Development of a flow method for the determination of phosphate in estuarine and freshwaters—Comparison of flow cells in spectrophotometric sequential injection analysis

Raquel B.R. Mesquita[a,b], M. Teresa S.O.B. Ferreira[a], Ildikó V. Tóth[c], Adriano A. Bordalo[b], Ian D. McKelvie[d], António O.S.S. Rangel[a,*]

[a] *CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*
[b] *Laboratory of Hydrobiology, Institute of Biomedical Sciences Abel Salazar (ICBAS) and Institute of Marine Research (CIIMAR), Universidade do Porto, Lg. Abel Salazar 2, 4099-003 Porto, Portugal*
[c] *REQUIMTE, Departamento de Química, Faculdade de Farmácia, Universidade de Porto, Rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[d] *School of Chemistry, The University of Melbourne, Victoria 3010, Australia*

A B S T R A C T

A sequential injection system with dual analytical line was developed and applied in the comparison of two different detection systems *viz*; a conventional spectrophotometer with a commercial flow cell, and a multi-reflective flow cell coupled with a photometric detector under the same experimental conditions. The study was based on the spectrophotometric determination of phosphate using the molybdenum-blue chemistry. The two alternative flow cells were compared in terms of their response to variation of sample salinity, susceptibility to interferences and to refractive index changes. The developed method was applied to the determination of phosphate in natural waters (estuarine, river, well and ground waters). The achieved detection limit (0.007 μM PO_4^{3-}) is consistent with the requirement of the target water samples, and a wide quantification range (0.024–9.5 μM) was achieved using both detection systems.

Keywords:
Sequential injection
Phosphate determination
Estuarine and river water
Spectrophotometry
Multi-reflective flow cell

1. Introduction

Phosphate is an important routine parameter in water analysis, being simultaneously an essential macronutrient and a possible pollutant, when its concentration is abnormally high. The quantification of phosphate in different water bodies is important since the increase of phosphate concentrations in surface waters is usually linked to diffuse sources (like agricultural run-offs).

Methods applicable to phosphate determination in a diverse range of natural waters (estuarine, river, well and ground waters) with highly varying intrinsic characteristics, are needed. Additionally, effective methods must handle the problems arising from the salinity gradient, should be fast, with low reagent consumption and should provide the possibility of real time assessment. Flow analysis techniques meet these requirements, which make them a valuable tool for monitoring processes.

Flow methods for the determination of phosphate in waters have been extensively investigated as listed in comprehensive reviews by Estela and Cerdà [1], Motomizu and Li [2] and Monbet and McKelvie [3]. Among the different flow analysis techniques proposed in the last twenty years, sequential injection analysis (SIA) has gained particular attention for water analysis [4]. Samples and reagents are sequentially aspirated to a holding coil, and subsequently transported to the detector by flow reversal. The protocol sequence, responsible for those actions, is computer controlled. SIA methods have recognised merits of robustness and versatility enabling the possibility to change the determination conditions without physical reconfiguration of the manifolds. In this scenario, several SIA methods have been reported for the spectrophotometric determination of phosphate [5–20] as listed in Table 1. However, these methods were generally developed for the application to a single type of water (even when other samples were also used). Within these applications, some were designed to cope with the low concentration of the analyte and proposed distinct strategies to handle the matrix differences and the interferents. For seawater samples, Ma et al. [5] adopted a strategy to directly concentrate phosphomolybdenium blue from the water sample to an HLB cartridge, this way minimizing salinity effect of the samples, but compromised sampling frequency. For wastewater and micro algae medium [11], a mixing chamber was introduced into a manifold to enhance the mixing conditions, and to achieve adjustable dilution of the samples coupled with the standard addition pro-

* Corresponding author. Tel.: +351 225580064; fax: +351 225090351.
E-mail address: aorangel@esb.ucp.pt (A.O.S.S. Rangel).

Table 1
Sequential injections systems for the determination of phosphate.

