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# Sequential injection determination of orthophosphate as ion associate of 12-molybdophosphate with Astra Phloxine

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ABSTRACT

A simple and fast reaction between 12-molybdophosphate heteropoly anion and the polymethine dye Astra Phloxine was used for the development of a new SIA method for the determination of orthophosphate. The suggested method has higher sensitivity and a broader calibration range than existing SIA methods. It can be used to detect phosphate in the range from 0.2 to 10 $\mu$mol L$^{-1}$ with a detection limit of 0.1 $\mu$mol L$^{-1}$ and an acceptable throughput of 20 samples h$^{-1}$. The comparably low molybdate (5.6 mmol L$^{-1}$) and dye (0.1 mmol L$^{-1}$) concentrations led to an improvement in the stability of the base line. Inter-day reproducibility was better than 5%, while the intra-day RSD was in the range 0.8–3.5%. The method was used for the analysis of natural water samples and phosphorus-containing chemicals having a low content of orthophosphate.

## 1. Introduction

Molybdophosphate heteropoly blue (phosphomolybdenum blue) and various ion association complexes (IA) of basic dyes with 12-molybdophosphate heteropoly anion (12-MPA) are two commonly used analytical forms for the determination of orthophosphate. The formation of phosphomolybdenum blue has been the basis for many flow injection (FI) procedures used to determine phosphate. Despite their higher molar absorptivity, IAs have not been widely used in flow-based systems for phosphate determination [1,2], mainly because low soluble aggregates of IA can be adsorbed onto the inner walls of FI system components (PTFE tubing, joints, flow cell, etc.), leading to a drift in the baseline [3].

Usually, with the formation of an IA by a basic dye and a heteropoly anion, the spectra of the dye and its IA with the heteropolyanion (HPA) or isopolymolybdate anions used as a reagent are very close to one another. Therefore, it is necessary to separate the dye excess and its IA with isopolymolybdate ions from the IA dye–HPA. This can be done using extraction, flotation and centrifugation [4], but such procedures complicate the determination, reduce reproducibility and prolong the determination. The best alternative method is based on the discoloration of the excess of triphenylmethane dye, which occurs through sufficient acidification due to the selective protonation of only dye cations not bound in an IA with HPA [2]. Malachite green has been the most widely used reagent among other dyes [5]. Nevertheless, besides the aforementioned disadvantages, such reactions are somewhat slow [6] and can lead to high blanks.

It was recently shown that polymethine dyes are capable of forming stable IAs with HPAs [7–9]. This reaction is accompanied by a marked change in the color of the solution. Thus, the absorbance of the IA can be measured directly and without preliminary separation of the dye. The color of the IA develops immediately after mixing reagents and remains constant for several hours. The method is much more sensitive than "phosphomolybdenum blue" or "malachite green" procedures, and its selectivity is significantly better for many species. High concentrations of chloride [10] and many other ions do not interfere with the determination, and phosphate can be determined in highly salty waters such as samples from the Dead Sea [7]. The absence of additional steps, in addition to the very rapid formation of colored substance, makes the determination highly reproducible.

The formation of IA by Astra Phloxine (AP) and the reduced form of 12-MPA was used for the development of an automated procedure for the determination phosphate by cyclic-injection analysis [9]. The configuration of the flow system used allowed for negligible dispersion, a fact that explains why the molar absorptivity obtained was nearly the same as in the batch method, that is,

