Sequential Injection Standard Addition System with a Mixing Chamber: Determination of Orthophosphate in Waters

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Abstract

A sequential injection system based on the standard addition method using a single standard solution was developed. Successive additions of known amounts of analyte are made to the sample in a mixing chamber placed in a side port of the selection valve; other in-line sample treatments were also performed in the mixing chamber. The proposed method was evaluated by performing experiments with the bromothymol blue dye, and subsequently applied to the spectrophotometric determination of orthophosphate in wastewater, micro algae medium and fish farming water samples. The sample concentration is obtained after plotting a linear graph of analytical signal as a function of the standard mass added to the sample. For the determination of orthophosphate, relative standard deviations were lower than 4 % and the results were in agreement with those obtained by the reference method.

Keywords Sequential injection, standard addition method, mixing chamber, spectrophotometric determination of orthophosphate

1. Introduction

During the last decade, a large number of sequential injection analysis (SIA) [1] systems, making use of different chemistries and techniques, were successfully developed. If compared to flow injection systems, this powerful methodology allows considerable saving of reagents and a significant decrease on the chemical waste produced. In addition, different analysis can be performed using the same manifold by simple reconfiguration of the sequence of events from the computer keyboard. Moreover, apart from the physical limitation imposed by the number of ports of the multiposition valve, there is apparently no limit to how many solutions (reagents, samples and standards) and devices (reactor coils, mixing chambers, gas-diffusion/dialysis units, pre-concentration columns and detectors) that can be gathered around the valve. SIA is thus widely accepted as a new and economical approach for enhancement of instrumental wet-chemical analysis.

Several papers report the possibility of performing different sample in-line pre-treatment in the side channels of the selection valve [2-7]. In most of them, this pre-treatment mainly regards performing sample dilutions and/or appropriate mixing between sample and reagent solution(s). Some of those systems make use of a mixing chamber to perform such tasks [3,4,7] since this approach enables complete mixture of solutions which is one of the major limitations pointed out to sequential injection system [8]. Others report the development of sequential injection manifolds where standard addition procedures were carried out in a reaction coil placed in a separate port [6] or on the way to the detector [9-10]. The advantages of performing the analytical flow calibration measurements in a background of sample matrix are widely recognized and many automated versions of this method have been developed throughout the years [11]. Recently, Lugo et al. [12] proposed a closed-loop flow injection system in which the mixing of the standard and sample was carried out in a small chamber, and discussed the importance of the generalized standard addition method in the area of instrumental analysis, where a good set-up of the chemical conditions is required. The method required successive additions of known amounts of a standard solution to the sample and a linear regression calculation enabled the original concentration to be evaluated regardless of the matrix effect, while also providing information on the magnitude of this effect. This idea had already been discussed by Agudo et al. [13] that pointed out the fact that the use of external calibration methods was useless in the presence of matrix effects and developed a simple, flexible automatic procedure to implement a standard addition method using also an open/closed flow loop. Systematic errors arising from the sample matrix could be detected in the multiple-addition method from changes in the slope of the absorbance-concentration plot. The automatic loop was the key part of the manifold and provided mixing and homogenisation of sample, standard and carrier prior to injection into the FI system for enzymatic determination of the glucose and fructose.

In 2002, Silva and Masini [6] established a SI procedure where the automation of the sample in-line dilution and the standard addition method were done at the same time, in a auxiliary reaction coil located in a side port. Although based on different premises, the feasibility to perform analytical calibrations with only one standard solution injected in a auxiliary dilution coil, had already been proposed by Baron et al. [2] in one of the earliest papers published regarding SI procedures.

