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Sequential injection spectrophotometric determination of orthophosphate in beverages, wastewaters and urine samples by electrogeneration of molybdenum blue using tubular flow-through electrodes

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Abstract

A novel and automated sequential injection procedure is proposed for the spectrophotometric determination of orthophosphate without requiring unstable chemical reducing species used in the classical molybdenum blue method. The flowing methodology is based on the on-line generation of the detectable species by electrochemical reduction of the 12-molybdophosphoric acid complex using a stainless steel tubular flow-through working electrode. The established method is linear up to 20 mg/l P, with coefficients of variation ($n = 10$) of 2.4 and 1.8% for 2.0 and 10 mg/l P, respectively. The versatility of the sequential injection method to analyse samples containing high orthophosphate levels has been demonstrated by the implementation of a dilution chamber as well as flow-reversal techniques, yielding relative standard deviations ($n = 17$) better than 2.0% for standards containing 200 and 800 mg/l P. The proposed analyser features an extremely wide dynamic range (viz., 0.3–800 mg/l) as well as improved tolerance to silicate interference, so that Si/P ratios higher than 50 are tolerated at the 5% level. Electrochemical conditions, reagent concentrations and physical variables have been thoroughly investigated. The method has been applied to the determination of orthophosphate in wastewaters as well as beverages and biological samples containing high concentrations of the target analyte. The *t*-test comparison of the means for the developed sequential injection system with electroreduction and both the molybdenum blue classical spectrophotometric batch procedure and inductively coupled plasma-optical emission spectrometric detection selected as external reference methods revealed that there is no evidence of significant differences between the obtained results at the 95% confidence level.

Keywords: Phosphate; Electroreduction; Molybdenum blue; Sequential injection analysis; Wastewater; Beverages; Urine

1. Introduction

Phosphorus is an essential nutrient to all organisms [1], and therefore, determination of orthophosphate is considered of great importance in various fields [2,3]. The chemical basis of spectrophotometric procedures for monitoring orthophosphate in various matrices lies in the reaction between the target analyte and molybdate in acidic medium in order to form a heteropolyacid species. In most applications, detection is undertaken either on the molybdophosphate reduction product, i.e. molybdenum blue method [4], or the yellow vanadomolybdate complex [5,6], the former being currently adopted in official methods [7], due to its improved sensitivity. Several reducing agents have been reported and reviewed in the literature for molybdenum blue detection [8], being ascorbic acid [7,9,10], and tin(II) chloride [11–14] the most widely used in both batch and flow injection procedures. With regard to sequential injection analysis (SIA), it should be stated that the detection and determination limits achieved for orthophosphate determination are typically comparable to those obtained by flow injection analysis (FIA), with similar sample volumes and less reagent consumption, although with a lower sampling throughput [15–17].

One indispensable condition for any analytical method to be implemented in a flowing stream analyser for environmental monitoring or process analysis is the stability of the reagents, and thus, unattended measurements for a prolonged period of time may be warranted. In the case of the molybdenum blue method, the instability of the

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reducing solutions [7,18] usually demands frequent reagent preparation or re-calibration of the system. The drawbacks derived from reagent instability can be sometimes circumvented by on-line generation in a flowing system of either the reagent or the product to be determined. Electrogeneration is a common practice in flow-injection chemiluminescence methods, coulometric flow titration and flow injection amperometric analysis, basically involving the in situ generation of reagents [19]. Yet, the on-line electrogeneration of the detectable product and further spectrophotometric detection has not been yet exploited. Moreover, it should be emphasised that working electrodes are usually non-tubular flow-through solid-state electrodes, such as those made of glassy carbon, graphite paste, platinum or other metals, or modified electrodes, which require dedicated wall-jet or thin-layer flow-through cells.

The aim of this work is to benefit, on the one hand, from the advantages of the automated SIA systems (namely, robustness, versatility, easy handling and sample/reagent saving) [17,20–22] and, on the other hand, from those regarding electrogeneration, avoiding preparation, preservation and use of unstable reducing reagents aiming to design an automated SIA orthophosphate analyser capitalised on the electrogeneration of the monitored species. The proposed method is based on the on-line electroreduction of the yellow molybdophosphate heteropolyacid in a tubular stainless steel working electrode, assembled in the flow manifold prior to spectrophotometric detection. The potentials and versatility of the developed assembly for monitoring orthophosphate at a wide concentration range exploiting on-line dilution schemes, relying upon flow-reversal techniques or mixing chambers, are demonstrated via the analysis of environmental (namely, wastewaters) and biological (viz., urine) samples as well as various orthophosphoric acid containing beverages.