Detection system	Sample	P fraction	Reagents	Dynamic range	RSD	LOD	Determination rate (h^{-1})	Reference date
Spectrophotometric	Seawater	Soluble reactive phosphorus	Molybdate, ascorbic acid	0.034–1.134 μM	2.50%	1.4 nM	6–10	[5]
Fluorimetric	Coastal waters	Phosphate	Molybdate, rhodamine (plus ammonia determination)	0.5–5.0 μM	–	0.05 μM	100	[6]
Fluorimetric	Coastal waters	Filterable Reactive phosphate (FRP)	Molybdate, rhodamine	0.5–5.0 μM	–	0.05 μM	270	[7]
Fluorimetric	River and marine waters	Reactive phosphate	Molybdate, rhodamine (plus ammonia determination)	0.3–4.0 μM	–	0.3 μM	120	[8]
Spectrophotometric	Urine	Phosphate	Molybdate, tin(II) chloride	0.01–30 mM	3.90%	–	30	[9]
Spectrophotometric	Beverages, wastewater, urine	Orthophosphate	Molybdate	Up to 20 mg P L^{-1}	2.4%, 1.8%	100 μg P L^{-1}	18	[10]
Spectrophotometric	Water samples	Orthophosphate	Molybdate, ascorbic acid used in standard addition	62.5–250 ng P (added)	4%	24 μg P L^{-1}	14	[11]
Turbidimetric	Urine	Phosphate	Calcium carbonate inhibition, calcium phosphate crystallisation	0.1–0.8 mg L^{-1}; 0.2–1.5 g L^{-1}	0.97–1.90%; 1.1–2.0%	0.01 mg L^{-1}; 14 mg L^{-1}	12; 15	[12]
Spectrophotometric	Water samples	Phosphate	Molybdate, ascorbic acid	19.9–196.0 μg L^{-1}	1.22%	9.92 μg L^{-1}	54	[13]
Spectrophotometric	Wastewater	Phosphate	Molybdate, vanadate (plus Si determination)	0.026–0.485 mM	2.10%	7.4 μM	30	[14]
Spectrophotometric	Waters and sediments	Phosphate	Molybdate, ascorbic acid	0.2–7 mg L^{-1}	–	0.1 mg L^{-1}	75	[15]
Spectrophotometric	–	Orthophosphate	Molybdate, ascorbic acid	4–40 mg L^{-1}	–	4 mg L^{-1}	48	[16]
Spectrophotometric	Natural water and effluent stream	Phosphate	Molybdate, ascorbic acid	0–70 mg L^{-1}	0.90%	0.5 mg L^{-1}	18	[17]
Spectrophotometric	Tap and well waters	Phosphate	Molybdate, tin(II) chloride (plus Fe(II) determination)	0.1–1 mg P L^{-1}	4.10%	20 μg P L^{-1}	7	[18]
Spectrophotometric	Wastewater	Phosphate	Molybdate, vanadate	Up to 12 mg P L^{-1}	<1.4%	0.2 mg P L^{-1}	23	[19]
Spectrophotometric	Natural and wastewater	Orthophosphate	Molybdate, vanadate, malachite green, tin(II) chloride	Up to 18.0, 0.4, 4.0 mg P L^{-1}	2.1, 18, 1.7%	0.15, 0.01, 0.01 mg P L^{-1}	30	[20]

cedure. For a lake and a tap water sample, Wu and Růžička [13] applied the kinetic stopped flow measurement mode, which can also contribute to the reduction of sample matrix effects in the applied Lab-on-Valve SIA mode. The same stopped flow strategy was used earlier by Mas-Torres et al. [14], to simultaneously measure phosphate and silicate in waste waters, although under certain conditions the mutual interference of the two analytes could be detected. Another interesting approach presented by the same research group [18] was based on the use of the sample solution as carrier to detect multiplicative interferences in the determination.

The use of the same flow manifold and protocol sequence for water samples of different origins does not usually lead to good quality results, due to both chemical and physical interferences. For example, large differences in sample salinity may induce shifts in the equilibrium of chemical reactions, and also may produce interferences in the spectrophotometric detection, by creating light refraction in the reaction interfaces, seriously affecting the analytical signal [21].

In this work, we propose the use of a multi-reflective cell (MRC) [22] coupled to a sequential injection system to tackle this problem. In these conditions, the SIA manifold provides the necessary "a la carte" in-line sample treatment and the miniaturized spectrophotometer (MRC with a LED light source and detector) provides detection with minimum schlieren effect. To highlight the merits of the flow cell, a manifold that allows the comparison with a conventional flow cell (CFC) placed in a UV–Vis spectrophotometer is presented. The SIA methodology was based on the widely used phospho-molybdenum blue reaction. This reaction has been reported as highly sensitive and selective with few possible interferences, namely As(V), Si and Ge [23]. The best conditions for the colorimetric reaction were assessed and the minimization of possible interferences was effectively carried out in-line. The developed system, was effectively applied to estuarine (salinity gradient), river, well and ground waters.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and deionised water (specific conductance less than 0.1 $\mu S\,cm^{-1}$).

The 20 $g\,L^{-1}$ ascorbic acid solution was weekly prepared by dissolving 2.0 g of ascorbic acid (Normapur, France) in 100 mL of deionised water and was kept in a dark glass flask.

The molybdate reagent was weekly prepared by dissolving 1.6 g of ammonium heptamolybdate-tetra-hydrate from Merck, Germany (16 $g\,L^{-1}$), 40 mg of potassium antimony(III) oxide tartrate hemi-hydrate from Sigma, Germany (0.4 $g\,L^{-1}$) and 0.75 g of tartaric acid from Merck, Germany (7.5 $g\,L^{-1}$) in deionised water, followed by addition of 10 mL of 6 M sulphuric acid from Merck, Germany (0.6 M). After homogenisation, deionised water was added to 100 mL and the solution was kept in dark glass flask.

Phosphate stock solution (29.4 mM) was prepared by dissolving 0.40 g of potassium di-hydrogen phosphate (from Merck, Germany) previously dried in 100 mL of deionised water and stored in a refrigerator. Phosphate intermediate standard solutions of 595 μM and 11.9 μM were prepared every fortnight by appropriate dilution of the stock solution and stored in the refrigerator. Working standards, 0.024–9.52 μM, were prepared weekly by appropriate dilution and stored in the refrigerator when not in use.