Going one step further, the aim of the present work was to demonstrate the use of a mixing chamber for sample preparation, standard addition and multiple collection of sample aliquots in a sequential injection system. This small chamber was placed in a side port of the selection valve to enable both in-line sample preparation and standard addition. One of the disadvantages of this approach is the increased amount of time of the analytical cycle, as the chamber must be properly washed after each determination. To surpass this difficulty, replicate determinations were performed from successive aliquots of same pre-treated sample, drawn up after thorough mixing of the sample and reagent solutions, therefore reducing the amount of time needed to rinse the mixing chamber. Due to its versatility,

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this manifold can be used for numerous analytical purposes, enabling different determinations.

The proposed method was evaluated by performing experiments with the bromothymol blue (BTB) dye, and subsequently applied to automate the spectrophotometric methodology for determination of orthophosphate in water samples of diverse origin.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with analytical reagent grade chemicals. To minimise bubble formation inside the flow system, solutions were prepared with previously boiled deionized water with specific conductance lower than 0.1 μS cm^{-1}. A stock standard solution of orthophosphate with concentration corresponding to 50 mg P L^{-1} was obtained by dissolving 0.110 g of previously dried KH_2PO_4 (Merck, Germany) in water and diluting to 500 mL. The working standard solution (12.5 mg P L^{-1}) was prepared by dilution of the stock solution. A reagent solution containing 1.6% (m/v) ammonium molybdate and 0.037% (m/v) antimony tartrate was prepared daily from $(NH_4)_6Mo_7O_{24}.4H_2O$ (Merck, Germany) and $K(SbO)C_4H_4O_6$ (Merck, Germany) in aqueous medium. A 0.7% (m/v) ascorbic acid solution, prepared daily, was obtained by dissolution of the solid ($C_6H_8O_6$ from Merck, Germany) in deionized water. To prevent the deposition of the molybdenum blue [14] in the reactor and in the flow cell walls, a 1 M NH_4Cl/NH_3 solution was used. A solution of 3.5 M H_2SO_4 (Merck, Germany) was also prepared. A 0.4 g L^{-1} Bromothymol Blue (BTB) in a 0.01 M sodium tetraborate solution was used in preliminary studies of the in-line standard addition system.

2.2. Apparatus

The developed sequential injection system is outlined in Figure 1. A syringe pump (BU 1 S, Crison, Alella, Spain) equipped with a 5 mL syringe was used as a liquid driver. This device was connected by the holding coil (HC) to the central channel of a ten port electrically actuated selection valve (Valco VICI C25-31180E, Houston, USA).

Absorbance measurements were carried out at 710 nm and corrected by subtracting the readings at 450 nm to suppress the effect of changes in the refractive index effect, using an Ocean Optics, PC 2000-ISA spectrophotometer connected to a 200 μm fibre optic cable and a DH-2000 Deuterium - Halogen light source from Top Sensor Systems (Eerbeek, The Netherlands). Facing the fibre optic, a Hellma 178.710-QS flow-through cell (10 mm light path, 80 μL inner volume) (Mülheim/Baden, Germany) was placed in a Ocean Optics CUV cell support.

A 386 personal computer (Samsung, Korea; SD700) equipped with an Advantec PCL818L interface card, running a homemade software written in QuickBasic 4.5, controlled the selection valve position and the syringe pump direction and speed. Data acquisition was achieved by SpectraWin (version 4.2) through an external trigger signal made from the Advantec PCL818L interface card. The mean acquisition frequency was 25 s^{-1}.

A mixing chamber (MC), with an internal volume of *ca.* 750 μL, made of acrylic and containing a magnetic bar, was placed over a magnetic stirrer. To increase sensitivity, the reaction coil was placed inside an I.S.Co GTR190 thermostatic bath (Milano, Italy) at 37 °C. All tubing connecting the different components of the sequential injection system were made of Omnifit PTFE (Cambridge, UK) with 0.8 mm i.d.

