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Use of solid phase extraction for the sequential injection determination of alkaline phosphatase activity in dynamic water systems

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ABSTRACT

In this work, a solid phase extraction sequential injection methodology for the determination of alkaline phosphatase activity in dynamic water systems was developed. The determination of the enzymatic activity was based on the spectrophotometric detection of a coloured product, *p*-nitrophenol, at 405 nm. The *p*-nitrophenol is the product of the catalytic decomposition of *p*-nitrophenyl phosphate, a non-coloured substrate. Considering the low levels expected in natural waters and exploiting the fact of alkaline phosphatase being a metalloprotein, the enzyme was pre-concentrated in-line using a NTA Superflow resin charged with Zn^{2+} ions. The developed sequential injection method enabled a quantification range of 0.044–0.441 unit mL^{-1} of enzyme activity with a detection limit of 0.0082 unit mL^{-1} enzyme activity (1.9 μmol L^{-1} of *p*NP) and a determination rate of 17 h^{-1}. Recovery tests confirmed the accuracy of the developed sequential injection method and it was effectively applied to different natural waters and to plant root extracts.

1. Introduction

Phosphorus (P) is an important nutrient required by all organisms since it is present in the nucleic acids (DNA and RNA) and in phospholipids, located in the membranes. Furthermore phosphorous plays an essential role in energy metabolism as ATP, ADP, AMP, and PPi [1]. In dynamic water systems such as natural waters, phosphorous is present in two soluble sources: dissolved inorganic phosphate, orthophosphates ($H_2PO_4^-$ and HPO_4^{2-}) and organic phosphorus compounds. Orthophosphates are available for direct assimilation by organisms such as bacteria, algae (both micro and macro) and plants. However, organic phosphorus compounds need to be mineralized in order to be part of the soluble orthophosphate pool [2].

When a shortage of dissolved inorganic phosphate in waters compared to other nutrients (namely *N*) arises, phytoplankton and bacteria have the ability to obtain phosphorus from dissolved organic compounds, if available, as an alternative source for their metabolism. This feature results from the production of extracellular enzymes such as alkaline phosphatase (AP) that hydrolyze phosphate monoesters releasing inorganic phosphate and organic matter [3]. Thus, alkaline phosphatase may play an important role in the availability and recycling of phosphorous. Therefore it is regulated by inorganic phosphate concentrations and internal phosphorous levels, which makes this enzyme an excellent indicator of phosphorous status [4–6]. Routine assessment of alkaline phosphatase activity (APA) in waters is usually attained by incubating the enzyme with specific substrates, namely methyl-umbelliferyl phosphate (MUF-P), *p*-nitrophenyl phosphate (*p*NPP), or monofluorophosphate, and quantifying the resulted product with different detection methods, such as fluorescence, spectrophotometry and potentiometry, respectively. Therefore, APA determination is not only quite laborious but also time consuming due to the incubation step, which can go from 30 min [4] to 28 h [7]. The lower limits aimed, such as the expected in dynamic water systems, the higher incubation time needed.

The purpose of this work was the automation of the spectrophotometric APA determination based on catalysis of the *p*-nitrophenyl phosphate in inorganic phosphate and *p*-nitrophenol, a coloured product of the catalysis, in natural waters from dynamic water systems (estuarine and river waters with respective interstitial water and well water). Due to the expected low values of alkaline phosphatase in natural waters, an enzyme preconcentration step would be required to carry out the determination in an expeditious way. Solid phase extraction (SPE) can be an attractive approach to solve this problem, as it permits an efficient preconcentration with no consumption of organic solvents. The amount of solid material used can be minimized by packing a column for a reusable approach. Some problems