2. Experimental

2.1. Reagents and solutions

All chemicals were of analytical-reagent grade (Merck, Darmstadt, Germany) and stored in polyethylene bottles, except for orthophosphate standard solutions and samples, which were stored in glass containers. Doubly de-ionised water (18.2 MΩ cm) obtained from a Milli-Q system (Millipore, Bedford, MA) was used throughout. A 0.6 M H_2SO_4 solution was used as a carrier stream. Working standard solutions of orthophosphate were obtained by proper dilution of a 10 g/l PO_4^{3-} stock solution in water. A 0.1 M ammonium molybdate stock solution was prepared from $(NH_4)Mo_7O_{24}\cdot 4H_2O$ dissolved in a 0.6 mol/l sulphuric acid medium. All solutions and samples were at room temperature when conducting the experiments.

2.2. Instrumentation and flow system

The sequential injection set-up designed for orthophosphate monitoring following electrochemical generation of reduced heteropolyacid species is shown schematically in Fig. 1. It comprises the following components: a compatible PC for controlling the SI-instrumentation, a 5 ml syringe pump (Type 738-Crison, Alella, Barcelona) with adjustable dispensing rate, a valve module with an 8-port multiposition valve (Type 2060-Crison), an autosampler (Type 2040-Crison) with 40 positions, a Metrohm 641 VA potentiostat, and a Hewlett-Packard HP-8452A diode array

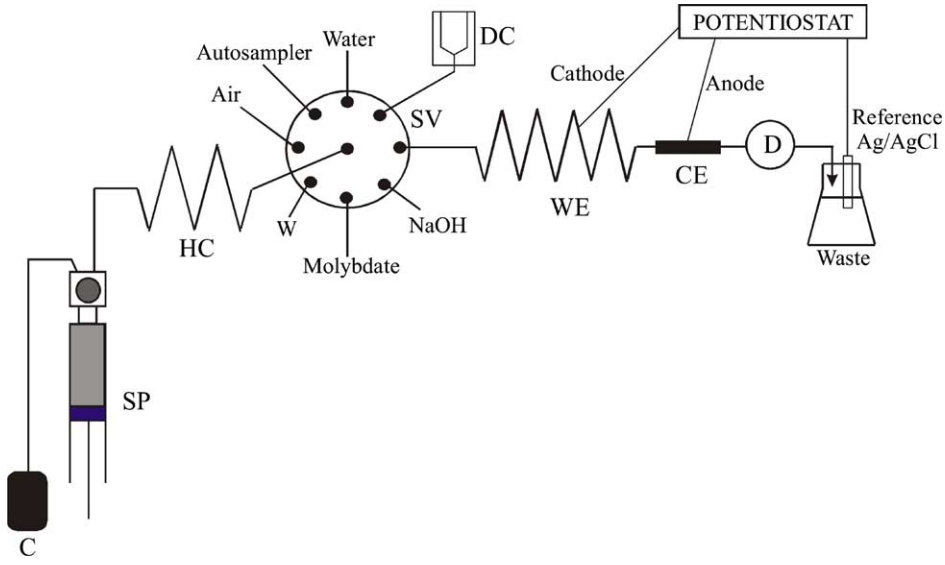

Fig. 1. Schematic illustration of the sequential injection set-up devised for the spectrophotometric determination of orthophosphate based on the electrochemical generation of molybdenum blue—C: carrier; SP: syringe pump; HC: holding coil; SV: selection valve; DC: dilution chamber; WE: working electrode; CE: counter electrode; D: detector; W: waste.

spectrophotometer. A stainless steel HPLC tube (135 cm length, 0.8 mm i.d.) has been used as a cathodic working electrode, a graphite tube (3.3 cm length, 1 mm i.d.) as a counter electrode and a conventional AgCl/Ag electrode (6.0727.000 Metrohm, Herisau, Switzerland) as reference electrode. The working and counter electrodes were electrically separated by a short Tygon tube (1.4 cm length), and the AgCl/Ag electrode was immersed into the acidic waste containing reservoir, placed at the outlet of a 10 mm path-length flow-through quartz cell. In order to dilute samples with high orthophosphate content, a dilution chamber with 3.0 ml of maximum effective capacity [23] was employed.

