ultimate optimized the PMoB method. This has resulted in a perplexing multitude of suggestions and assumptions about the properties of the

phosphomolybdenum blue species. As summarized in a recent comprehensive review [1] in the presence of nonmetallic reductants, PMoB forms that exhibits a spectrum with a single peak and a molar absorptivity $\varepsilon = 7000$ at λ 700 nm, and this spectrum changes upon heating to yield PMoB with $\varepsilon = 26{,}000$ at λ 880 nm. Metallic reductants (such as $SnCl_2$) form PMoB with a spectrum with twin peaks $\varepsilon = 19{,}000$ at λ 700 nm and $\varepsilon = 16{,}000$ at λ 620 nm. In the presence of Sb and a nonmetallic reductant (such as ascorbic acid), SbPMoB is formed, with a twin peak spectrum $\varepsilon = 20{,}000$ at λ 880 nm and $\varepsilon = 15{,}000$ at λ 710 nm.

Even more variance is seen in data on the molar absorptivities of PMoB that range between $\varepsilon = 1000$ and $\varepsilon = 32{,}000$ depending on the wavelength and reducing agent used (Fig. 6 and Table 3. in Ref. [1].). This disarray documents the complexity of the chemical reactions on which PMoB assay is based and, as the authors of this outstanding review observed: “These significant differences (in spectral characteristics) are noteworthy, since there is, of course, only one actual ‘molar absorptivity’ for a particular compound at a given wavelength”. The question is whether we are dealing with so many different compounds or a blend of several species [6]. Inevitably, further questions come to mind.

Can the PMoB method be optimized without obtaining data on the initial reaction rates, and without examination of spectra of reaction products in presence and *absence* of phosphate? (see initial G. Christian quote).

Why is 880 nm the most frequently used wavelength for measuring absorbance of PMoB (which appears to be blue), and why has almost any wavelength within the 500 nm–900 nm range been used as well?

Since it is generally accepted the PMoB assay is best performed in acid solutions (pH 0 to 1), what are the reasons for this method to fail outside this range?

Since it has been established that suitable concentrations of molybdate and acidity of solution are mutually dependent, is it the suitability of their *ratio* that provides a guide for the optimization of this method?

There is also no explanation why sulfuric acid is almost always used, while hydrochloric and nitric acids are overlooked [1].

In the confusing multitude of proposed PMoB based assays, developed during last 70 years, the seminal work of Murphy and Riley published in 1962 [11], provides the foundation on which the majority of presently used, flow-based assays, are founded. This is because of the use of ascorbic acid in the presence of Sb, enhances the rate of formation of molybdenum blue, thus making the method suitable for automation by Continuous Flow Analysis (CFA) and by Flow Injection. Yet another lasting impact on the present concept of PMoB chemistry was Murphy and Riley's finding, that Sb is incorporated into the complex as $PSb_2Mo_{12}O_{40}$, which exhibits a twin peak spectrum at 710 nm and 910 nm, while the PMoB spectrum obtained without Sb has a single peak at 850 nm (Fig. 1 in Ref. [11].). This finding was later confirmed by Going and Eisenreich [12] and it is now accepted (Fig. 9 in Ref. [1]) although at present, the majority of methods that use ascorbic acid combined with potassium antimony tartrate employ 880 nm for measurement of phosphate content. It remains, however unexplained why the reported molar absorptivity obtained at 880 nm, for the Murphy-Riley method varies from $\varepsilon = 21{,}600$ to $\varepsilon = 25{,}670$ [1].

Since one of the aims of this work is to develop an assay suitable for trace analysis of phosphate in sea water, the optimization is in this study focused on the Murphy-Riley method because it is widely used in chemical oceanography.