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associated with SPE, namely low reproducibility, solid-phase swelling and compaction, and washing can be minimised using sequential injection analysis (SIA). In SIA, the high level of automation [8] decreases reproducibility problems, furthermore solutions can flow through the column in opposite directions, thus minimizing possible compaction of the solid support. Pre-concentration and elution can also be easily achieved by selecting an appropriate sequence protocol, in which different solutions placed around the selection valve, namely conditioning and eluting buffers can be sent to or aspirated from the column and sent to the detector. The chosen solid phase material was Nitrilotriacetic Acid (NTA) Superflow resin, commercialized for protein purification when charged with nickel ions [9]. Nitrilotriacetic acid is an aminopolycarboxylic acid that can complex metal ions when fully deprotonated [10]. Thus, the NTA resin was charged with Zn^{2+} (instead of the commonly used nickel ions) in order to retain the enzyme since the enzyme requires that ion on its active site. In fact, alkaline phosphatase is a homodimeric metalloenzyme containing one Mg^{2+} and two Zn^{2+} ions in the active site. Magnesium is an important structural stabilizer of the enzyme, whereas the two zinc ions are directly involved in catalysis. One plays an important role in binding both the substrate and phosphate while the other stabilizes the amino acid responsible for the nucleophilic attack on the phosphate [11].

As far as we know, this is the first time that AP has been pre-concentrated with NTA resin charged with zinc ions. Moreover, we believe this was the first use of solid phase extraction coupled to pre-concentration for alkaline phosphatase activity determination. Although previous works have reported the determination of alkaline phosphatase activity using flow analysis techniques [12–23], they all relate to flow injection and only one was applied to water samples (seawater) [15]. Furthermore, within those reported works no extraction/pre-concentration was attained.

The developed SIA methodology was successfully applied to natural waters, namely estuarine, river, interstitial and well waters. Concomitantly, AP in root samples was also assessed in order to evaluate the potential relationship between plant root and soils [24,25].

2. Materials and methods

2.1. Reagents and solutions

Solutions were prepared with analytical grade chemicals and deionised water (specific conductance less than 0.1 $\mu s\,cm^{-1}$), previously boiled.

Alkaline phosphatase (AP) from bovine intestinal mucosa was purchased from Sigma Aldrich (EC 3. 1. 3. 1). Enzyme long term stock solution of 179.3 unit mL^{-1} was prepared according to the instructions of the product supplier [26], and kept at 2–8 °C in a storing buffer composed by: 10 mmol L^{-1} Tris–HCl (pH=8), 2.5 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 0.15 mmol L^{-1} $ZnCl_2$, and 50% glycerol.

The diethanolamine buffer was daily prepared by dissolving 2.6 mg of magnesium chloride hexahydrate in water, adding 2.4 mL of diethanolamine (d=1.09, Merck) and diluting to 25 mL of water to final concentrations of 1.0 mol L^{-1} diethanolamine and 0.5 mol L^{-1} of magnesium chloride. The pH was adjusted to 9.8 using a 4 mol L^{-1} HCl solution, obtained by proper dilution of the concentrated acid (d=1.19; 37%). A 5 fold dilution of the diethanolamine buffer was used in the sequential injection manifold (DB_{SI}).

Enzyme stock solution 0.441 unit mL^{-1} was prepared by proper dilution of the long term stock solution (179.3 unit mL^{-1}) in diethanolamine buffer. The working solution of 0.2 unit mL^{-1} of AP, and the AP standards in the range 0.022–0.441 unit mL^{-1} were daily prepared by appropriate dilution in diethanolamine buffer.

The substrate solution, *p*-nitrophenyl phosphate (Calbiochem) 30 mmol L^{-1}, was also daily prepared by dissolving 28 mg in 2 mL of water.

The elution buffer NPI-250 (NTA—QIAGEN handbook [9]) used in preliminary studies, EB_{PS}, was obtained by dissolving 0.136 g of KH_2PO_4, 0.351 g of NaCl and 0.340 g of imidazole in 20 mL of water to final concentrations of 50 mmol L^{-1} KH_2PO_4, 300 mmol L^{-1} of NaCl and 250 mmol L^{-1} of imidazole. The pH was adjusted to 8.0 using NaOH.