2.2. Sample collection and preparation

Different water samples were collected and analysed: estuarine waters (with a salinity gradient along the estuary), river waters, well waters and ground water.

The estuarine water samples were collected from three different Portuguese estuaries located in NW Portugal: Ave (41.3°N, 08.7°W), Cávado (41.5°N, 08.7°W) and Douro (41.1°N, 08.6°W) rivers. For

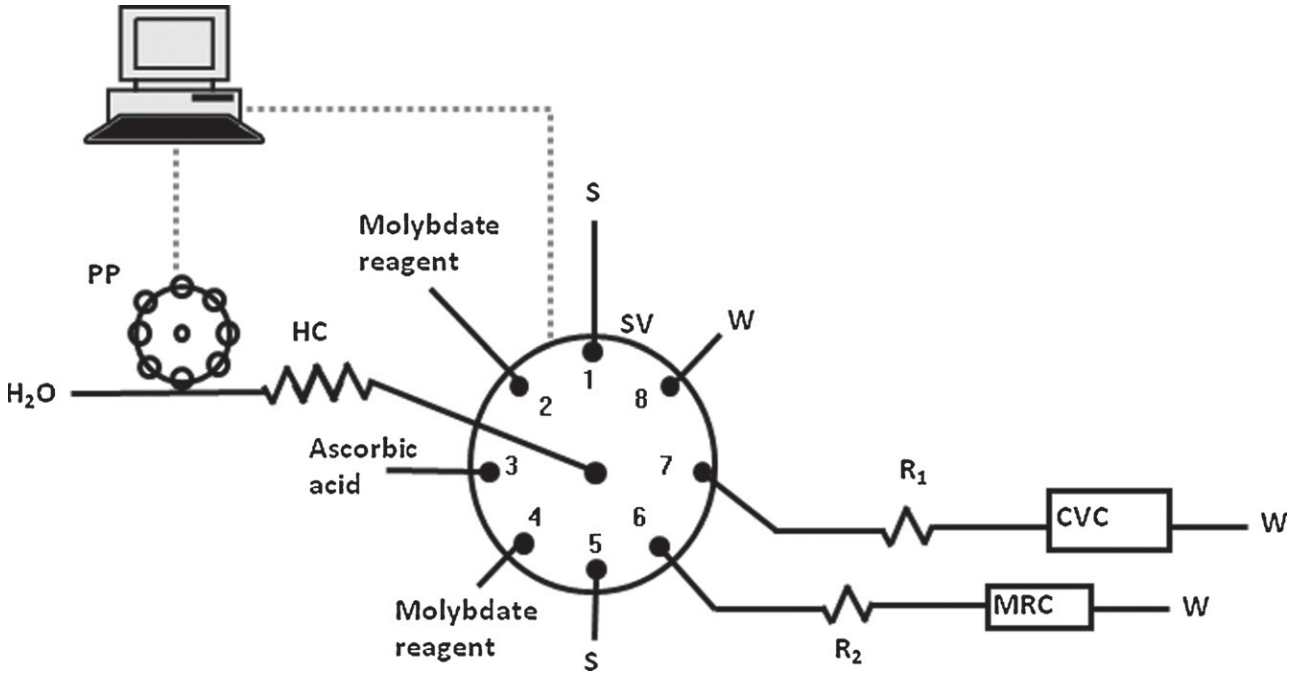

Fig. 1. SIA manifold for the spectrophotometric determination of phosphate: molybdate reagent, ammonium heptamolybdate-tetra-hydrate 16 $g\,L^{-1}$, potassium antimony(III) oxide tartrate hemi-hydrate 0.40 $g\,L^{-1}$, tartaric acid 7.5 $g\,L^{-1}$ and sulphuric acid 0.60 M; ascorbic acid, 20 $g\,L^{-1}$; S, sample or phosphate standard; W, waste; PP, peristaltic pump; HC, holding coil, 400 cm long; R_i, reaction coils, 270 cm long; SV, eight port selection valve; CFC, spectrophotometer at 660 nm with a conventional flow cell; MRC, spectrophotometer with a multi-reflective flow cell and LED light source (660 nm).

each estuary, the water was collected from the surface at three different locations: at close to the ocean (location 1) and two other upstream (locations 2 and 3), location 3 having the less influence from the ocean. (Maps of locations can be found in Electronic Supporting Information.)

Samples were also collected from the same rivers, but further upstream from the river mouth to ensure that they were not influenced by the estuary, as indicated by the measured salinity values.

Well and ground waters were also collected in the north of Portugal. All the water samples were introduced directly into the SIA system. Therefore, results on phosphate analysis correspond to the fraction of Total Reactive Phosphorous [24].

2.3. Sequential injection manifold and procedure

The sequential injection manifold used for the colorimetric determination of phosphate with the two possible detection systems is depicted in Fig. 1.