2.4.2. Optimization of phosphomolybdenum blue method

For trace analysis, optimization is aimed at maximizing the slope of the calibration line, and at minimizing the limit of detection (LOD) by minimizing the reagent and analyte blank. Obviously, systematic optimization is based on the investigation of the influence of the principal components of an assay on the slope of calibration line. It follows from the current literature that it is the acidity of the molybdate reagent that is the critical component because it determines the suitable range (pH 0 to 1) for the PMoB assay. Therefore, in this study, acidity and molybdate concentrations were varied, while concentrations of all other reagents were kept the same. Because it is the composition of reactants *in the flow* cell, that determines the rates of the chemical reaction and their outcome, all quoted concentrations of reagents (in molarities and normalities) represent the concentrations of the reactants in the flow cell during the incubation period. In this study, the input reagents are diluted 4-fold as they are merged and delivered to the flow cell (Fig. 4) resulting in the input reagent that combines ascorbic acid with surfactant yields the concentration of 0.75% (43 mM) ascorbic acid and 0.75% sodium dodecyl sulphate (SDS) in the flow cell (Table 1). Similarly, the molybdate reagent was composed of various concentrations of ammonium molybdate, various concentrations of strong acids and a constant concentration of potassium antimony tartrate that results in 0.03 mM KSb tartrate in the flow cell. The input concentration of the orthophosphate standard solutions are, referred to in ppb P units in order to distinguish them from the phosphate concentrations in the reactant mixture. Thus e.g. 31 ppb P yields 0.5 μM P in the reactant solution, because P standards are diluted by reagents (Standard + reagents 1 + 1, step 3 in Fig. 4.) [13]. The physical parameters (temperature: 22 °C, incubation time: 30 s, mixing ratios (steps 2 and 3 in Fig. 4) as well as timing of the data and spectra collection at 880 nm with a reference at 550 nm were kept constant throughout all experiments.

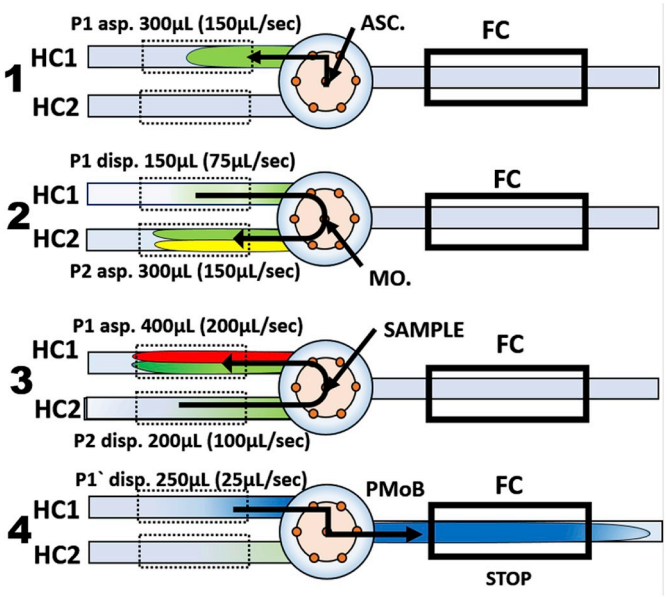


Fig. 4. Flow programming for two reagent batch type determination of PMoB molar absorptivity and for optimization of PMoB assay. HC1 and HC2 are temperature-controlled holding coils. P1 and P2 are milliGAT bi-directional pumps, FC is a 20 cm path length temperature-controlled flow cell. Carrier is deionized water.

2.4.3. Flow programming, data collection and [H^+] diagrams

In order to identify the conditions under which the slope of the calibration line is maximized, a flow programming must be designed that reproduces conditions that ensue that the known concentrations of reactants are homogenously mixed by the time they reach and are held within the flow cell. Therefore, the flow program for optimizing the PMoB assay (Fig. 4.) comprises of the following steps [14]. Aspirated ascorbic acid reagent (step 1), is mixed with molybdate reagent in the proportion of 1 + 1 in HC2 (step 2), followed by step 3, where 200 μL of the phosphate sample is mixed with 200 μL of a mixture of the ascorbic acid/molybdate reagents in HC1 (effecting a 4-fold dilution). Next (step