The elution buffer (buffer E in the NTA—QIAGEN handbook [9]) used in the sequential injection method for eluting the enzyme in denaturing conditions, EB_{SI}, was prepared dissolving 24 g of urea, 0.69 g of KH_2PO_4 and 0.79 g of Tris–HCl, in 50 mL of water. The pH was adjusted to 4.5 (with HCl) and the final concentrations were: 8 mol L^{-1} urea, 100 mmol L^{-1} of KH_2PO_4 and 100 mmol L^{-1} of Tris–HCl.

The buffer solution used for the assays with plant roots, B_{MOPS}, was based in the work of George et al. [25] where the MES (2-(*N*-morpholino) ethanesulfonic acid) reagent was replaced by MOPS (3-(*N*-morpholino) propanesulfonic acid), a structural analog expected to have better compatibility with the NTA resin [8]. Stock solutions of 25 mmol L^{-1} of cysteine and 255 mmol L^{-1} of MOPS were obtained by dissolution of the solids: 0.2 g cysteine to a final volume of 50 mL and 2.7 g of MOPS to a final volume of 50 mL. The buffer for plant roots assays, B_{MOPS}, 5 mmol L^{-1} of cysteine and 15 mmol L^{-1} of MOPS, was prepared daily by proper dilution of the stock solutions cysteine and MOPS, respectively.

A stock solution of the coloured product, *p*-nitrophenol (*p*NP) 560 μmol L^{-1}, was prepared by dissolving 15.6 mg of the solid in 200 mL of water. The working standards in the range 19–280 μmol L^{-1} were obtained by proper dilution of the stock solution.

Nitrilotriacetic Acid Superflow resin, highly cross-linked 6% agarose, 60–160 mm of bead diameter, 50% suspension in 30% ethanol (30510, Qiagen, UK) was used for solid phase extraction/preconcentration of the enzyme. For metal charging the column a 2% Zn^{2+} solution was obtained from the solid $ZnCl_2$.

2.2. Preparation of the NTA beads column

An acrylic column with 3 mm i.d., 20 mm length and 140 μL inner volume was used to pack the NTA resin (60–160 μm). Filters (10 μm pore size, MoBiTec M2210) were placed at both ends to prevent resin leaking. Three milligrams of NTA resin were introduced in the acrylic column with a Gilson micropipette, and connected to the SI system (Fig. 1).

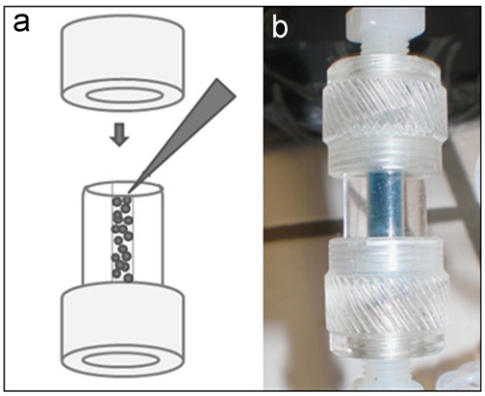

Fig. 1. NTA resin micro column: (a), schematic representation of the packaging process; (b), photography of the column connected to the selection valve.

The column was prepared only once and used throughout all the work, corresponding to about 1600 determinations (≈ 4 months work). The NTA resin was daily charged with the 2% Zn^{2+} solution by passing about 1 mL of the solution through the column.

2.3. Sample collection and preparation

Different water samples were collected for the determination of alkaline phosphatase activity (APA) and inorganic phosphate: river, estuarine, interstitial, and well. Well waters were collected using a Van Dorn bottle of 2 L capacity. Estuarine and river waters were collected in polyethylene plastic bottles of 1 L capacity at about 20 cm depth. The collected water samples from estuaries included not only surface water but also pore water (interstitial water). To collect interstitial water, sediment was collected from the estuary bottom with a petit–ponar sampler (a clamshell-type scoop activated by a counter-lever system), and then water from the sediment was aspirated with a plastic syringe (≈ 25 mL). All water samples were directly introduced into the system without any previous treatment.

Root samples were obtained from different plants and the sample preparation was based on the procedure described by George et al. [25]. The freshly collected roots were immersed in liquid nitrogen and stored. Then, prior to its use, the frozen roots were triturated and suspended in 5 volumes of buffer (B_{MOPS}). The obtained suspension was centrifuged (14,000 rpm, 15 min) and the supernatant was collected for APA measurements.