Solutions were propelled by a Gilson Minipuls 3 peristaltic pump, with PVC pumping tube. This was connected to the central channel of an eight port selection valve (Valco VICI 51652-E8). All tubing connecting the different components was made of PTFE (Omnifit), with 0.8 mm i.d.

A personal computer (Samsung SD 700) equipped with a PCL818L interface card, running homemade software written in QuickBasic 4.5, was used to control the selection valve position and the peristaltic pump direction and speed.

A conventional spectrophotometer (Hitachi UV-Vis, 100-40) was set at the wavelength of 660 nm and equipped with a Hellma 178.711-QS flow-cell (10 mm light path, 30 μL inner volume). While the optimal detection wavelength for ascorbic acid reduced phosphomolybdate is ca. 880 nm [25], the spectral absorption band is very broad, and a wavelength of 660 nm was selected for the spectrophotometer in order to allow direct comparison with the other detection system used, which was especially designed multireflective flow cell (MRC) previously described by Ellis et al. [22]. The light source in this detector was a red LED (λ_{max} at 660 nm) light source connected to a 12 V power supply regulated to 5 V using a multimeter. The output voltage was set to 0 V while the LED was on and using deionised water. For detailed description of the detector the readers should refer to the original paper [22]. Analytical signals were recorded on a Kipp & Zonen BD chart recorder.

The sequence of steps with the respective time and volumes for the determination of phosphate is shown in Table 2.

The first step is the aspiration of sample (step A), followed by the aspiration of the molybdate reagent and ascorbic acid (steps B and C). Mixing is then promoted by the reversal of the flow while propelling the plugs towards the detector (step H).

For the linear dynamic range of 0.025–0.25 μM, four extra steps (steps D–G) were added. Steps D and E enable to obtain a double plug of sample and reagent, respectively, and the steps F and G result in a stopping period before the determination. The flow was stopped in the reaction coil for 30 s to ensure higher sensitivity.

No adsorption of molybdenum blue on the surface of the Teflon tubing was observed, and no carryover between peaks was detected under the working conditions presented in Table 2. Yet, the flow system was rinsed with a diluted (0.05 M) NaOH solution at the end of a working week.

2.4. Reference procedure

For the accuracy assessment, samples were analysed using the vanadomolybdophosphoric acid colorimetric method (APHA 4500-P C) [25]. The assays were performed in triplicate and quantified from standard curves prepared daily.

3. Results and discussion

3.1. Study of the determination parameters

Optimization of the colorimetric reaction was carried out using the UV–Vis spectrophotometer with the conventional flow cell. The wavelength was selected to 660 nm, corresponding to the maximum emission of the LED to be used subsequently.

3.1.1. Aspiration sequence to the holding coil

According to the molybdenum blue chemistry, the intensely coloured compound results from the reduction of the phosphomolybdic acid by ascorbic acid, APHA 4500-P E [25]. The phosphomolybdic acid results from the reaction between orthophosphate and the mixture of ammonium molybdate/potassium antimonyl tartrate in acidic medium.

Two possible sequences were investigated: sample–molybdenum solution–ascorbic acid and ascorbic acid–molybdenum solution–sample. The results obtained when the sample was aspirated first showed an 8.5% increase in sensitivity, so this order was chosen.

3.1.2. Reagent concentration

The molybdate reagent solution is composed of ammonium heptamolybdate, potassium antimony(III) oxide tartrate and sulphuric acid. Firstly, the sulphuric acid concentration was fixed and the concentration of heptamolybdate and antimony(III) oxide tartrate was studied. This test was carried out by maintaining a concentration proportion of 40:1 (ammonium heptamolybdate tetra-hydrate:potassium antimony(III) oxide tartrate hemi-hydrate) as reported by Morais et al. [11]. The concentration of ammonium heptamolybdate tetra-hydrate was varied from 8 to 18 g L^{-1}, resulting in a concentration range of 0.20–0.45 g L^{-1} for the antimony(III) compound. The highest sensitivity was observed for 16 g L^{-1} of ammonium heptamolybdate tetra-hydrate with

Table 2
Protocol sequence for the determination of phosphate.

Step	SV position	Time (s)	Flow rate (mL min^{-1})	Pump direction	Volume (μL)	Action
A	1	12/7.5[a]	3.9	a	780/488[a]	Aspiration of sample/standard
B	2	1.5	2.9	a	72	Aspiration of molybdate reagent
C	3	3.5	3.9	a	228	Aspiration of ascorbic acid
D[b]	4	1.5	2.9	a	72	Aspiration of molybdate reagent
E[b]	5	7.5	3.9	a	488	Aspiration of sample/standard
F[b]	6/7[c]	17	3.9	b	1105	Propel to reaction coil
G[b]	6/7[c]	30	0	–	0	Stop flow
H	6/7[c]	70	3.9	b	4550	Propel to detector (λ = 660 nm) and system washing

[a] Time and respective volume for the linear dynamic range 0.025–0.25 μM.
[b] Steps added in case of the dynamic range 0.025–0.25 μM.
[c] The position was different accordingly with the detector.

Table 3
Summary of the chemical and physical parameters studies.