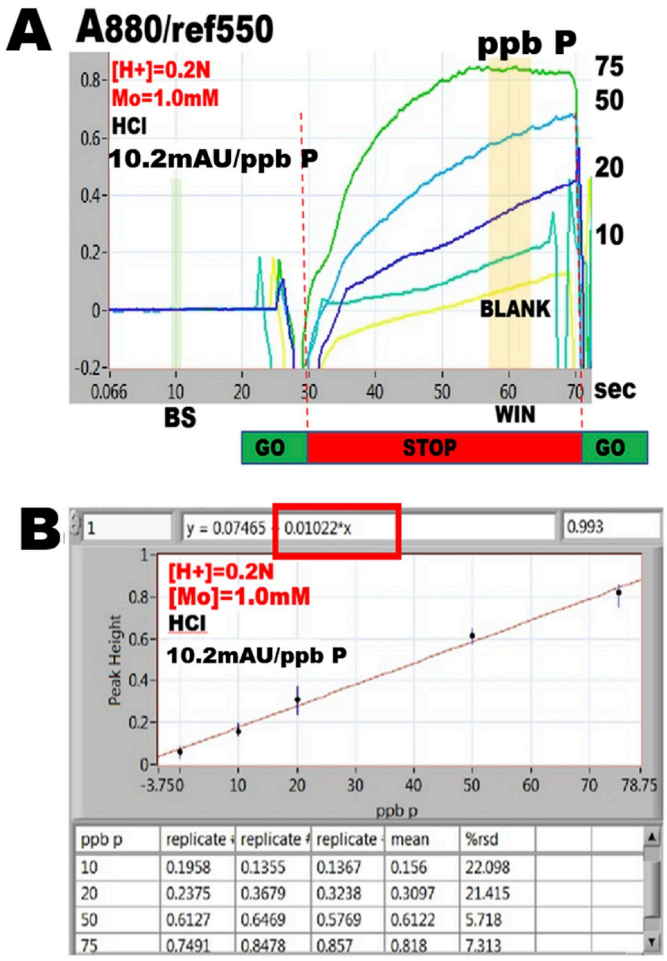


Fig. 5. A. Stopped flow response curves obtained by analyzing orthophosphate standards in the range 0–75 ppb P. BS - baseline absorbance measurement, WIN - calibration data collection window where absorbance is measured. **B.** Calibration graph obtained from data collected in A.

4) the reactant mixture is then transported into the flow cell where the flow is stopped for a 30-s long incubation time, during which the reaction progress is recorded (Fig. 5A) and, at the end of the incubation time, the absorbance is measured (WIN, shown in the orange box in Fig. 5A.) along with the absorbance spectrum of the reactants at that time. Repetition of this protocol with phosphate standards in the range 0–75 ppb P yields the slope of the calibration line, expressed in mAU/ppb P unit (Fig. 5B.).

The $[H^+]$ diagrams, obtained by plotting acidity, against slope of calibration line prepared for sulfuric (Fig. 6A), hydrochloric (Fig. 6B.) and nitric acids [15] reveal that the maximum slope is reached at suitable combinations of acidity $[H^+]$ and concentration of molybdate [Mo]. At lower acidities, the production of MoB manifests itself as an increase in absorbance in the spectrum of a reagent blank. The maximized slope of the calibration line is very similar over the entire range of $[H^+]$ and [Mo] concentrations presented in the $[H^+]$ diagrams, and is independent of the type of acid used in this study. Using the maximized slope associated with optimized spectra that do not show a MoB blank (Fig. 8B) and (N3, N4, N5 in Ref. [16] and S5, S6 in Ref. [17]), we find an averaged slope of 10.6 mAU/ppb P. Since this slope was determined using a 20 cm long optical path, in solutions of phosphate standards diluted with reagents in a ratio of 1 + 1, it yields for P (a.w. 31), a molar absorptivity ε = 32,900 measured at 880 nm with reference at 550 nm.

The spectrum of reactants under the optimized conditions exhibits a single, well defined maximum at 880 nm, with a shoulder showing only