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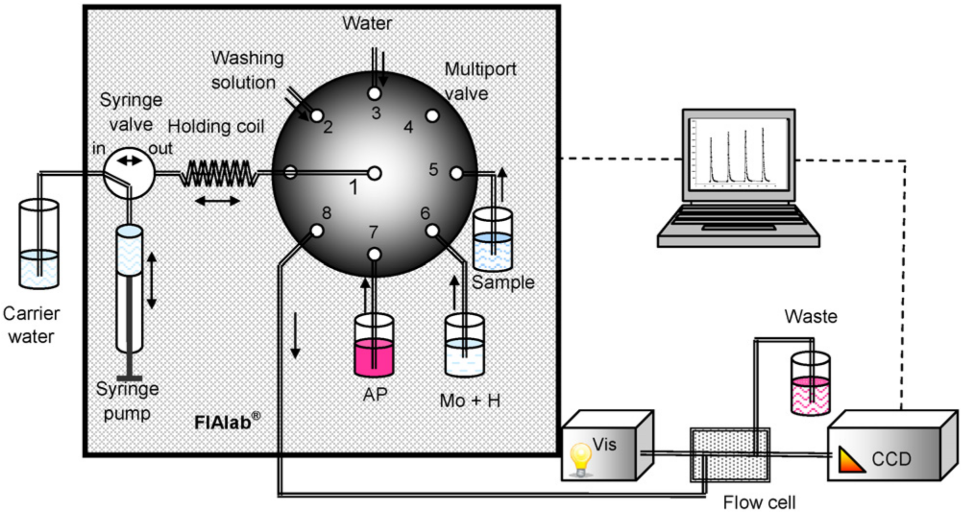

**Fig. 1.** Scheme of the SIA manifold for the determination of phosphate. CCD: charge-coupled detector; Vis: tungsten lump; AP: Astra Phloxine, $C_{AP}$ = 0.1 mmol L$^{-1}$; Mo + H: $C_{Mo(VI)}$ = 5.6 mmol L$^{-1}$, $C_H$ = 0.2 mol L$^{-1}$; holding coil length 20 cm; λ = 574 nm; flow rate 25 μL s$^{-1}$.

$1.6 \times 10^5$ mol$^{-1}$ L cm$^{-1}$. The detection limit achieved was 5 μg L$^{-1}$ at a sample volume of 0.3 mL. The method was distinguished by a high consumption of reagents (0.6 mL) and a slow sampling rate of 6 analyses per hour.

Sequential injection analysis (SIA) is a versatile flow method using programmable flow as well as possibility of flow redirection. Among the major benefits of SIA systems are reduced consumption of reagents, robust hardware and flexible control software, which provides convenient optimization and operation of the system.

The determination of phosphorus is very important for monitoring rivers, lakes and oceans. Increased phosphorus concentrations resulting from overfertilization or release from industrial and municipal sources cause eutrophication and other adverse effects [1]. Phosphate concentrations in oligotrophic systems can be less than 10 μg L$^{-1}$ [11], making them difficult to analyze using FI procedures based on the formation of phosphomolybdenum blue. Moreover, due to its slowness, the phosphomolybdenum blue method may overestimate orthophosphate by erroneously including acidic hydrolysable organic or condensed phosphorus compounds [12].

The many existing FI and SI procedures for the determination of phosphate are based on slow reactions [1,2]. Thus their use in FI systems leads to a significant increase in dispersion and a decrease in sensitivity. In this work, the very fast reaction of 12-MPA with Astra Phloxine was used to develop a simple, low-cost, sensitive and selective SIA method for the determination of orthophosphate. The applicability of the method was demonstrated through the analysis of real water samples.

## 2. Materials and methods

### 2.1. Reagents

All reagent solutions were prepared from analytical grade chemicals. Water was purified on a Milli-Q system (Millipore, USA). A phosphorus(V) stock solution containing 0.1 mol L$^{-1}$ of phosphate was prepared by dissolving $KH_2PO_4$ in water. A 0.1 mol L$^{-1}$ molybdate stock solution was prepared by dissolving $Na_2MoO_4 \cdot 2H_2O$ recrystallized from water–ethanol, standardizing by evaporation and weighing, and then storing in a polyethylene bottle. Astra Phloxine (Basic Red 12, 1,3,3-trimethyl-2-[3-(1,3,3-trimethyl-2-indolinylidene)propenyl]-3*H*-indolium chloride) was purchased from Hangzhou Dayangchem Co., Ltd. (China) and used without purification. A 1 mmol L$^{-1}$ Astra Phloxine stock solution was prepared by dissolving 0.444 g of AP in 10 mL of ethanol and diluting to 1000 mL with purified water. All solutions as well as the water used as a carrier were degassed in an ultrasonic bath for 20 min prior to analysis. Standard solutions of potassium pyrophosphate, adenosine 5′-triphosphate, sodium phenylphosphate, sodium tripolyphosphate, and sodium glucose-6′-phosphate were prepared directly before analysis.