Parameter	Studied range	Selected condition
$[(NH_4)_6Mo_7O_{24}.4H_2O)]$ (g L^{-1})	8–18	16
$[C_4H_4KO_7Sb]$ (g L^{-1})	0.20–0.45	0.40
$[H_2SO_4]$ (M)	0.45–0.90	0.45
$[C_6H_8O_6]$ (g L^{-1})	10–50	20
$V_{Molybdate\ reagent}$ (μL)	51–163	72
$V_{ascorbic\ acid}$ (μL)	195–325	228
V_{sample} (μL)	488–910	780

0.40 g L^{-1} of the antimony(III) compound so these reagent conditions were chosen.

The concentration of sulphuric acid was also studied; the acid is required to ensure that the pH was <1 in the final reaction mixture as well as to increase the solubility of the reagent components. Concentrations of 0.45, 0.60 and 0.90 M of sulphuric acid were tested. With a concentration of 0.45 M it was not possible to dissolve the reagents, and a minimum concentration of 0.60 M was required to obtain full dissolution. Higher acid concentrations resulted in a decrease in sensitivity, so 0.60 M of sulphuric acid was the chosen concentration.

The ascorbic acid concentration was also studied within the range 10–50 g L^{-1} and the highest sensitivity was obtained for 20 g L^{-1}, so that was the concentration chosen. A summary of all studied parameters and the chosen values is shown in Table 3.

3.1.3. Linear dynamic range 0.25–9 μM

For obtaining the maximum sensitivity in this application range, the study was initiated by testing the influence of the volume of the molybdate reagent (sample volume set to 488 μL, within the range 1–4 μM). The tested volumes were: 51, 72, 98, and 163 μL. A volume of 72 μL gave increased sensitivity and better linearity, and was the chosen volume.

Optimization of the ascorbic acid volume was performed within the range 195–325 μL. The volume of ascorbic acid which provided the highest sensitivity was 228 μL, so this was the volume chosen. This result can be explained by the limited overlapping (or mutual dispersion) between the reagent and sample solutions induced by the increase of the ascorbic acid volume introduced between them.

The sample volume was studied and a volume of 780 μL was chosen from the range 488–910 μL as it gave the highest sensitivity. With this reassessment of sample volume, a linear dynamic range of 0.25–9.5 μM was obtained.

3.1.4. Linear dynamic range up to 0.25 μM

Considering that some uncontaminated natural waters may have phosphate concentrations below 0.25 μM [26], different approaches were tested to achieve a lower limit of quantification.

3.1.4.1. Effect of different reagent sequences—the double sample plug. To obtain a linear dynamic range of 0.05–0.25 μM it is necessary to maximize the volume of sample and, at the same time, to guarantee efficient mixing with the reagents. To achieve both conditions, the sample was introduced as two plugs, with one on either side of the reagent plugs. A program sequence including the double plugs for both sample and molybdate reagent was tested. The order of aspiration with the two extra plugs was: sample–molybdate reagent–ascorbic acid–molybdate reagent–sample. The sample volume of each plug was set to about 500 μL and the volumes of molybdate reagent and ascorbic acid were maintained at the same values derived from the previous study (Section 3.1.3).

3.1.4.2. Effect of reaction time—stop-flow. In order to further increase sensitivity, an increase in the reaction time was tested. After the aspiration of all the plugs, the mixture was propelled towards the detector and then stopped in the reaction coil. To ensure that all the plugs were in the reaction coil, the coil length was set to 270 cm, a value that was obtained from previous tests employing a dye solution. Several stop times were tested ranging from 0 to 90 s and a stop time of 30 s was chosen. While sensitivity increased with the increased stop time, the linearity of the calibration curve decreased, so a 30 s stop time was selected as a compromise between sensitivity and linearity of response.

Under the optimized conditions with the double sample plug set up it was possible to reach the detection limit of 0.007 μM, and the quantification limit of 0.023 μM.

3.2. Comparison of the two possible detection systems

The conditions obtained from previous studies (Section 3.1) for the linear dynamic range of 0.25–9.5 μM phosphate were used (unless otherwise stated) in order to compare the relative merits of the two detection cells.

3.2.1. Schlieren effect

In flow analysis, the phenomenon known as the schlieren effect, results from the deflection of the light beam (signal refraction) caused by the created concentration gradient [21] if the refractive indices of carrier sample and reagents are significantly differ-