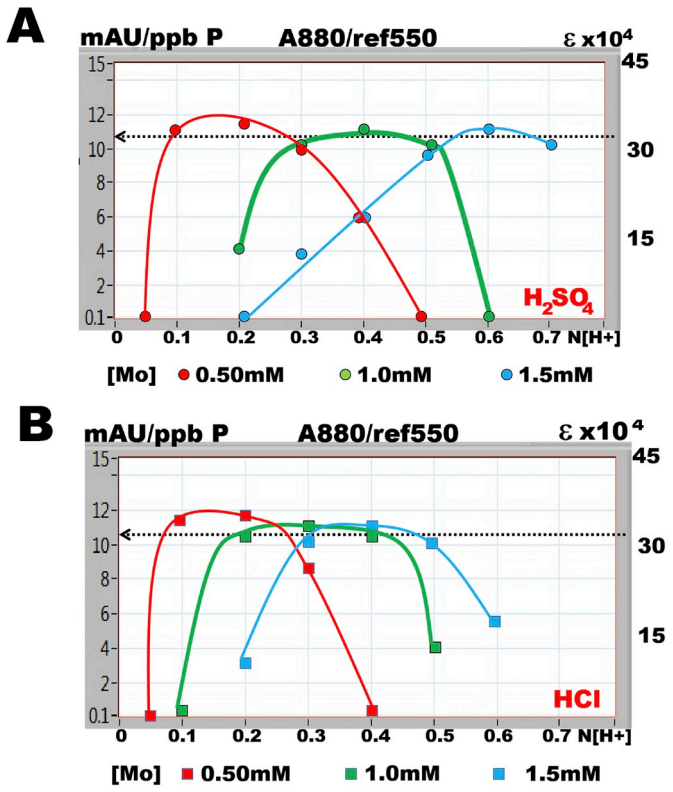


Fig. 6. $[H^+]$ diagrams for sulfuric (A) and hydrochloric (B) acids. [Mo] is concentration of molybdate in the flow cell. Slope of calibration line associated with points on the diagram is expressed in mAU/ppb P. Points on the green line for hydrochloric acid are associated with spectra on the following figures. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

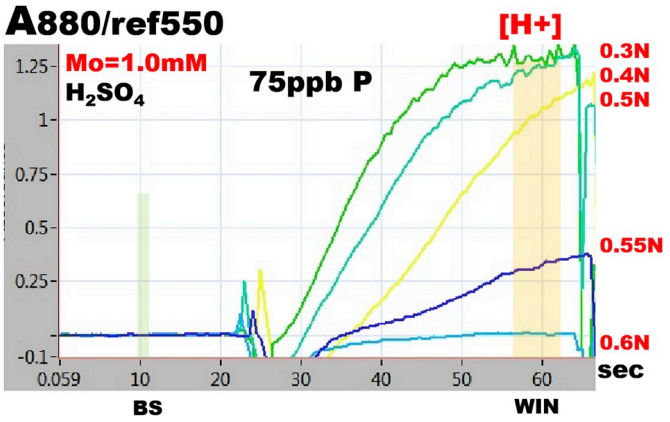


Fig. 7. Influence of acidity of molybdate reagent on formation of PMoB.

a minor elevation at 710 nm. The spectrum in the absence of phosphate (labelled as "BLANK"), is flat above 550 nm wavelength (Fig. 8B.) and (N3, N4. N5 in Ref. [16] and S5, S6 in Ref. [17].). Surprisingly, the range of optimized acidities is rather narrow. Thus e.g. in the case of the hydrochloric acid (Fig. 6B.) with a given concentration of [Mo] 1.0 mM (green line), the optimized range of acidity is 0.2 N–0.4 N. Therefore, the statement that PMoB assay is "best carried out at pH 0 to 1" [1] is misleading, because it omits the necessity of using a suitable $[H^+]$ & [Mo] concentration combination.

The answer to what limits the narrow range of acidities associated with any given [Mo] concentration is revealed by inspection of the reaction rates (Fig. 7.) and of the spectra captured at the end of incubation period (Fig. 8 and 9.).