### 2.2. Apparatus

The SIA manifold was arranged using the following equipment: a FIAlab® 3500 system (FIAlab® Instrument Systems Inc., Bellevue, USA) consisting of a syringe pump (syringe reservoir 5 mL) and a central eight-port Cheminert selection valve (Valco Instrument Co., USA).

The manifold was equipped with a USB 2000 fiber-optic charge-coupled detector (Ocean Optics Inc., Dunedin, USA) with LS-1 tungsten lamp (Ocean Optics Inc., Dunedin, USA) as a VIS light source and a micro-volume Z-flow cell of 20 mm optical path length. All manifold tubing was made of 0.75 mm i.d. polytetrafluoroethylene (PTFE), except that used for the aspiration of water as a carrier, which was 1.5 mm i.d. The whole SIA system was controlled by the latest version of FIAlab program for Windows 5.0.

### 2.3. SIA procedure

The SIA manifold configuration used for the determination of orthophosphate is shown in Fig. 1. The analytical cycle begins by filling the piston pump syringe with 2000 μL of the carrier solution (water), which is drawn into the syringe at a flow rate of 100 μL s$^{-1}$. The syringe pump outlet (two-way valve) is then set into the out position, the flow rate changed to 25 μL s$^{-1}$, and 80 μL of reagent (5.6 mmol L$^{-1}$ molybdate and 0.1 mol L$^{-1}$ sulfuric acid) using port 6 of the multiport valve, 30 μL of 0.1 mmol L$^{-1}$ solution of AP (port 7), 60 μL of sample solution (port 5), and 70 μL of water (port 3) are sequentially aspirated into the holding coil. For better mixing, 40 μL of water are initially dispensed and then aspirated through port 4. The entire volume is forced at 25 μL s$^{-1}$ through a Z-flow cell using port 8. The spectrometer reference scan is made after a 1 s delay and then absorbance scanning begins. The response signal is measured at 574 nm. The system is washed after every five cycles by aspirating 300 μL of 50% ethanol into the holding coil through port

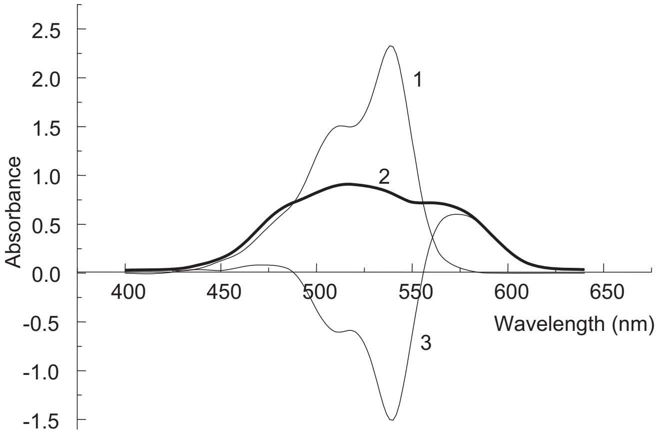

**Fig. 2.** The absorption spectra of Astra Phloxine (1), IA AP–12-MPA (2), difference spectrum (3). $C_{P(V)} = 4.0\,\mu mol\,L^{-1}$, $C_{Mo(VI)} = 2.8\,mmol\,L^{-1}$, $C_H = 0.2\,mol\,L^{-1}$, $C_{AP} = 2.8\,\mu mol\,L^{-1}$, $l = 1$ cm.