Instrumental control, implementation of sequential injection protocols, and data acquisition were performed via the software package Autoanalysis [24], which also allowed the treatment of the transient signals recorded. The manifold was built from poly(tetrafluoroethylene) tubing of 0.8 mm i.d., except the connection between the syringe pump and the carrier reservoir as well as the holding coil, which were made from 1.0 mm i.d. to allow high flow-rate performance.

2.3. Analytical procedure for the sequential injection determination of orthophosphate

The protocol sequence adopted for analysis of samples containing low orthophosphate concentrations (<20 mg/l P) starts with the consecutive aspiration of 300 μl of molybdate reagent (0.06 M Mo/0.6 M H_2SO_4), and 300 μl of sample solution into the holding coil. The interdispersed zones are thereafter directed to the working electrode using the carrier solution, whereupon a stopped-flow modality for 120 s is applied to perform the electroreduction at −0.53 V (versus AgCl/Ag). The on-line generated molybdenum blue is then propelled to the flow-through cell for spectrophotometric detection at 760 nm, using a reference wavelength of 470 nm to minimise the Schlieren effect interferences. The holding coil, tubular working electrode and detection line are finally rinsed with a 300 μl plug of 1 M NaOH, yielding the system ready for the subsequent analysis cycle.

Whenever concentrations of the target analyte above 20 mg/l P are detected, as found in urine samples and beverages such as coke and beer, dilution protocols prior to the aforementioned procedure are initialised. Initially, a 100 μl air zone is aspirated into the holding coil aiming to control the sample dispersion, followed by an appropriate sample/water volume ratio. The syringe pump is next set-up to dispense an appropriate carrier volume to the mixing chamber until the withdrawal of the air segment is observed. After homogenisation of the plugs into the dilution tank for 15 s, a minute, well-defined volume of diluted sample is dispensed to waste in order to rinse the line connecting the mixing chamber with the multiposition valve. Hence, the system is ready to start the sequential injection protocol with injection of the on-line diluted sample.

3. Results and discussion

3.1. Selection of electrodes and configuration of the potentiostatic flowing system

Several experiments aimed to establish the nature and most adequate location of the electrodes in the proposed SIA set-up were initially carried out. Preliminary investigations were made using a graphite tube as a working electrode (cathode), a glassy carbon electrode (commonly implemented in anodic stripping voltammetric procedures) as an anodic counterelectrode, and finally, an Ag/AgCl reference electrode, commonly used in potentiometric methods.

This potentiostatic system was chosen, on the one hand, due to the fact that the assayed experiments in batch proved the viability of electroreduction of molybdophosphoric acid at a graphite electrode and, on the other hand, due to its ready availability in laboratories and straightforward implementation in flowing networks. The tubular working electrode was located in the flow manifold as close as possible to the flow-through cell aiming to minimise the dispersion of the electrogenerated compound. The remaining two electrodes were placed in the waste reservoir containing a 0.6 M sulphuric acid solution, and hence, electrical contact with the working electrode is made through the tubing acting as a waste carrier. The results obtained with this configuration were not satisfactory, due to the low yield of the electrochemical reaction, and therefore, the cathode and anode were assembled in series in the flow manifold. Moreover, the latter electrode was replaced by a flow-through tubular graphite electrode, similar to that used as a cathode. The type and location of the reference electrode remained constant. Although this configuration provided a sensitivity improvement along with better repeatability, the carryover effect between consecutive samples, attributed to the porosity of the graphite working electrode, hindered its applicability to real samples. According to the above results, the likelihood of electroreduction of molybdophosphoric acid over stainless steel was next assessed and successfully demonstrated in a batch mode. Hence, the graphite electrogenerator (cathode) was replaced by a stainless steel tubing, commonly used in HPLC equipments. The final arrangement of the potentiostatic system is depicted in Fig. 1.

3.2. Working potential and electroreduction time

In order to evaluate the effect of the working potential on the electrogeneration of molybdenum blue, several experiments were carried out. For a 0.06 mol/l molybdenum concentration and a fixed reduction time (viz., 150 s), the electrogeneration potential and medium acidity were varied within the interval ranging from −0.23 to −1.13 V and 0.3–1.2 M of sulphuric acid, respectively. The results obtained show an increase of the analytical signal whenever the cathodic potential becomes more negative, revealing a higher yield of the redox reaction. Nevertheless, the formation

of hydrogen bubbles increases with decreasing the cathodic potential as well as increasing the sulphuric acid concentration. The release of gas in the flow system precludes the proper mixing between sample/reagent stacked plugs and deteriorates the repeatability of the spectrophotometric signals. Bearing in mind the above-mentioned facts, a cathodic potential of −0.53 volts, and a working medium of 0.6 M sulphuric acid were selected, since under these experimental conditions the generation of bubbles into the flow conduits was negligible. The sulphuric acid concentration is similar to the optimum reported for the selective formation of molybdophosphoric acid [16], and presents the concomitant advantage of minimising the coating effect in comparison with more concentrated acid solutions.