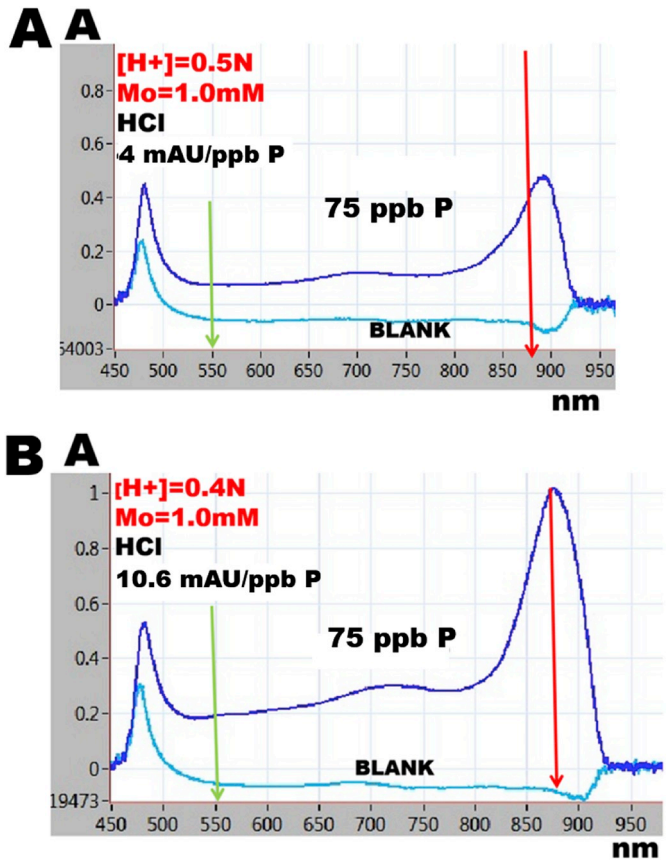


Fig. 8. Spectra recorded in presence (75 ppb P) and absence (BLANK) of phosphate. A when $[H^+]$ = 0.5 N HCl in 1.0 mM molybdate reagent, and B when $[H^+]$ = 0.4 N HCl in 1.0 mM molybdate reagent.

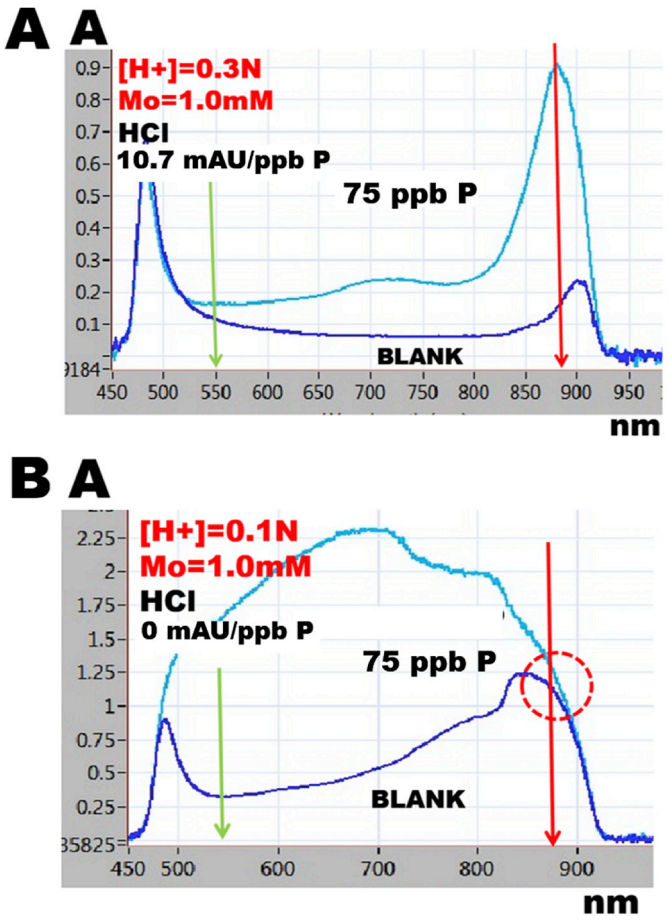


Fig. 9. Spectra recorded in presence (75 ppb P) and absence (BLANK) of phosphate. **A** when $[H^+]$ = 0.3 N HCl in 1.0 mM molybdate reagent, and **B** when [H+] = 0.1 N HCl in 1.0 mM molybdate reagent.

With *increasing acidity*, the formation rate of the PMoB complex decreases, as seen by the decrease in the rate of reaction progress in Fig. 7, obtained with hydrochloric acid at $[H^+]$ ranging from 0.2 to 0.5 N and [Mo] = 1.0 mM. As confirmed in Fig. 6B (green line), the optimum range of acidity does not extend above 0.4 N HCl with [Mo] = 1.0 mM. The spectra of the reactants confirms this observation as it is seen that the absorbance at the 880 nm maximum is lower at $[H^+]$ = 0.5 N (Fig. 8A.) and maximized at [H+] = 0.4 N (Fig. 8B.).

As acidity decreases, initially a small peak from MoB formation appears in the spectrum of BLANK with 0.3 N (Fig. 9A.) and the formation of this reagent blank (MoB) further accelerates, and the peak broadens as the acidity decreases, distorting the spectrum of the PMoB (Fig. 9B.).

The reaction rate responses and spectra obtained for sulfuric, nitric and hydrochloric acids follow the same pattern shown in Refs. [16,17] for [Mo] concentrations ranging from 0.5 mM to 1.5 mM. It follows that the range of acidities where the slope of the calibration line is maximized, is limited by two processes; decrease in the formation rate of PMoB, and increase in the formation rate of the reagent blank (i.e. MoB). However, it is important to note that $[H^+]$ diagrams presented here, and the resulting molar absorptivities, are valid for the following conditions: an incubation time of 30 s, and use of molybdate reagent containing KSbtartrate, which accelerates the formation rate of PMoB in the presence of the ascorbic acid and sodium dodecyl sulphate.