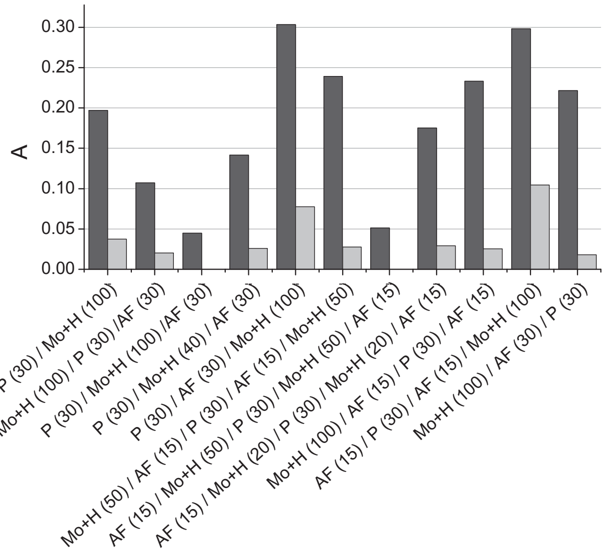

**Fig. 3.** Dependence of the obtained signal on the measurement sequence. Grey columns designate intensity of the signal for the blank. Volume of specie in μL is given in parentheses. $C_{P(V)} = 5\,\mu mol\,L^{-1}$, $C_{Mo(VI)} = 4\,mmol\,L^{-1}$, $C_H = 0.2\,mol\,L^{-1}$, $C_{AP} = 0.05\,mmol\,L^{-1}$, $\lambda = 574$ nm, $l = 2$ cm.

2 by first dispensing 200 μL and after a delay of 10 s the remaining volume through a Z-flow cell using port 8.

## 3. Results and discussion

### 3.1. Color reaction

The reduction of the yellow-colored 12-molybdophosphate heteropoly anion with common reducing agents such as ascorbic acid or hydrazinium sulfate is a slow reaction. Therefore, in the FI methods, the absorbance of phosphomolybdenum blue is often measured before the reduction of the 12-MPA is completed. Increasing the length of the reaction coil in FIA manifold or the residence time in the holding coil in an SIA manifold leads to higher dispersion, which can strongly lower the sensitivity. Therefore, a compromise between sensitivity and duration of the analysis must be sought. Recalculation of the absorbance measured using a conventional FIA method [13] to the molar absorptivity leads to a value of $2 \times 10^3\,mol^{-1}\,L\,cm^{-1}$, which is one magnitude lower than that obtained for the batch spectrophotometric method.

The reaction of AP with 12-MPA is accompanied by a sharp change in color from rose to red-violet. Below a P(V) concentration of $1\,\mu mol\,L^{-1}$, supersaturated solution of IA is formed. The color of the IA develops instantaneously after mixing of reagents. As a result of the reaction between HPA and AP, as well as for other polymethine dyes [7,8], the spectrum of the IA differs significantly from that of the dye under certain conditions (Fig. 2). An intensive red-shifted band appears in the spectrum of IA AP–12-MPA at 574 nm.

Recently, the optical properties of organic nanoparticles have attracted growing attention [14,15]. Cyanine, porphyrine and merocyanine dyes in particular stand out among a large family of organic nanoparticles. The self-association of the dyes in solution is a frequently encountered phenomenon in the chemistry of polymethine dyes owing to a strong dipole–dipole interaction between the molecules. Aggregates in solution exhibit distinct changes in the absorption band when compared to the monomeric species. The bathrochromically shifted J-bands and hypsochromically shifted H-bands of the aggregates have been explained in terms of the molecular exciton coupling theory, i.e., the coupling of transition moments of the constituent dye molecules [14–23].