To evaluate the influence of the effective surface of the generating electrode on the reaction yield, a stainless steel wire was introduced inside the working electrode, which enabled a 1.6-fold increase of the active surface. The results obtained reveal that the signal increases proportionally with the increase of the effective surface area.

Electrochemical reduction of molybdophosphoric acid on stainless steel electrodes is proven to be a relatively slow process. The effect of the electroreduction time (up to 480 s) for the blank signal and various standards with orthophosphate concentrations ranging from 1 to 20 mg/l P is shown in Fig. 2. As can be observed, the higher the stopped-flow time the higher analytical signals are recorded. For concentrations ≤20 mg/l P and times <150 s an almost linear relationship between peak height and residence time is observed. Stopped-flow times longer than 250 s (especially for concentrations >10 mg/l P) are recommended for fulfilment of steady-state conditions.

Table 1
Comparison of the analytical signals recorded using flow-reversal strategies or stopped-flow approaches and an electroreduction time of 120 s[a]

Concentration (mg/l P)	A	B	C	D
1	130 ± 2	110 ± 4	112 ± 3	111 ± 4
2	185 ± 4	187 ± 4	190 ± 6	166 ± 6
5	296 ± 5	304 ± 6	313 ± 5	302 ± 6
10	364 ± 7	368 ± 8	411 ± 9	418 ± 9
15	474 ± 8	445 ± 9	448 ± 10	510 ± 12

Results are expressed as the mean of three injections ± standard deviation. A: 120 s stopped-flow; B: displacements of 0.1 ml (6 times), 10 s stopped-flow each time; C: as B but with forward–backward movements of 0.15 ml; D: 20 steps of 0.01 ml, 6 s stopped-flow each step.

[a] Sample and reagent zones are injected into the working electrode before initialisation of the flow-reversal protocols.

In order to enhance the interdispersion of sample and reagent zones, the application of forward–backward movements, which also ensures the renovation of the liquid in contact with the surface of the generating electrode, was evaluated. The results obtained in the former experiments are listed in Table 1 for several concentrations of orthophosphate, various displacement lengths and different number of forward–backward movements. According to the presented data, none of the former approaches produces a significant sensitivity improvement in relation to that obtained

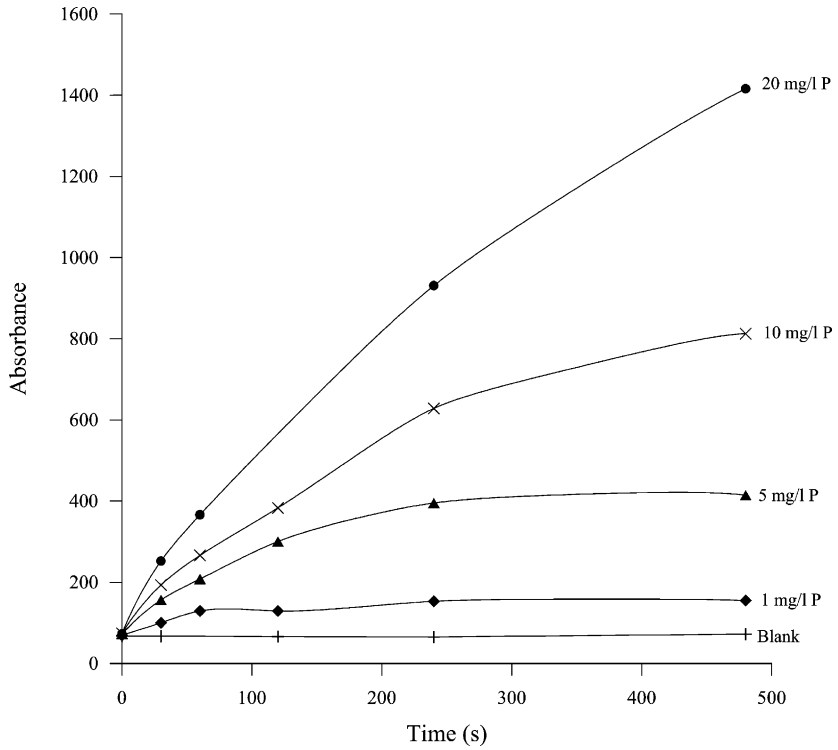

Fig. 2. Dependence of the analytical signal on the electroreduction time at a working potential of −0.53 V. Experimental conditions: 300 μl sample, 300 μl reagent (0.06 mol/l Mo), 0.6 mol/l sulphuric acid and flow-rate of 2.0 ml/min.