2.4.4. Optimization of PMoB assay

The importance of using a suitable combination of acid and molybdate concentrations was recognized a long time ago, by Going and Eisenreich [12] and confirmed by Pai et al. [18], who recommended using $[H^+]$/[Mo] *ratios* as a tool for optimization of PMoB method. However, this concept was recently rejected (ref. [1] Section 6). "Because it fails to define any chemical property of MB system and is extremely misleading".

The $[H^+]$ diagrams for hydrochloric (Fig. 6B.) and nitric acid [16] are identical and the averaged value of all optimized $[H^+]$/[Mo] ratio for these monoprotic acids is 300, while for sulfuric acid (Fig. 6A.) is higher ($[H^+]$/[Mo] = 400), because sulfuric acid is not fully dissociated in this range of acidities. In summary, while use of $[H^+]$/[Mo] ratio for optimization of reagent composition would be convenient, it will be misleading if used outside the $[H^+]$&[Mo] range of $[H^+]$ diagrams investigated in this work. Therefore, use of $[H^+]$ diagrams is preferable for the method optimization. It should also be noted that because hydrochloric acid can be purified phosphate by isothermal distillation it is more suitable for this assay than sulfuric acid.

To maximize the sensitivity of the assay, the following steps should be followed.

1. Adjust step 3 of the assay protocol (Fig. 4 and A and B in Ref. [14]) to mix the sample with the reagents in proportion 3+1 (sample + reagent), effectively increasing the amount of sample in the reaction mix in the flow cell.
2. From the $[H^+]$ diagram select a [Mo]&$[H^+]$ combination within the optimized range. Greater acidity is preferable to avoid interference from silica.

As an example (Fig. 10.), the reaction mixture *in the flow cell* is composed of [Mo] = 1.0 mM, $[H^+]$ = 0.4 N HCl, 0.03 mM KSb tartrate as well as 0.75% (43 mM) ascorbic acid and 0.38% sodium dodecyl sulphate (8× dilution, Table 1.). The calibration experiment (Fig. 10A.)

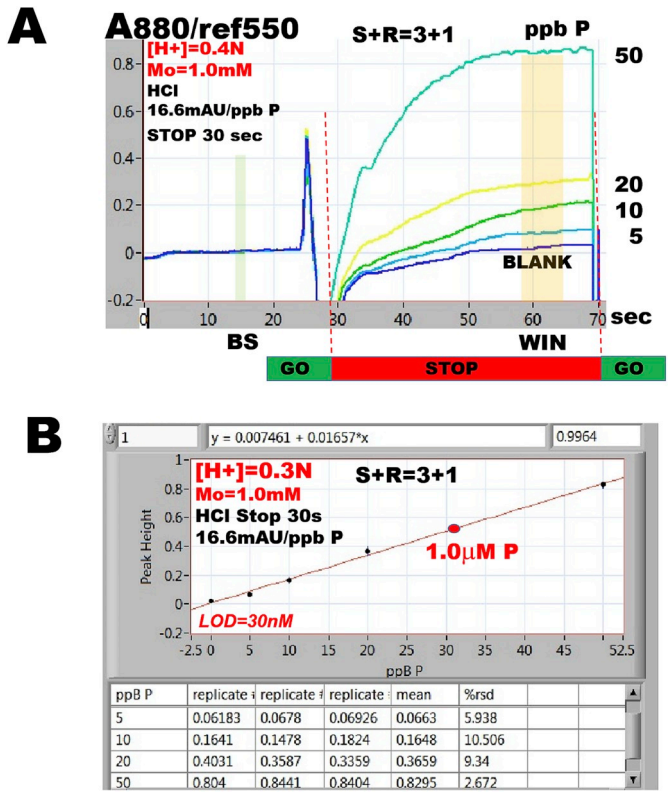


Fig. 10. Maximized sensitivity of phosphate assay. **A.** Stop flow response curves obtained by analyzing orthophosphate standards in the range 0–50 ppb P. **B.** Calibration graph obtained from data collected in A.