The self-association of polymethine dyes in aqueous solution can be enhanced by either decreased temperature or increased dye concentration. As indicated in [23], inorganic salts could have a significant influence on the formation of the J-band in the absorption spectra of aqueous solutions of polymethine dyes, which was explained by a decrease in the solubility of the salt formed. A stable supersaturated solution of IA is formed during the reaction between AP and HPA. Close contact between IA molecules in low-soluble aggregates favors the noncovalent bonding interaction of the AP cations with each other. It is known that in the absence of HPA, AP can form aggregates only in a medium having a very low polarity containing more than 95% of hexane in dichloroethane [17]. It can be therefore supposed that large hydrophobic HPAs are capable of forming low-soluble particles of IA, thus significantly facilitating the formation of new well-ordered nanoclusters.

### 3.2. SIA procedure: optimization of manifold parameters

The optimal conditions for the formation of the IA $(AP)_3PMo_{12}O_{40}$ had been found previously [7] and were used in this study as a starting point for the optimization of the flow variables. In batch conditions, 12-MPA and, consequently, IA are formed completely when starting with a molybdate concentration of 2–3 $mmol\,L^{-1}$. At this concentration of molybdate, the optimal range of the $H_2SO_4$ concentration is equal to 0.06–0.14 $mol\,L^{-1}$. Due to the high stability of the obtained IA, the concentration of the dye used can be low, only slightly exceeding the amount based on the stoichiometry of the reaction. All the reagents used in the procedure are highly stable in time.

The sequence of mixing reagents and analyte in the holding coil has a strong influence on the signal obtained. A mixed reagent containing molybdate, acid and dye is unstable. Therefore, the solutions of acidified molybdate and dye must be injected separately. It can be seen from Fig. 3 that the best sequences for mixing include one in which the analyte is initially mixed with a low volume of concentrated dye solution. The use of the molybdate solution for the same purpose is limited. At a molybdate concentration higher than 6 $mmol\,L^{-1}$, the formation of isopolymolybdate anions, which are also capable of forming colored IA with AP, is strongly increased.

The various chemical and SI variables shown in Fig. 1 were optimized using the univariate method at a fixed P(V) concentration of 6.7 $\mu mol\,L^{-1}$ (Table 1). During the optimization of the chemical variables, we sought conditions in which the absorbance of the blank would remain at a reasonable level. Demineralized water was

**Table 1**
Optimized chemical and physical parameters of the SIA manifold.

| Parameter | Optimized range | Optimal value |
|---|---|---|
| Acid concentration, mol L$^{-1}$ | 0.1–0.3 | 0.2 |
| Molybdate concentration, mmol L$^{-1}$ | 4.0–7.2 | 5.6 |
| Dye concentration, mmol L$^{-1}$ | 0.02–0.50 | 0.1 |
| Volume of sample, μL | 20–80 | 60 |
| Volume of Mo + H reagent, μL | 40–100 | 80 |
| Volume of dye, μL | 20–40 | 30 |
| Flow rate through the detector, μL s$^{-1}$ | 15–50 | 25 |

chosen as the carrier solution in view of the fact that zone of colored product was isolated and the acidified molybdate solution was used.

The effect of the concentration of molybdate ions on the response was examined (Fig. 4a). A maximum of the difference between the absorbances of solution containing phosphate and blank solution was observed in the range from 5.6 to 7.0 mmol L$^{-1}$. A molybdate concentration of 5.6 mmol L$^{-1}$ was chosen as optimal because the influence of the formation of IA between AP and isopolymolybdate anions was at its smallest within the above-mentioned range.

It can be seen from Fig. 4b, that the maximal analytical signal was obtained for concentrations of the acid which varied in the range from 0.15 to 0.20 mol L$^{-1}$. The concentration 0.20 mol L$^{-1}$ was selected as optimal, because it allowed for a low blank and the best signal/noise ratio to be achieved.

Increasing the AP concentration in the range 0.02–0.5 mmol L$^{-1}$ steadily increased the absorbance (Fig. 4c). A dye concentration of 0.1 mmol L$^{-1}$ was chosen as the most suitable for the further study. When higher dye concentrations were used, the obtained response was poorly reproducible and the blank signal had tendency to increase. In addition, increasing the sorption of the IA required more frequent washing of the system, thus increasing the duration of the analysis.