2.3. Sequential injection procedure

The SI standard addition method consists of adding different volumes of a single standard solution to the sample in a mixing chamber (MC) located in a side port of the selection valve. The total amount of volume dispensed to the MC was 700 μL; this volume was kept constant in every determination. To modify the sample as little as possible, small volumes of the standard, ranging from 5 to 40 μL, were added to the sample in a MC. Water was aspirated so that a total volume of 40 μL (standard plus water) was assured in the holding coil (HC). The original concentration was calculated from a linear graph of absorbance as a function of the standard mass added to the sample. The mass added was previously determined as the product between the concentration and the volume added of the orthophosphate standard solution. Finally, the concentration value was calculated from the Equation (1):

$$Cx = \left| \frac{-b/m}{Vx} \right| \qquad \text{Equation (1)}$$

where Cx is the sample concentration, V_x the sample volume, and b and m the intercept and slope of the analytical graph, respectively.

This methodology was applied to the determination of orthophosphate, following the protocol of flow and timing sequence listed in Table 1.

The analytical cycle can be divided in three parts: MC washing (Table 1, steps a-c); sample treatment in the MC, that includes standard addition, acidification and dilution (Table 1, steps d-j); and spectrophotometric determination of orthophosphate in waters (Table 1, steps k-t).

Initially the MC is washed with water and emptied before the beginning of a new sample pre-treatment steps, to remove previous sample remains. The second part starts with the sequential aspiration of well-defined plugs of acid, sample, standard solution and deionized water to the HC. The flow is then reversed and the stacked zones are sent to the MC. The treated sample is drawn from the MC to fill the connection to the selection valve. After flushing the HC, the system is ready to perform the spectrophotometric determination of orthophosphate. Buffer cleaning solution (NH_4Cl/NH_3), deionized water, treated sample, molybdate and acid ascorbic solutions are sequential aspirated to the HC and subsequently propelled to the detector. At this point, the mixture is stopped for 100 s at thermostatic bath, to enhance the colour reaction development. Finally, the stacked zones are impelled to the detector and the measurements carried out. The last steps (Table 1, steps k-t) could be repeated up to three times, allowing successive determinations of the same sample without the need to rinse the MC.

The sample volume used, and consequently the dilution factor, depends on orthophosphate content level. This was established by the absorbance value obtained without the addition of the standard. The maximum value was set to 0.2 AU in order to reduce the spectrophotometric errors of the absorbance values obtained after addition of the standard solution.

For samples with low orthophosphate content, maximum sample volume possible (635 μL) was used. Minor changes in the program had to be made, namely in the sample treatment steps in MC (Table 1, steps d-j). Thus being, to ensure that in the

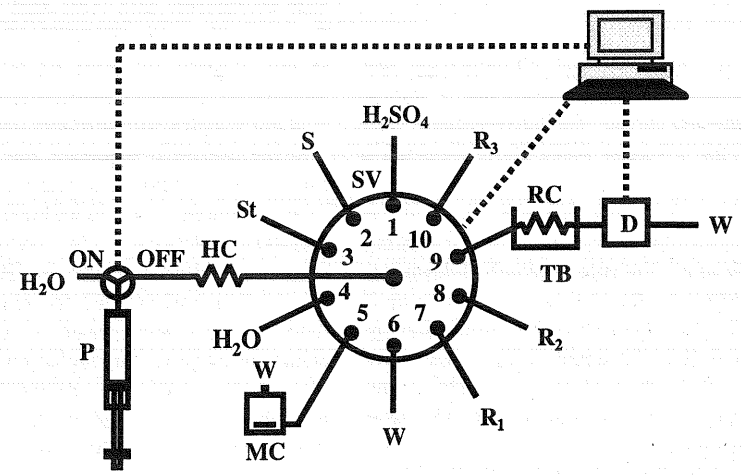

Figure 1. Sequential injection manifold for the orthophosphate determination in waters. S – sample; St – standard solution (12.5 mg P L^{-1}); R_1 – 1.6% ammonium molybdate and 0.037% antimony; R_2 – 0.7% L(+) ascorbic acid; R_3 – 1 M NH_4Cl/NH_3; W – waste; HC – holding coil (4 m); RC – reaction coil (140 cm); P – syringe pump; MC – mixing chamber; SV – selection valve; TB – thermostatic bath (37 °C); D – detector.