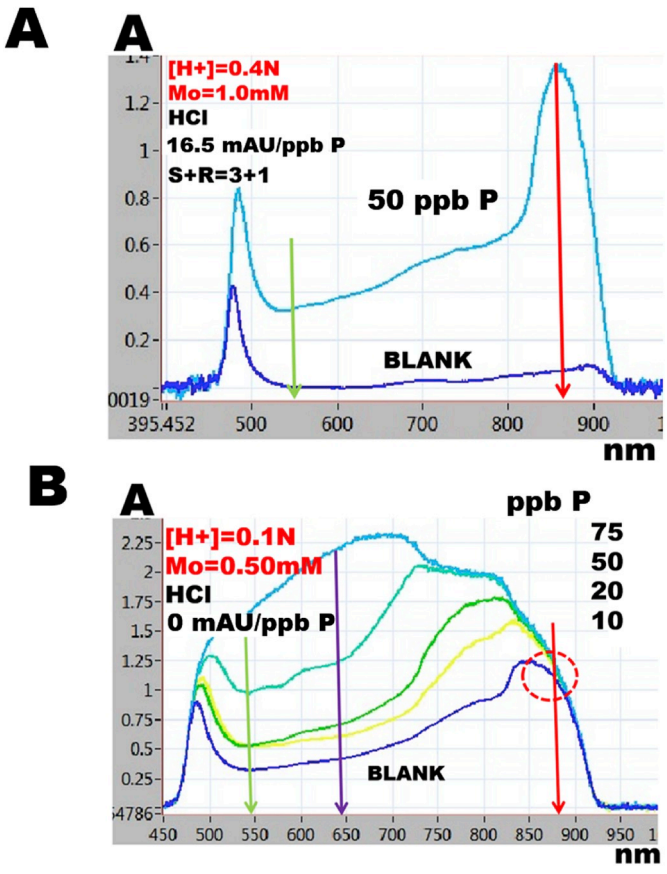


Fig. 11. A. Spectra recorded at optimized conditions in the absence (BLANK) and presence of 75 ppb phosphate. **B.** Spectra of blend of PMoB and MoB obtained at sub optimized conditions in absence of phosphate (BLANK) and with increasing concentrations up to 75 ppb P. Note that at 880 nm wavelength absorbance is no longer a function of phosphate concentration.

yields a calibration line (Fig. 10B.) with a slope of 16.5 mAU/ppb P and a LOD = 30 nM P. To achieve this limit of detection, both reagent and analyte blank must be eliminated. The MoB reagent blank is absent due to the optimized [Mo]&[H^+] combination as confirmed by the spectrum (BLANK in Fig. 11A), while analyte blank was eliminated by using high purity reagents.

Spectra obtained at sub optimized conditions (Fig. 11B.) may suggest that a calibration could be performed at some other wavelength than 880 nm as long as the calibration line can be reliably reproduced. And indeed, many "suitable" wavelengths have been reported in the literature [1]. However, even if the calibration sub-optimal conditions are reproducible, the sensitivity of the method will be lower, as documented by the lower values of molar absorptivities in the literature [1]. Furthermore, differences in the matrix of the standards and samples at sub-optimal conditions may cause undetected errors, if acidity or ionic strength of samples would differ from acidity of standards.

3. Discussion and conclusion

The [H^+] diagrams illustrate the influence of acidity on the formation of the various molybdate species, and therefore it would have been more appropriate to use a pH scale, rather than normality of acids to plot the diagrams. However, even though such an approach is rigorous, it is quite impractical because there is no way in which the pH can be measured precisely enough in the mixture of reactants within the flow cell (for pFI and/or CFA). The advantage of the scale based on normality of [H^+] is simple, that it is directly linked to the concentration of acid used in the preparation of the reagent. The diagrams also confirm that optimized conditions for sulfuric acid are located at higher acidities and higher [H^+]/[Mo] ratios than those of hydrochloric or nitric acid, because sulfuric acid is not fully dissociated at these concentrations.