The effect of sample and reagents volumes on the determination was studied in the ranges shown in Table 1. In each case studied, the volumes which corresponded to the maximum absorbance were chosen as optimal. A change in the flow rate through the detector only slightly influenced the analytical signal. To achieve better throughput, the rate of 25 μL s$^{-1}$ was chosen.

Under optimal conditions (Table 1), a linear calibration curve was obtained over the range 0.2–10 μmol L$^{-1}$ of P(V) with a correlation coefficient of 0.9992. Aspiration of a series of standard orthophosphate solutions resulted in the flow signals shown in Fig. 5. The regression equation was $A^{574} = (0.032 \pm 0.002) + [(5.81 \pm 0.07) \times 10^4]\cdot C_P$ (mol L$^{-1}$). The RSD was 0.8–3.0% ($n = 8$) over the range of P(V) concentrations studied and the limit of detection (3 s) was 0.1 μmol L$^{-1}$ with a sample throughput of 20 h$^{-1}$.

The stability of the base line was controlled by repeating measurements throughout the day and by a comparison with the results obtained on the following day (Table 2). Inter-day reproducibility was better than 5%, while the intra-day RSD was in the range 0.8–3.5%. It can thus be concluded that a change in the blank signal was insignificant within at least a two-day period.

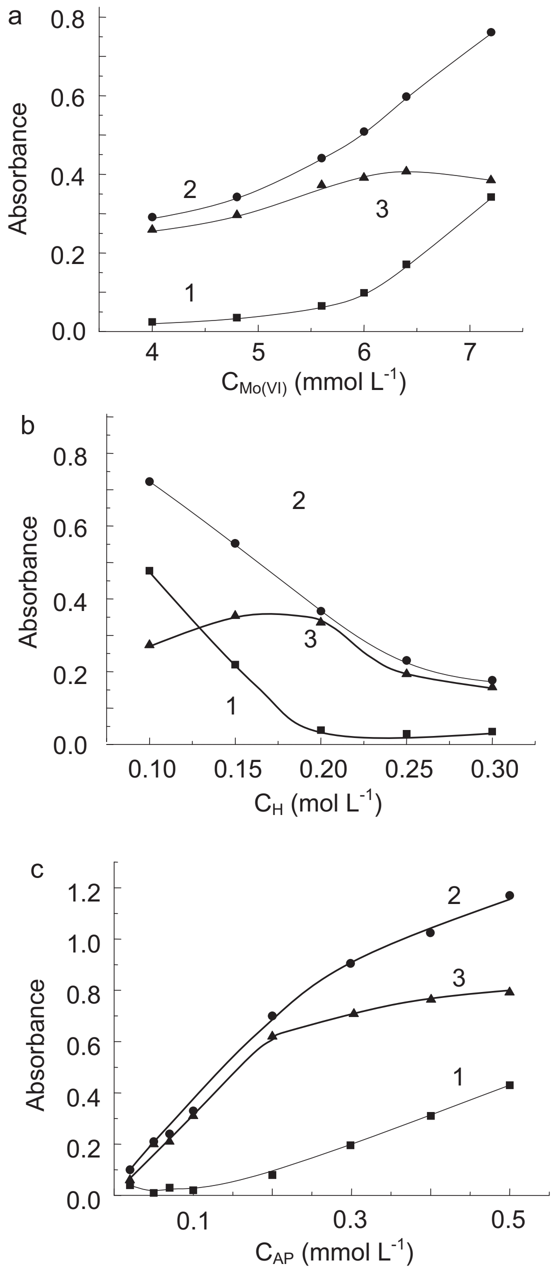

**Fig. 4.** Influence of concentrations of molybdate (a), acid (b), and Astra Phloxine (c) on the formation of IA AP–12-MPA. 1 – blank; 2 – IA; 3 – difference between 2 and 1. $C_H = 0.2$ mol L$^{-1}$, $C_{Mo(VI)} = 5.6$ mmol L$^{-1}$, $C_{AP} = 0.1$ mol L$^{-1}$, $C_{P(V)} = 5$ μmol L$^{-1}$.