Table 1. Flow system protocol for the determination of orthophosphate in waters.

Step	SV position	Commutation valve position	Volume (µL)	Flow rate (mL min^{-1})	Description
a	5	Off	1500	15	Dispense water to wash MC
b	5	Off	1000	5	Draw up MC content
c	6	Off	1200	15	Dispense HC to waste
d	1	Off	25	0.5	Draw up H_2SO_4 solution
e	2	Off	200*	0.5	Draw up sample
f	3	Off	†	0.5	Draw up standard
g	4	Off	§	0.5	Draw up water
h	5	Off	700	2	Dispense HC content to MC
i	5	Off	180	5	Draw up MC content to fill the connection tubing
j	6	Off	395*	15	Dispense to waste
k	10	Off	100	5	Draw up NH_4Cl/NH_3 solution
l	4	Off	300	5	Draw up water
m	5	Off	115	2	Draw up treated sample from MC
n	7	Off	30	2	Draw up molybdate solution
o	8	Off	60	2	Draw up ascorbic acid solution
p	9	Off	295	2	Propel HC content to RC
q	9	On	2000	1.2	Stop period
r	9	Off	3000	5	Propel RC content to detector, signal acquisition
s	9	On	690	15	Piston adjustment
t	9	Off	0	1	Commutation valve OFF

† the increments of orthophosphate to the sample are obtained by adding different volumes (between 0-40 µL) of the standard solution; § volume of water between 40-0 µL; * dependent on sample concentration.

700 µL dispensed to the MC (Table 1, step h), 635 µL were in fact of undiluted sample, a large sample volume (1000 µL) was drawn up before the aspiration of the H_2SO_4 solution. Consequently, the dispensed volume to wash the HC (Table 1, step j) had to be increased (1200 µL).

Samples presenting large amounts of suspended particles and from micro algae artificial sea-water medium [15] were filtered through a 6 µm, 125 mm filter paper (Whatman, England) in order to withdraw solid particles and cells from the solution prior to its introducing in the sequential injection system.

2.4. Reference method

Ammonium molybdate reacts in acidic medium with orthophosphate in the presence of potassium antimonyl tartrate to form a heteropolyacid compound, that is reduced to molybdenum blue with ascorbic acid [16]. The absorbance is measured at 880 nm, and the orthophosphate concentration is determined using a calibration graph with linear dynamic range from 0.15 to 1.25 mg P L^{-1}.

3. Results and discussion

3.1. Development of the sequential injection standard addition system

Precision of the SI standard addition methodology was assessed using a BTB solution as the standard and a sodium tetraborate buffer as sample and carrier. The measurements were performed at 610 nm and the refractive index effect corrected at 450 nm.

In the beginning of this work, two different liquid driver systems were tested: a peristaltic pump and a syringe pump. With a flow rate of 0.5 mL min^{-1}, the volume standard deviations (n=10) for both devices were less than 0.1 μL for volumes up to 40 μL. Considering that in this standard addition methodology the mass of the analyte as well as the sample volume have to be precisely determined and since the use of a peristaltic pump requires daily tube calibration to achieve this purpose, this work proceeded using the syringe pump.

One of the disadvantages of placing the MC in a side port of the selection valve is the increased amount of time necessary for each analytical cycle as the chamber must be properly washed after each determination. As the total MC volume is considerably higher than the sample pre-treated volume needed to perform one determination, it is possible to withdraw several aliquots allowing replicate measurements from the treated sample and avoiding successive washing of the MC. In the following experiments, three aliquots were aspirated from the chamber.

The precision of the standard addition methodology was assessed by 5 consecutive calibration curves with additions of 10, 20, 30 and 40 μL of BTB. The calibration curve obtained was: Absorbance = $0.0101(\pm0.0001)V_{BTB} + 0.001(\pm0.003)$; r^2 = $0.9997(\pm0.0003)$.