by merely applying a stopped-flow mode for 120 s, owing to the compensation of the benefits of the flow-reversal strategy with the higher dispersion of the on-line generated heteropolyacid species. Hence, the sensitivity of the SIA methodology strongly depends on the electroreduction time selected, and this step together with those corresponding to the sample renewal and rinsing of the system with alkaline solutions between consecutive injections to prevent molybdenum blue coating are the limiting processes for the sampling rate. In order to improve the sampling throughput without deterioration of the analytical performance, the electroreduction time was fixed at 120 s, which provided a reduction yield of ca. 75% for 10 mg/l P.

3.3. Investigation of chemical variables

Amongst the various spectrophotometric methods implemented in flow systems for orthophosphate monitoring, namely, the vanadomolybdate [25], the Malachite Green [26] and the molybdenum blue [12] methodologies, the latter one involving the chemical reduction of the 12-molybdophosphoric acid complex, mainly using ascorbic acid or tin(II) chloride, is widely exploited. The optimum concentrations of molybdate and sulphuric acid reported for heteropolyacid formation according to the criteria of lower background signal and better signal to noise ratio were 0.07 M Mo and 0.8 M H_2SO_4, respectively [16]. Though the higher the acidity of the reaction medium the higher the tolerable concentration level of silicate is typically encountered [27], the sulphuric acid concentration was fixed in the present SIA system at 0.6 M H_2SO_4 to minimise the generation of hydrogen in the flow conduits. Using this acid concentration, the spectrophotometric signals were found to increase with molybdate concentration up to a concentration of 0.06 M Mo, above which become to slowly saturated up to a concentration of 0.12 M Mo. In order to ensure low background signals, which are the consequences of the electrochemical reduction of the excess of molybdate, a concentration of 0.06 M Mo was finally chosen for further investigations.

3.4. Investigation of physical parameters

Both sample and reagent volumes were optimised taking into account that the volume of the tubular generating electrode was 680 μl. Under the optimised chemical conditions described in the foregoing section, the highest sensitivity with negligible blank signals was encountered exploiting a binary sampling modality with sample and molybdate zones of 300 μl, as detailed in the protocol of the experimental section. The flow-rate for both sample/reagent aspiration and transportation of the on-line generated reduced species into the flow-through cell was investigated over the range 0.5–5.0 ml/min. In order to prevent undue dispersion of the electrogenerated species in the way to the detector, without deterioration of the coefficients of variation, a flow rate of 2.0 ml/min was adopted for the various steps of the analytical procedure.

3.5. Analytical features of the method

Under the aforementioned chemical and physical conditions, a linear calibration graph was established over the range 0.3–20 mg/l P (sensitivity $= 23.5 \pm 0.7$ l/mg, $r = 0.9965$, $n = 6$). Dynamic working ranges at higher orthophosphate concentrations are straightforwardly attainable by implementation of on-line dilution strategies, as described in the following section.

The detection limit calculated as the concentration corresponding to the mean of the blank signal (assessed by 10 consecutive injections), plus three times its standard deviation was 100 μg/l P. Repeatability was estimated from 10 consecutive injections of two standard solutions. Relative standard deviations of 2.4 and 1.8% were obtained for standards containing orthophosphate concentrations of 2.0 and 10 mg/l P, respectively. The analytical throughput for a typical protocol sequence without further sample pre-treatment was 18 h^{-1}.

3.6. Adaptation of the SIA manifold to the analysis of samples containing high levels of orthophosphate

The sensitivity of the methodology for analysing samples with orthophosphate contents slightly higher than 20 mg/l P may be easily adapted to the particular assay by modification of the experimental variables, such as electroreduction time, sample volume or reagent concentrations. Yet, sample pre-treatment by application of dilution steps is required when the concentration of the target species exceeds more than five times the superior limit of the working range. In the present work, different dilution strategies exploited in an on-line fashion, based on the implementation of a mixing chamber or flow-reversal approaches, are assessed.