### 3.3. Interference study and application

The effects of other anions and cations which might coexist with orthophosphate in natural waters were studied. The tolerance limit was set as the maximum amount causing an error of ≤±5% in the absorbance. $Na^+$ (5 g L$^{-1}$), $K^+$ (8 g L$^{-1}$), $Mg^{2+}$ (2.4 g L$^{-1}$), $Ca^{2+}$ (2 g L$^{-1}$), $SO_4^{2-}$ (10 g L$^{-1}$), $HCO_3^-$ (0.1 g L$^{-1}$), $Cl^-$ (7 g L$^{-1}$), $NO_3^-$ (3 g L$^{-1}$) and 10 mg L$^{-1}$ $NO_2^-$, $Cd^{2+}$, $Cu^{2+}$, $Pb^{2+}$, $Fe^{3+}$, $Mn^{2+}$, $Zn^{2+}$ resulted in no significant interference with the determination of phosphate at 0.15 mg L$^{-1}$ level. By using 0.01 mol L$^{-1}$ tartaric acid together with molybdate reagent, we also succeeded in masking Si(IV) up to 20 mg L$^{-1}$. Preliminary reduction of As(V) with thiosulfate (2 mmol L$^{-1}$, 15 min, $C_H = 0.2$ mol L$^{-1}$) makes negligible its influence on the determination of P(V) up to the As(V) concentration of 0.04 mmol L$^{-1}$.

The applicability of the method was tested by analyzing different samples of natural waters. The results are given in Table 3. A comparison with the results of analysis by using the phosphomolybdenum blue method [24] showed the accuracy and sufficient reproducibility of the proposed SIA method.

The content of phosphate in several representatives of phosphorus-containing compounds was evaluated by the proposed method and compared with the results obtained by batch phosphomolybdenum blue method (Table 3). The short contact time

**Table 2**
Intra-day and inter-day precision and accuracy data for the determination of phosphorus(V) (μmol $L^{-1}$ ± confidence limits for $n = 8$ and 95% confidence level).

| Taken, μmol $L^{-1}$ | Intra-day[a] | | | Inter-day[b] | | |
|---|---|---|---|---|---|---|
| | Found, μmol $L^{-1}$ | Found, % | RSD, % | Found, μmol $L^{-1}$ | Found, % | RSD, % |
| 6.7 | 6.66 ± 0.20 | 99.4 | 3.5 | 6.67 ± 0.25 | 99.5 | 4.5 |
| 5 | 5.01 ± 0.07 | 100.2 | 1.7 | 5.09 ± 0.07 | 101.7 | 1.7 |
| 3.6 | 3.59 ± 0.08 | 99.7 | 2.7 | 3.66 ± 0.11 | 101.6 | 3.7 |
| 2.2 | 2.14 ± 0.05 | 97.3 | 2.4 | 2.24 ± 0.06 | 102.0 | 3.4 |

[a] Eight measurements were made throughout the day.
[b] Four measurements were made every two days.