The values in parentheses correspond to the standard deviations of the different parameters. These values point out that this methodology could be applied to carry out the standard addition method with a good precision. The signal output of one of the calibration curves is outlined in Figure 2.

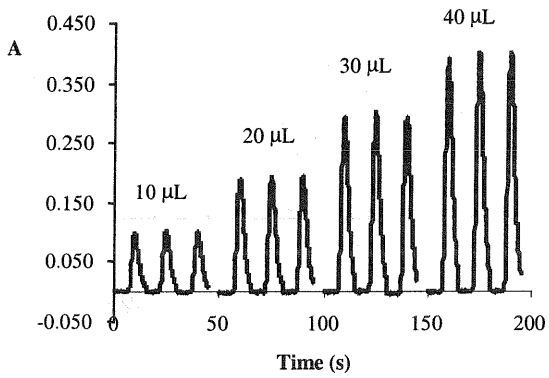

Figure 2. Signal output for a sequential injection standard addition run using BTB as the standard solution and sodium tetraborate buffer both as sample and carrier.

3.2. Application to orthophosphate determination

After the development of the in-line standard addition method, the system was applied to the determination of orthophosphate in waters, based on the reaction of orthophosphate with ammonium molybdate and potassium antimony tartrate in acidic medium to form an heteropoly acid, that was later reduced to molybdenum blue by ascorbic acid. This analysis was chosen to demonstrate the versatility and potential of the developed SI system as several reagents solutions are necessary, proper time control is required for enhanced sensitivity and orthophosphate is assessed in water samples with different matrices.

In the analytical sequence, the sample was first acidified and then a known amount of analyte was added. An aliquot of the treated sample was withdrawn from the MC, and then molybdate/antimony and ascorbic acid solutions were sequentially drawn up into the HC. The valve was switched to the detector position and the reaction mixture propelled to the detector after a stop period of 100 s at 37 °C to enable colour enhancement providing higher reaction sensitivity.

Using the manifold depicted in Figure 1, optimisation procedures were carried out by studying the influence of some parameters on the calibration curve and maintaining the others on pre-set values.

3.2.1. Chemical parameters

Molybdate and antimony concentrations were studied simultaneously keeping the ratio Mo/Sb (44:1) constant and similar to that of the reference method used [16]. Thus being, molybdate and antimony concentrations were varied between 0.5-1.8% and 0.011-0.041%, respectively. An aqueous solution with 1.6% of molybdate and 0.037% of antimony was selected as a compromise between sensitivity and the blank signal, produced as a consequence of the molybdate self-reduction, since both increased with higher concentrations.

After setting the molybdate and antimony concentrations, the content of the ascorbic acid solution was assessed. The 0.7% ascorbic acid solution was chosen after testing several aqueous solutions containing concentrations between 0.2% and 1.3%. Higher concentrations gave rise to high blank signals and lower concentrations led to poor sensitivity.

The acidity has an important role on both sensitivity and selectivity of the molybdate reaction [17,18]. An increase in the acid concentration level reduces silicate interferences but leads to a decrease in the reaction sensitivity. Moreover, low acid concentrations, even in the presence of phosphate, lead to self-reduction of molybdate [18-20]. Initially, in an attempt to use a fewer reagent solutions, the ammonium molybdate and the antimony tartrate solution was prepared in sulphuric acid. However, after a short period of time, solutions became blue (due to molybdate self-reduction) and thus their use was disregarded. To overcome this problem, a fixed volume (25 μL) of several sulphuric acid concentrations ranging from 1 M to 4.5 M was added to the sample in the MC. The 3.5 M H_2SO_4 solution was chosen because sensitivity did not decrease significantly (6%) up to this point and, as expected, the blank signals were higher when lower acid concentrations were used.

In order to prevent accumulation of molybdenum blue in the reactor and the flow cell [14], a 100 μL plug of 1 M NH_4Cl/NH_3 solution was included in the analytical sequence, to wash the manifold immediately after the molybdenum blue formation.