In order to limit this study to a manageable scope, we used a fixed temperature (22 °C), incubation time (30 s), concentration of potassium antimony tartrate and the composition of the reducing reagent (ascorbic acid and sodium dodecyl sulphate), as these were optimized during our previous work [6]. Of all these parameters, the short incubation time is quite different compared to times used by the CFA method and therefore its suitability needs to be confirmed. As Fig. 12A shows, in the presence of Sb, the formation of PMoB increases rapidly within first 30 s of incubation time and then gradually declines, while in the absence of Sb the absorbance continuously increases, albeit at a slower rate. Interestingly, and unexpectedly, Sb enhances the formation rate of *both* PMoB and of the MoB (BLANK). The spectra obtained after a long incubation time of 240 s (Fig. 12B.) in the presence of Sb reveals the presence of MoB (BLANK), and a shift of the peak maximum from 880 nm to a lower wavelength, which explains why absorbance monitored at 880 nm (Fig. 12A) falls as the incubation time increases. Obviously, there is no benefit in prolonging the incubation time beyond the 30 s that was used in this study, because the formation of PMoB in the presence of Sb has reached equilibrium (Fig. 5A, 7, 10A). The similarity of the molar absorptivity of PMoB, ε = 32,900 obtained from [H^+] diagrams with value obtained by calibration experiments (ε = 34,100, Fig. 10B.) are also confirmed the equilibrium reached completion in 30 s.

While optimization of the PMoB method for trace analysis of phosphate, which was the objective of this work, was achieved, many unknowns about PMoB chemistry remain hidden in the "black box". While this study implies a simple situation, interaction between reaction rates of formation of PMoB and of the reagent blank (MoB), the literature offers a far more complex picture, where various PMoB

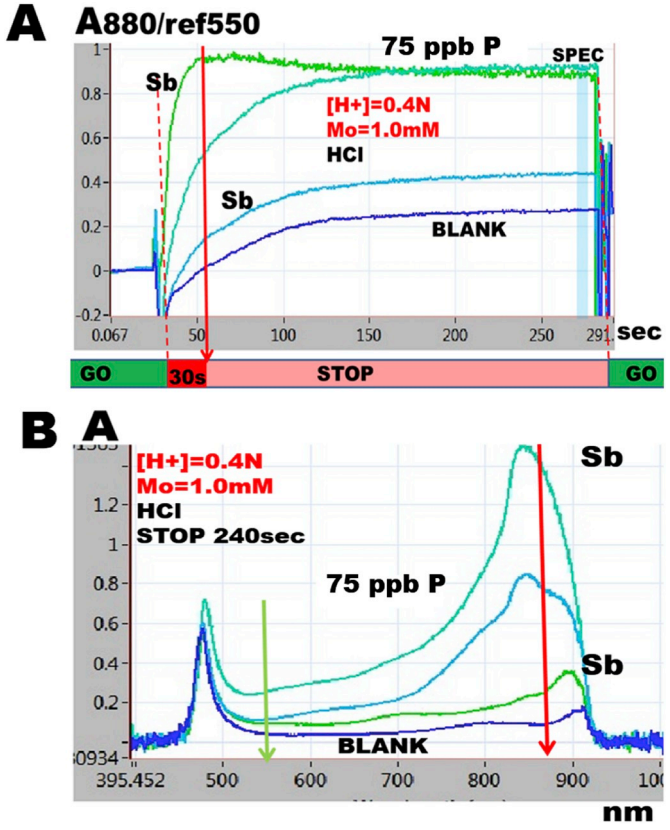


Fig. 12. A. Influence of potassium antimony tartrate (graphs labelled Sb) on formation of PMoB (75 ppb P) and MoB (BLANK), at the short incubation time (30 s) used in this study, and at long incubation time up to 400 s. **B.** Spectra recorded at the end of long incubation period in presence and absence of potassium antimony tartrate.

species are formed in the presence of various reductants and with and without the intervention of Sb (Fig. 6.in Ref. [1]). Whether the different values of molar absorptivities reported in the literature are caused by measuring a blend of colloidal suspension [6] produced at sub-optimal conditions, or are due to formation of different PMoB compounds still has to be resolved. Also, the influence of yet another component of the PMoB method, the various surfactants, that are almost always used in any flow system, remains largely unexplained and unexplored [6], and therefore should be investigated. Hopefully, further studies of the fascinating chemistry of phospho- and silico-molybdates will continue, with assistance of programmable Flow Injection technique and the associated instrumentation that made this initial work possible.

In conclusion, experimental data presented here, confirm that Flow Injection can be programmed to function in a batch mode, and is therefore suitable for determination of molar absorptivities [9], and for investigation of reagent based assays, by monitoring reaction rates and spectra of ongoing chemical reactions.

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