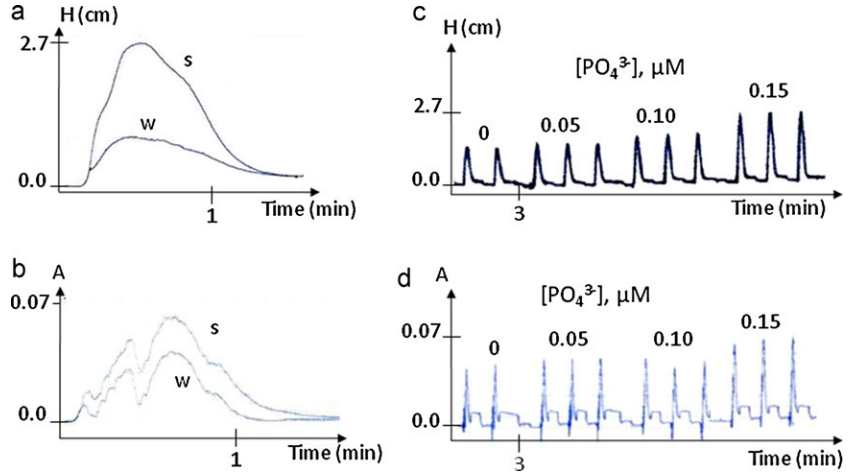

Fig. 2. SIA traces showing a comparison between the sample (s) and the deionised water (w) peaks: (a) with the MRC, (b) with the CFC; and comparison of a partial calibration curves (c) with the MRC, and (d) with CFC.

ent. The interface between these solutions can produce optical lenses and so a signal resulting of light deflection is registered, schlieren effect. If this signal is concomitant with the one to be measured at the same wavelength (light absorption), erratic results are obtained, with more evident effects at low analyte concentrations. This problem is particularly important in SIA systems because, unlike FIA manifolds, no confluence points are used to improve the mixing. Different strategies were effectively applied to minimize the impact of this phenomenon on the accuracy and precision of spectrophotometric determinations, Dias et al. suggested the use of dual wavelength measurement [21] and McKelvie et al. [27] applied the matrix matching strategy for salinity compensation. The MRC was specially designed by Ellis et al. [22] to minimize the schlieren effect. To assess this capacity under SIA based flow conditions, sample and blank were injected and the signals (amplified view in Fig. 2a and b) were compared. The results presented on the figure correspond to the double plug program (lower linear dynamic range) as this particular effect is more pronounced when increased numbers of plugs are injected. SIA peaks were produced using an MRC and the conventional flow cell, and it can be observed that, when using a CFC, the signals are much more prone to erratic signals close to the baseline (Fig. 2c and d).

These results indicate that the SIA-MRC can be used in matrices with quite different refraction indices, with no significant optical interference in the main signal. Additionally, the use of a MRC contributes to the miniaturization of the overall apparatus. This detection system proved to be advantageous for the estuarine water samples.

3.2.2. Chemical interferences

Distinctively from other spectrophotometric or chemiluminometric determinations of phosphate, the molybdenum blue method is only subject to a small variety of interferences under flow analysis conditions. Interfering species in the determination of phosphorous by the phosphomolybdenium blue method are As(V), Si and Ge [23], which also react with molybdate to form the corresponding acids which are reduced to the respective heteropoly blues by ascorbic acid.

Considering that the proposed method was directed at natural waters (estuarine, river and well waters) the most likely interference would be from silica. This is a chemical interference therefore it was not expected to produce different behaviour in the two detection system.

This interference was assessed at phosphate concentrations of 0.25 μM, with different amounts of silicate added. Using the CFC detection system, there was no significant interference up to a silicate concentration of 32.9 μM, however with the MRC detection system significant (>5%) interference was found in the presence of 1.3 μM silicate. The interference, caused by the formation of molybdosilicate heteropoly acid complex at reduced pH can explain this effect, which becomes more pronounced using the detector cell with higher sensitivity. Estuarine waters are expected to be affected by sea water with values up to 60 μM of silica [28]. The presence of tartaric acid has been reported to effectively reduce silicate interference [23] so it was added to the molybdate reagent. Different concentrations of tartaric acid were used (1.7, 3.2, 6.4, 7.5 g L^{-1}) for a silicate concentration of 65.7 μM. As expected, with an increase in the tartaric acid concentration, there was a decrease of the percentage of interference. The percentage interference dropped from 18%, with no tartaric acid, to 2%, with the 7.5 g L^{-1} tartaric acid.

So, even though only some estuarine samples were likely to have silicate values up to 60 μM of silica, 7.5 g L^{-1} of tartaric acid was included in the molybdate reagent. The reason for adding the tartaric acid to the molybdate reagent instead of to the ascorbic acid solution was based on the aspiration order, to ensure that it contacts directly with the sample.

The possible interference from nitrate was also studied, at the concentration level of 200 μM of nitrate, and resulted in <3% interference on the signal of a 0.25 μM phosphate standard solution. This nitrate concentration is about the maximum found in these estuarine waters [29].

3.2.3. Salinity interference

As the aim of the developed method was the application to water samples with a wide salinity range, i.e.: estuarine waters, a study of salinity interference was of high priority. The salinity interference was assessed by comparing calibration curves using standard solutions with different salinity values; these solutions were prepared by adding sodium chloride to the standards. The salinity values were adjusted to an intermediate and a maximum level of salinity in estuarine waters, of 9‰ and 19‰, respectively [30], although it is recognised that salinities at 35‰ can be found in estuaries at the seaward extent, being also dependent on the tidal river flow conditions. The calibration curve with pure standards was compared to the calibration curve with standards with adjusted salinities. The estimated slopes of the curves were assessed at the confidence intervals at 95%. The quality of the regression was tested by residual analysis (i.e. randomness and normality) and by the coefficient of determination (i.e. R^2, which was above 0.981 in all cases). For the CFC there is only overlapping of the limits while for the MRC there is an almost perfect overlapping of the entire interval. So the salinity affects more the spectrophotometric detection with the conventional cell (Fig. S1 in Electronic Supporting Information).