3.2.2. Physical parameters

The lengths of the HC and RC were pre set at 4 m and at 140 cm, to prevent the sample and reagents from entering in the syringe conduit and to provide efficient mixing between sample and reagents, respectively.

The flow rates were set as described in Table 1. A flow rate of 0.5 mL min^{-1} was adopted during the standard addition, sample dilution and acidification steps (Table 1, steps d-j) as higher flow rates could affect the precision of the standard addition method. Flow rates for propelling the reaction mixture to the detector (Table 1, step r) between 1.7 mL min^{-1} and 10 mL min^{-1} were tested. A flow rate of 5 mL min^{-1} was selected since a better mixture between sample and reagents is achieved, leading to an increase on the sensitivity.

The volume of the treated sample drawn up from the MC was varied between 80 and 140 µL. A 115 µL was chosen, as sensitivity increased approximately 15% up to this volume. The influence of volume of molybdate/antimony solution was also assessed. After testing reagent volumes between 30 and 85 µL, the smaller volume was chosen since it provided better sensitivity. The ascorbic acid volume was set at 60 µL.

In order to accelerate the reduction of the blue heteropoly compound, known to be the rate-determining step [21], different temperatures between 25 and 60 °C were tested using an in-line thermostatic bath. A temperature of 37 °C was selected since the sensitivity increased more than 40% up to this point. Higher temperatures gave rise to upper blank signals and bubbles formation.

To enhance colour development, the use of a stop period, between 0 and 150 s, prior to data acquisition was studied. As an increase on sensitivity occurred continuously, a stop period of 100 s was selected as a compromise between sensitivity and sampling rate. Additionally higher stop periods could lead to an increase of silicate interference as the formation of the silicomolybdenum blue complex is slower than that of phosphomolybdenum complex [17].

3.2.3. Interferences

The molybdate reaction is subjected to the interference of silicate and arsenate, since both can form similar complexes with molybdate. Under the optimised conditions, no interference of silicate was registered up to a Si/P concentration ratio of 0.3.

Regarding arsenate, the reported As/P ratio in environmental samples is very low. Moreover, in areas of arsenic pollution, arsenate interference is readily avoided by addition of thiosulphate to reduce arsenate to arsenite, which is non-reactive to molybdate reagent [17].

3.2.4. Detection limit

To calculate the detection limit, the sample was replaced by deionized water and increasing standard additions were made to the MC to obtain a calibration curve. After 6 consecutive calibrations, the standard deviation of the intercept was calculated and multiplied by 3, and the corresponding concentration was obtained from the ratio between the average of the calibration curves and the sample volume [22]. For a sample volume of 635 µL, the limit of detection was 0.024 mg P L^{-1}.

3.3. Application to water samples

Several samples with different matrix composition (waste waters, micro algae medium and fish farming waters) were analysed by the developed standard addition flow procedure (Figure 3) and by the reference method. The results and the corresponding relative deviations, are presented in Table 2.

To assess the accuracy of flow methodology, linear relationship of the type $C_S = C_0 + SC_r$ (being C_S the sequential-injection results and C_r those provided by reference method) was established. The following results were obtained: $C_0 = 0.002$ (± 0.158) and $S = 0.983$ (± 0.035), with a correlation coefficient of 0.9989. This result means that there is no statistical difference between the two sets of results [23], since the slope and correlation coefficient are close to unity and intercept value is near zero.