2.4. Sequential injection manifold and procedure

The sequential injection (SI) manifold used for the determination of alkaline phosphatase activity is depicted in Fig. 2.

Solutions were propelled by a Gilson Minipuls 3 (Villiers-le-Bel, France) peristaltic pump with a PVC pumping tube, connected to the central channel of an eight-port electrically actuated selection valve (Valco VICI 51652-E8, Houston, USA). All tubing connecting the different components of the sequential injection system was made of Teflon from Omnifit (Cambridge, UK), with 0.8 mm id. A Hitachi (Tokyo, Japan) 100–40 UV–vis, with a Hellma (Müllheim/Baden, Germany) 178.711-QS flow cell (10 mm light path, 40 μL inner volume) was used as detection system (λ at 405 nm). Analytical signals were recorded using a Kipp & Zonen BD 111 (Delft, The Netherlands) chart recorder. A personal computer (Samsung SD 700, Korea) equipped with a PCL818L interface card, running with a homemade software written in Quick basic 4.5, controlled the selection valve position and the pump rotation sense and speed.

The sequences of the steps and respective time and volumes for the determination of alkaline phosphatase activity are given in Table 1.

For the determination of APA, the sample was aspirated and sent to the NTA beads column (steps A and B), where the alkaline phosphatase enzyme was retained. After that, in order to remove the enzyme that was not retained in the NTA beads, the column was washed (step C).

Then, the substrate, *p*NPP, was aspirated and sent to the NTA resin column to incubate with the immobilized enzyme (steps D and E). While the enzymatic catalysis was occurring, the holding coil was washed (step F). Afterwards, the coloured product formed was aspirated from the column and sent to the detector for absorbance measurement (steps G and H).

After the APA assay, the enzyme retained in the NTA resin column should be eluted. Thus, elution buffer was aspirated and sent to the column (steps I and J). Then, the elution buffer was removed by aspiration back to the holding coil (step L). In order to prepare the NTA resin column for the next cycle, the aspirated volume in the step L ensured that not only the elution buffer was removed but that the column itself was filled with diethanolamine buffer. Finally, the holding coil was washed (step M) and the system was ready for the following cycle.

2.5. Calculation of the enzyme activity

The developed SI system enabled the determination of alkaline phosphatase activity (APA) by absorbance measurement of the coloured product formed. Therefore, the results could be presented either in enzyme activity units (Enz_{CC}), or in formed product concentration (NP_{CC}), the two most common ways of presenting results in enzymatic analysis.

2.5.1. Enzyme calibration curve (Enz_{CC})

A calibration curve based on the linear relationship A=f(APA) was established using enzyme activity standards. The APA of the samples was calculated by direct interpolation of the obtained absorbance value in the calibration curve.