3.6.1. Successive backward and forward movements

This alternative involves the aspiration of a certain sample volume (<300 μl) in the holding coil, and the performance of various automated flow-reversal movements aiming to disperse the sample segment into the carrier stream. The influence of the number of forward and backward movements and the lengths of the displacements on the analytical signal is depicted in Fig. 3. As can be seen, the dilution effect depends mainly on the length of the forward–backward movements rather than the number itself. Thus, for example, 10 forward–backward movements of 100 μl (i.e., 20 cm) each give rise to a decrease in the signal of 20%, whereas if this flow-reversal protocol is carried out by dispensing-aspirating a solution volume of 500 μl (corresponding to a length of 100 cm in the reaction coil), a decrease of 95% is attained. A 20-fold sample dilution is achieved exploiting the latter modality. Higher dilution factors require extremely time-consuming procedures, so that the following on-line dilution strategy is strongly recommended.

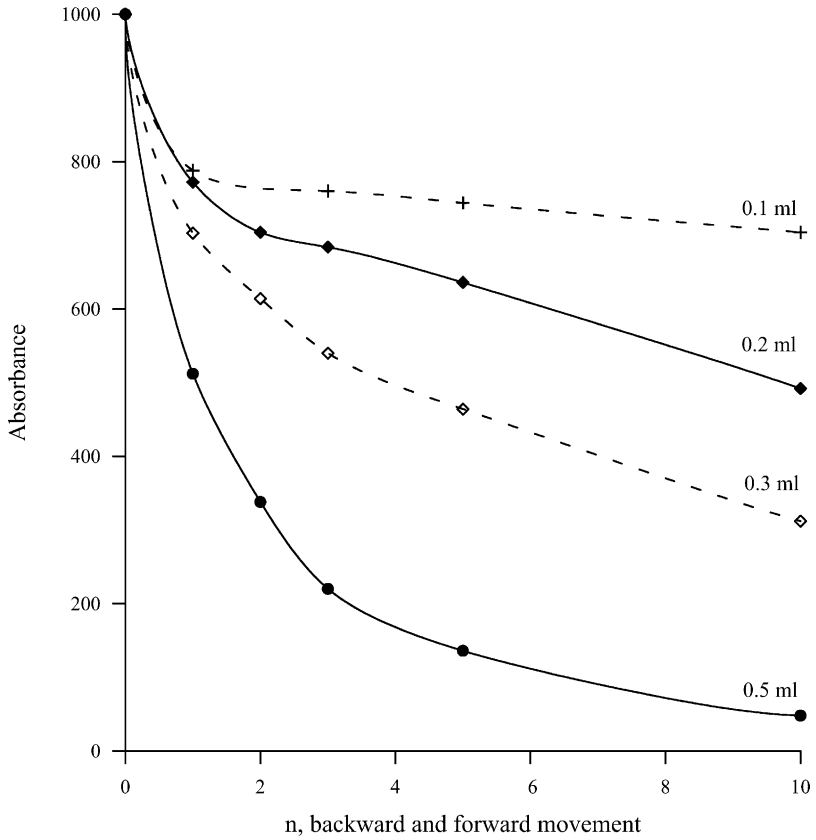

Fig. 3. Influence of the number and length of backward–forward movements on sample dilution. Experimental conditions: 125 μl sample (50 mg/l P), 300 μl reagent (0.12 mol/l Mo), 0.6 mol/l sulphuric acid, 120 s of electroreduction time and flow-rate of 2.0 ml/min.

3.6.2. Mixing chamber

The use of a small dilution tank with an inner volume of 3.0 ml and provided with a stirring bar is the most reproducible and reliable way of performing on-line controlled sample dilution within a wide concentration range. A maximum dilution factor of 100 was attained exploiting the methodology described in Section 2 relying upon the introduction of a 30 μl sample zone. The relative standard deviation ($n = 17$) for standards containing 200 and 800 mg/l P was better than 2.0% for both concentrations. When applying this strategy to dilute concentrated biological samples as well as soft drinks, the calibration graphs were obtained with standards treated similarly to the samples.