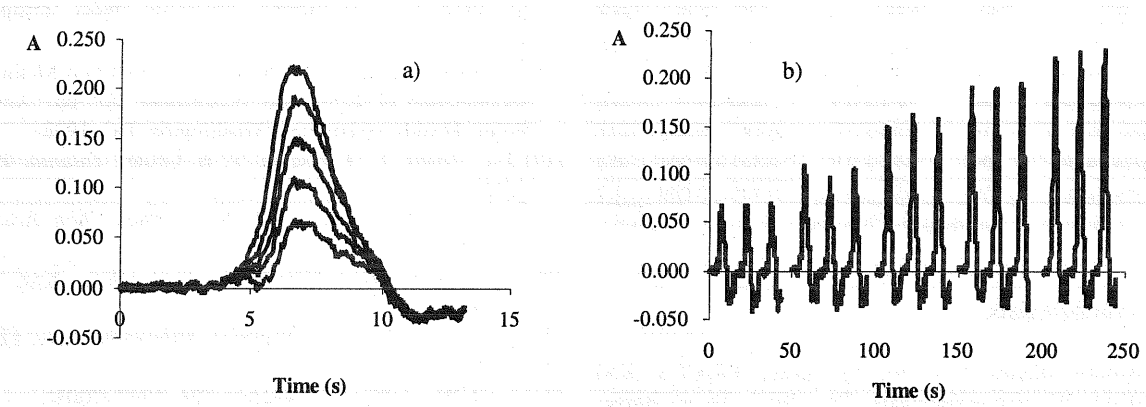

Figure 3. Output signals obtained for a SI standard addition run of a fish farming water sample. The signals presented correspond to addition of 0, 62.5, 125, 187 and 250 ng of P to the sample. a) Register pointing out the coincidence of the peak shape and the time corresponding to the maximum absorbance value. b) Signals perspective showing the repeatability of the methodology.

The precision of the SIA method was assessed from 5 consecutive determinations of three waste water samples. Relative standard deviations of 2.6, 3.6 and 3.5% were obtained for samples with orthophosphate concentrations of 0.154, 2.06 and 8.6 mg P L^{-1}, respectively.

4. Conclusions

The sequential injection system based on the addition of different volumes of a single standard solution to the sample using a mixing chamber placed in a side port of the selection valve allows to efficiently carry out the standard addition procedure with good precision and accuracy. Additional sample in-line treatments can also be made in the mixing chamber (e.g. acidification, dilution, addition of releasing agents and masking

of interference species) allowing the use of the treated aliquots in numerous applications.

Its use in the determination of orthophosphate demonstrated the capability to overcome problems associated with different physical characteristics of the sample matrices, since it was applied to wastewaters, micro algae medium and fish farming waters. Due to the flexibility of the in-line sample preparation, this methodology could also be used for the determination of total phosphorus, after off-line sample digestion; the sample volume introduced in the mixing chamber should be adjusted to the amount of phosphorus in the sample digest.

The disadvantage of increasing the time of the analytical cycle, to properly wash the chamber after each determination, was minimised by performing replicate determinations from successive aliquots of same pre-treated sample, and therefore reducing the amount of time needed to rinse the mixing chamber.

Tabela 2. Determination of orthophosphate (mg P L^{-1}) in waters by SIA methodology and the reference method

Sample source	Sample volume (μL)	SIA*	Ref. Method*	RD (%)
waste water	635	0.156 ± 0.001	0.157 ± 0.004	-0.6
waste water	35	7.1 ± 0.2	7.39 ± 0.08	-3.9
waste water	70	2.08 ± 0.09	2.14 ± 0.08	-2.8
waste water	25	8.7 ± 0.3	8.58 ± 0.02	+1.4
waste water	70	2.80 ± 0.02	2.75 ± 0.04	+1.8
waste water	35	6.7 ± 0.1	6.89 ± 0.02	-2.8
micro algae medium	70	3.35 ± 0.01	3.25 ± 0.05	+3.1
micro algae medium	40	4.3 ± 0.2	4.64 ± 0.3	-7.3
micro algae medium	635	0.20 ± 0.01	0.187 ± 0.002	+7.0
fish farming water	200	0.54 ± 0.02	0.541 ± 0.004	-0.2
fish farming water	150	0.91 ± 0.03	0.926 ± 0.009	-1.7

* Results expressed as the mean of three determinations ± standard deviation.

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