3.3. Features of the developed SIA method

A summary of the main analytical features: the dynamic concentration ranges for the typical calibration curves, limits of detection

Table 4
Features of the developed SIA method with both detection systems.

Detection system	Linear dynamic range (μM)	Calibration curve[a]	LOD (μM)	LOQ (μM)	RSD (%)[b]	Determination rate (h^{-1})	Effluent volume (mL)
Spectrophotometer and conventional flow cell	0.30–9.50	$A = 0.0869$ (±0.0035) $[PO_4^{3-}] - 0.039$ (±0.025); $R^2 = 0.999$ (±0.001)	0.089	0.297	0.6% (2.47 ± 0.02) 0.6% (7.33 ± 0.04)	37	4.6
Multi-reflective flow cell	0.25–7.10	$H^c = 15.2$ (±0.5) $[PO_4^{3-}] + 2.6$ (±1.4); $R^2 = 0.998$ (±0.001)	0.066	0.219	1.3% (2.92 ± 0.04) 0.2% (7.04 ± 0.01)	37	4.6

[a] Values in brackets correspond to the standard deviation, $n = 5$, of the equation parameters (inter day precision).
[b] Values in brackets correspond to the concentration and the standard deviation, $n = 10$ (intra day precision).
[c] Peak height (mm).

(LOD) and quantification (LOQ), repeatability (RSD), determination rate and effluent production, for the two detection systems, is shown in Table 4.

The typical calibration curves correspond to a mean of four calibration curves with the standard errors between brackets. The LOD and LOQ of both linear dynamic ranges were calculated as the concentration corresponding to three and ten times the standard deviation of the blank, respectively, according to IUPAC recommendation [31,32].

The repeatability was assessed by calculation of the relative standard deviation (RSD) obtained by the mean of ten consecutive injections of an estuarine water sample [31].

The determination rate was calculated based on the time spent per cycle. A complete analytical cycle must take into account not only the sum of times in the protocol sequence but also the time required to change the valve position and to activate the pump. The analytical cycle took 2.6 min for the double plug method (lower linear dynamic range) and 1.6 min for the single plug method (higher linear dynamic range).

The most significant conclusion that can be drawn from the observations in Table 4 is that lower detection and quantification limits are obtained with the multi-reflective cell. This may be attributed to the higher blank value and an increase in the blank variability obtained when using the spectrophotometer equipped with the commercial flow cell (Fig. 2). The detection limit can be further decreased if needed with the multi-reflective cell and using the double sample plug strategy presented Section 3.1.4.

The reagent consumption values for the single plug method values were: 1.52 mg of ammonium heptamolybdate-tetra-hydrate; 28.8 μg of potassium antimony(III) oxide tartrate hemi-hydrate; 0.54 mg tartaric acid; 4.24 mg sulphuric acid and 4.56 mg ascorbic acid.

3.4. Application to water samples

The developed SI method was used for the analysis of a variety of natural waters, with different sources and characteristics, and the results were compared to the ones obtained by the reference procedure.

3.4.1. Phosphate determination in natural waters

The estuarine and river water samples were collected in different locations of the Ave river, as mentioned in Section 2.2. Some of those samples were considered estuarine waters due to their proximity to the river mouth; one was considered river water due to the distance to the sea. Therefore the salinity values were significantly different ranging from 0.4‰ to 7.2‰.

Some well and ground waters were also analysed and their salinity values were negligible and were not listed. The values are reported in Table 5 as mean with the standard deviation (three replicate determinations for each sample).

The *F* test indicates that the variance detected in the reference method in most cases is greater than the one from the proposed methods. Therefore, unequal variances were considered [33] in the *t*-test for the comparison of the means. Calculated $|t|$ values in Table S1 (ESI) for the two detection systems indicate that the results obtained using the MRC based SIA system showed a better overall agreement with the reference method.

To evaluate accuracy, a linear relationship between the results obtained with the reference procedure and each one of the two possible detection systems, CFC and MRC, was established (Fig. S2 in the Electronic Supporting Information). The regression of C_{CFC} (μM) vs $C_{Ref.\ Met.}$ (μM) resulted in the linear equation: $C_{CFC} = 0.930\ (\pm 0.196) \times C_{Ref.\ Met.} - 0.004\ (\pm 1.199)$, $R = 0.953$. The linear relationship between C_{MRC} (μM) and $C_{Ref.\ Met.}$ (μM) resulted in the equation: $C_{MRC} = 0.966\ (\pm 0.133) \times C_{Ref.\ Met.} + 0.185\ (\pm 0.811)$, $R = 0.979$. The values in parenthesis represent the 95% confidence limits. In both cases the figures show that the estimated slope and intercept do not differ statistically from values 1 and 0, respectively. Therefore, no evidence of systematic differences can be pointed out between the two sets of results [33].