3.7. Evaluation of coating effect and investigation of interferences

Coating is a well-known drawback of the molybdenum blue methodology causing carryover effects as well as a decline of analytical precision. It results from the formation of a colloidal product that is readily adsorbed onto solid surfaces such as tubing or cuvettes used in dynamic systems [28]. Coating seems to be related to the different characteristics of the reduced complex formed at various pH, in particular, the products formed at low pH seem colloidal and tend to coat the inner walls [29]. Prolonged rinsing cycles are often used to minimise coating effects but at the expense of the sample throughput. Yet, in the present SIA assembly, coating resulted negligible by merely washing the flow set-up with a 1 M NaOH zone between consecutive injections.

A typical problem encountered in the determination of orthophosphate exploiting the molybdenum blue method is the additive interference caused by arsenate and silicate due to their ability to form similar blue complexes with molybdate [30]. Arsenate concentration is often much lower than orthophosphate concentrations unless handling samples from areas polluted by arsenic from hydrothermal activity. Thus, the arsenate interference at the level found in the samples analysed is negligible. Its interference can be, however, effectively overcome by the addition of thiosulphate into the reaction medium aiming to reduce arsenate to arsenite, which is non-reactive to molybdate reagent [29].

Unlike arsenate, silicate is often present in environmental samples at similar concentrations, or even higher, to those of orthophosphate. It should be stressed that a certain disagreement is found in the literature with respect to the maximum Si/P ratio tolerated at the 5% (or 10%) interference level, which ratio may differ in more than two orders of magnitude. This discrepancy on the overall magnitude of the silicate interference is attributed to the different experimental conditions, such as $[H^+]$/[Mo] ratio, pH, temperature, chemical reductants and presence/absence of Sb in the

Table 2
Determination of orthophosphate (mg/l P) in wastewaters exploiting both the standard APHA-AWWA-WPCF method [7] and the proposed SIA procedure with electrogeneration

Sample	Batch standard method	SIA method with electroreduction	Chemical composition
Inlet 1	5.9 ± 0.2	5.8 ± 0.l	BOD_5: 549 mg/l; pH: 7.0; COD: 959 mg/l; SS: 254 mg/l
Inlet 2	8.4 ± 0.3	8.0 ± 0.2	BOD_5: 316 mg/l; pH: 7.7; COD: 792 mg/l; SS: 499 mg/l
Outlet 1	1.27 ± 0.08	1.25 ± 0.05	BOD_5: 51 mg/l; pH: 6.7; COD: 198 mg/l; SS: 73 mg/l
Outlet 2	3.0 ± 0.1	2.97 ± 0.09	BOD_5: 80 mg/l; pH: 7.6; COD: 278 mg/l; SS: 146 mg/l
Tertiary inlet	1.14 ± 0.07	1.20 ± 0.04	BOD_5: 7.7 mg/l; pH: 7.1; COD: 30.3 mg/l; SS: 10.3 mg/l; nitrate: 130 mg/l; nitrite: 6 mg/l; ammonium: 14 mg/l
Lagoon 1	4.4 ± 0.2	4.1 ± 0.1	BOD_5: 9.0 mg/l; pH: 9.2; COD: 58 mg/l; SS: 29 mg/l; nitrate: 110 mg/l; nitrite: 2.5 mg/l; ammonium: 1.2 mg/l
Lagoon 2	2.7 ± 0.1	2.52 ± 0.08	BOD_5: 7.2 mg/l; pH: 8.9; COD: 47 mg/l; SS: 24 mg/l; nitrate 100 mg/l; nitrite: 2.1 mg/l; ammonium: 1.0 mg/l

The results are expressed as the mean of three determinations ± standard deviation. BOD: biochemical oxygen demand; COD: chemical oxygen demand; SS: suspended solids.

reagent, explored by various researchers [16,29,31]. Hence, the interfering effect of silicate on the electrogeneration of molybdenum was evaluated. Under the optimised experimental conditions, a maximum tolerance of 100 mg/l Si at the 5% level was obtained for a 2 mg/l P standard solution, whereas silicate was tolerated up to 300 mg/l for a solution containing 10 mg/l P. The tolerance to silicate in the absence of the typical masking agents used (namely, oxalic and tartaric acid) [15] may be explained by the selective reduction of the phosphomolybdic acid against silicomolybdate at the stainless steel working electrode for an electroreduction time of 120 s.