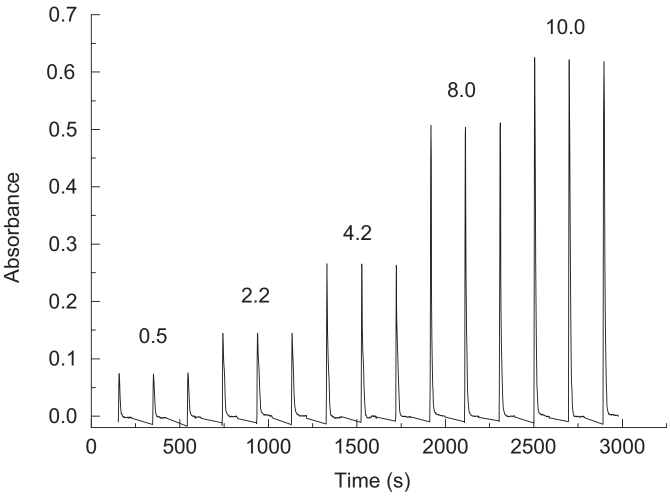

**Fig. 5.** Typical SIA signals for standard phosphate solutions obtained by SIA system in Fig. 1. Values indicate concentration of phosphate in μmol $L^{-1}$.

**Table 3**
Determination of phosphate in Slovak natural waters (μmol $L^{-1}$ ± confidence limits for $n = 7$ and 95% confidence level) and in phosphorus-containing chemicals (% ± confidence limits for $n = 5$ and 95% confidence level).

| Spring water/chemical | MB method | | Proposed method | |
|---|---|---|---|---|
| | $C_P$ | RSD, % | $C_P$ | RSD, % |
| Liptovsky jan | 3.73 ± 0.07 | 2.0 | 3.64 ± 0.10 | 3.1 |
| Lucky | 2.77 ± 0.04 | 1.7 | 2.71 ± 0.07 | 2.8 |
| Dudince | 4.16 ± 0.10 | 2.5 | 4.21 ± 0.16 | 4.0 |
| Potassium pyrophosphate | 0.044 ± 0.009 | 8.5 | 0.042 ± 0.003 | 3.0 |
| Adenosine 5′-triphosphate | 0.56 ± 0.06 | 4.3 | 0.55 ± 0.06 | 4.4 |
| Sodium phenylphosphate | 0.85 ± 0.05 | 4.4 | 0.83 ± 0.05 | 4.8 |

and comparatively low acidity of the reaction medium prevent the hydrolysis of studied compounds. The sensitivity of both methods was unsufficient to detect the phosphate in the sodium tripolyphosphate and sodium glucose-6′-phosphate chemicals.

## 4. Conclusions

A very simple and fast reaction between 12-MPA and the polymethine dye Astra Phloxine was used for the development of a promising new SIA method for the determination of orthophosphate. The use of a rapid reaction is advantageous in SIA, as it allows for a significant increase in the method throughput. In addition, the decreased residence time as well as the dispersion of a colored product, together with high molar absorptivity of IA AP–12-MPA, results in a method of high sensitivity. The effective molar absorptivity calculated from the slope of the calibration graph was $3.7 \times 10^4$ $mol^{-1}$ L $cm^{-1}$, which is approximately twenty times higher than the value obtained when using the phosphomolybdenum blue method in an FIA/SIA configuration. Narrower calibration ranges and higher detection limits (in μmol $L^{-1}$) have been found in preceding SIA methods for the determination of P(V): 1.7–13 (0.3) in [3] and 6.7–230 (3) in [25], respectively.

Some papers have shown comparably low detection limits based on the outstanding ability of the FIA method to detect very small values of absorbance with high reproducibility, but it should be noted that reproducibility and sensitivity can be greatly reduced through speciation analysis [26–28], simultaneous determination of P(V) and Si(IV) [29,30], the use of on-line digestion [31] or by taking the hydrolysis of organically bound phosphorus in natural waters into consideration [11]. The high selectivity of the proposed method and its broad calibration range allow for the monitoring of orthophosphate concentration in most types of natural waters.

The concentrations of molybdate and dye necessary for the formation of the IA were lower than in the MG method, which allowed for a significant decrease in the influence of coating effects of the IA on the stability of the baseline. One possible disadvantage of SIA methods is their slow analysis rate. However, in this work a relatively high sampling rate of 20 samples $h^{-1}$ was achieved. Reagent consumption was low, and the reagent solutions were stable over a long period of time. The method offers high tolerance to silicate by using tartaric acid as a masking agent.

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