3.4.2. Recovery studies for several different types of water samples

To further assess the efficacy of the developed system, recovery studies were performed on water samples previously collected from other estuaries in the north of Portugal (Section 2.2) and stored frozen.

Samples were spiked with volumes of 100 μL, 120 μL, 150 μL and 160 μL of phosphate standard solution (595 μM) were added to 10 or 20 mL of sample. The calculation of the recovery percentage was made according to IUPAC [34]. The analysis was carried out using both detection systems (Table S1 in Electronic Supporting Information).

The SIA methodology provided recovery ratios with an average of 98% (standard deviation 6.1%) and 100% (standard deviation 5.5%) for the CFC and MRC, respectively. A statistical test (*t*-test) showed that for a 95% significance level the recovery values did not differ from 100% as the calculated *t*-values were 1.639 and 0.096 with correspondent critical values 2.475 and 2.445 for the CFC and MRC,

Table 5
Application of the developed sequential injection method with the two possible detections, the conventional flow cell with the spectrophotometer (SIA-CFC) and the multi-reflective cell (SIA-MRC) to the phosphate determination in different water samples and comparison with the reference procedure (Ref. Met.); SD, standard deviation from 3 replicas, RD relative deviation.

Sample type	Sample ID	Salinity (‰)	Ref. Met.	SIA-CFC		SIA-MRC	
			$[PO_4^{3-}] \pm$ SD (μM)	$[PO_4^{3-}] \pm$ SD (μM)	RD (%)	$[PO_4^{3-}] \pm$ SD (μM)	RD (%)
Estuarine water (Ave river)	A1	5.9	6.94 ± 0.14	5.93 ± 0.05	−14.6	6.72 ± 0.02	−3.2
	A2	1.2	6.13 ± 0.28	5.87 ± 0.03	−4.2	6.16 ± 0.01	0.5
	A3	0.1	6.53 ± 0.01	6.34 ± 0.05	−2.9	6.35 ± 0.01	−2.8
	A4	7.2	4.75 ± 0.14	3.82 ± 0.02	−19.5	4.71 ± 0.03	−0.7
	A5	0.8	5.89 ± 0.14	5.60 ± 0.03	−4.9	5.86 ± 0.02	−0.5
	A6	0.1	6.54 ± 0.24	6.31 ± 0.05	−3.5	6.32 ± 0.01	−3.3
	A7	6.5	7.84 ± 0.14	7.40 ± 0.05	−5.6	8.05 ± 0.01	2.7
	A8	1.1	5.40 ± 0.14	5.59 ± 0.01	3.5	5.75 ± 0.02	6.6
	A9	0.5	6.94 ± 0.14	6.82 ± 0.04	−1.8	7.40 ± 0.01	6.6
Ground water	G1	–	3.53 ± 0.01	3.36 ± 0.06	−4.8	3.54 ± 0.03	0.3
	G2	–	8.41 ± 0.14	7.30 ± 0.03	−13.2	7.88 ± 0.01	−6.2
Well water	W0	–	4.23 ± 0.15	4.35 ± 0.04	2.9	4.58 ± 0.01	8.3
River water	R0	0.4	4.10 ± 0.01	3.05 ± 0.02	−25.6	3.68 ± 0.01	−10.3

respectively, thus indicating the absence of multiplicative matrix interference [33].

4. Conclusions

The developed method enabled the evaluation of two alternative detection cells under the same experimental conditions, and resulted in a SIA procedure for phosphate determination applicable to different types of water with high salinity and large matrix variability. The use of a sequential injection technique, to fully explore its versatility, proved to be a suitable choice. The approach of using two protocol sequences, resulting in two linear dynamic ranges, can be applied to samples with trace concentration of the analyte.

In comparing the two detection systems, the multi-reflective flow cell proved to be better for lower phosphate concentrations with an achievable detection limit of 0.007 μM.

With respect to the possible interferences of silicate, the interference was effectively minimized, in-line, by adding tartaric acid to the molybdenum reagent. The percentage interference, observed for 68 μM of silicate, decreased from 18% to 2% as a result.

When compared to previously described procedures based on the same principles (Table 1), the most significant advantage of the work described is the use of a single manifold for the determination of phosphate in different matrices (well, estuarine, river, and sea waters). The achievable detection limit compares favourably to the previously published SI alternatives (Table 1) without the need for a pre-concentration procedure. The analysed water samples were compared to a reference procedure and proved the accuracy of the developed method for both detection systems.

Acknowledgements

R.B.R. Mesquita thanks to FSE and Fundação para a Ciência e a Tecnologia (FCT, Portugal) the grant SFRH/BPD/41859/2007. I.V. Tóth thanks FSE and Ministério da Ciência, Tecnologia e Ensino Superior (MCTES) for the financial support through the POPH-QREN program. The authors thank FCT financial support through project PTDC/AMB/64441/2006, and Peter Ellis, Monash University, for the kind provision of the multi-reflection flow cell.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2011.06.002.

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