3.8. Application of the designed automated system to the monitoring of orthophosphate in wastewaters, soft drinks and biological matrices

The proposed SIA method has been satisfactorily applied to the determination of orthophosphate in wastewater samples collected from different locations (namely, inlet, outlet, tertiary treatment and lagoon) of wastewater treatment plants (EDAR-1 and EDAR-2, Palma de Mallorca). The chemical composition of each sample, including chemical oxygen demand (COD), biochemical oxygen demand (BOD), suspended solids (SS), pH, ammonium, nitrate and nitrite, together with the orthophosphate concentration determined by the proposed automated system without requiring additional on-line dilution protocols are listed in Table 2.

The experimental results obtained were compared with those of the batch reference method [7] using ascorbic acid as a reducing agent and potassium antimonyl tartrate acting simultaneously as a catalyst and masking agent. No statistical differences were observed between the results obtained by both methodologies using a *t*-test comparison of the means [32] at a significance level of 0.05 and 4 degrees of freedom.

Aiming to assess the potentialities of the on-line dilution scheme based upon the implementation of a miniaturised mixing chamber, beverages such as cola drinks and beers, as well as samples of clinical interest, e.g., urine, containing orthophosphate concentrations above 100 mg/l P were analysed as shown in Table 3. The results were contrasted with both the APHA-AWWA-WPCF standard method and direct phosphorous measurement using inductively coupled plasma spectrometry with optical emission detection (ICP-OES). The mean *t*-test showed no statistical differences between the proposed SIA procedure and both reference methodologies at the 95% confidence level.

Table 3
Analysis of biological samples and beverages containing high levels of orthophosphate (mg/l P)

Sample	Batch method	ICP-OES	SIA[a]
Urine			
1	245 ± 7		246 ± 6
2	311 ± 7		322 ± 7
3	447 ± 9		458 ± 8
4	194 ± 6		203 ± 6
5	1555 ± 35		1611 ± 28
6	312 ± 7		293 ± 8
7	216 ± 6		214 ± 5
Tonic water (Schweppes)	218 ± 5	215 ± 4	214 ± 4
Beer			
Aguila	248 ± 7	247 ± 6	248 ± 7
Budweiser	160 ± 5	156 ± 4	167 ± 4
Heineker	265 ± 7	246 ± 5	257 ± 7
Mahou	173 ± 6	164 ± 4	168 ± 5
Coronita	115 ± 4	107 ± 3	110 ± 4
Coke			
Coca-Cola	172 ± 4	169 ± 3	171 ± 4
Coca-Cola light	156 ± 4	153 ± 4	164 ± 6
Coca-Cola without caffeine	154 ± 5	147 ± 4	157 ± 6
Pepsi-Cola	168 ± 5	155 ± 4	165 ± 5

The results are expressed as the mean of three determinations ± standard deviation.
[a] On-line dilution using a miniaturised mixing chamber.

4. Conclusions

A novel and versatile SIA procedure with on-line sample pre-treatment has been developed for the spectrophotometric determination of orthophosphate at a wide concentration range without involving chemical reductants. The method is based on the electrochemical generation of the molybdenum blue complex in a stainless steel tubing, which acts as a working electrode (cathode) of a potentiostatic system. The validated method presents a series of advantages described as follows:

- It avoids preparation, preservation and use of unstable reagents, such as the typical reducing agents used for orthophosphate determination, i.e., tin(II) chloride or ascorbic acid. This feature makes the method especially robust for implementation in an automated and unattended analyser able to monitor the target analyte for long periods of time.
- The sensitivity of the methodology is straightforwardly adaptable to the analysis demands. In the case of samples with low orthophosphate content, sensitivity may be enhanced by increasing the electroreduction time. Samples containing orthophosphate concentrations above 20 mg/l P may be analysed by proper adjustment of physical and chemical variables (sample volume, reagent concentrations and electroreduction time) or by performance of on-line sample dilution exploiting either flow-reversal strategies or miniaturised mixing chambers.
- The inherent noteworthy characteristics of the SIA systems in comparison with the parent FIA, such as reagent/sample saving, flexibility, liquid driver robustness and ease of performing on-line sample pre-treatments (e.g., dilution) are proven applicable in the designed assembly.
- Coating effects are readily avoided using merely one rinsing operation in alkaline medium, and the method presents an acceptable tolerance to silicates at the 5% interference level.
- Finally, it should be outlined that the present methodology alike the batch method is able to determine reactive phosphate, i.e., the sum of free orthophosphate and that susceptible of acidic hydrolysis, as opposed to the total phosphorus measurements carried out by ICP-OES. Thus, this detection technique may be only exploited as a reference method in samples containing reactive orthophosphate as the major form of phosphorus.

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