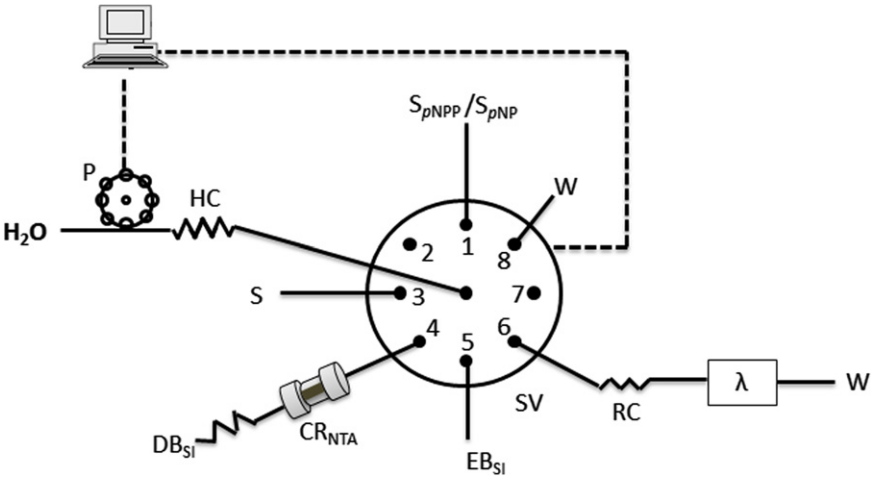

Fig. 2. Sequential injection (SI) manifold for the spectrophotometric determination of alkaline phosphatase activity: P, peristaltic pump; SV, eight-port selection valve; HC, 4.25 m holding coil; S, sample or enzyme standard; CR_{NTA}, column with NTA resin charged with Zn^{2+} ions; DB_{SI}, diethanolamine buffer (0.2 M diethanolamine buffer with 0.10 mM $MgCl_2$); S_{pNPP}, enzyme substrate *p*NPP 30 mM; S_{pNP}, *p*NP standard; EB_{SI}, elution buffer (8 M of urea, 100 mM of KH_2PO_4 and 100 mM of Tris–HCl); RC, 95 cm reaction coil; λ, spectrophotometer (405 nm); W, waste.

Table 1
Sequential injection protocol for the determination of alkaline phosphatase activity.

Step	SV position	Time (s)	Pump speed, Q ($\mu L\ s^{-1}$)	Pump direction	Volume (μL)	Description
A	3	8	62.5	a	500	Aspiration of sample/enzyme standard or aspiration of deionized water[a]
B	4	16	31.5	b	500	Propelling to NTA resin column
C	4	20	31.5	a	630	Aspiration of excess of sample/enzyme standard not retained in the resin column ensuring the buffering of the NTA resin
D	1	2.5	31.5	a	78	Aspiration of substrate *p*NPP or aspiration of *p*NP standard[a]
E	4	2	31.5	b	63	Propelling to NTA resin column for enzyme catalysis
F	8	12	62.5	b	756	Washing the holding coil
G	4	2.1	31.5	a	66	Aspiration of the product (*p*NP) formed in the resin column
H	6	45	62.5	b	2835	Propelling to the detector (λ=405 nm) colour measurement
I	5	2	62.5	a	126	Aspiration of eluting buffer
J	4	4	31.5	b	126	Propelling to NTA beads column to remove the enzyme adsorbed
L	4	7	31.5	a	220	Aspiration to wash the column and ensuring the re-buffering of the NTA resin
M	8	4	62.5	b	250	Washing the holding coil

[a] pNP calibration curve.

This calculation method is simpler and more direct for presenting the enzyme activity in water samples. However, it requires daily preparation of enzyme activity standards.

2.5.2. Product calibration curve (NP_{CC})

A calibration curve was obtained using the reaction product standards, corresponding to the linear relationship A=f([*p*NP]). By interpolating the absorbance value obtained for the water samples, the result is presented in concentration of formed product.

Furthermore, by interpolating the absorbance values obtained with the enzyme standards in the product calibration curve, a linear relationship *p*NP=f(APA) could be established. Thus, if the calculated product concentration, corresponding to a certain sample, was interpolated in the linear relationship *p*NP=f(APA), the APA of the samples would be calculated.

The indirect calculation of APA through the product calibration curve has the advantage of not requiring daily standards preparation. The product standards are stable for one month, and the established linear relationship *p*NP=f(APA) was valid for a set of product standards only re-established when the product standards were replaced. Furthermore, enzyme standards were only needed when a new set of product standards were prepared (once a month) representing an important decrease in enzyme consumption.

2.6. Sample characterization—Phosphate determination

Due to the probable relationship between the level of inorganic phosphate and the alkaline phosphatase activity, a previously developed system was adapted [27] and incorporated in the same manifold. In this way, the inorganic phosphate content of the water samples was also assessed.

3. Results and discussion

3.1. Preliminary studies—batch procedure

3.1.1. Activity assessment of the enzyme solution

In order to estimate the kinetics of the substrate degradation by alkaline phosphatase, the enzyme activity of the solution 0.2 unit mL^{-1} was assessed according to the diethanolamine assay [28]. The increase in absorbance, a result of the increase of coloured product obtained from the substrate catalysis, was measured for 5 min (ESI Fig. 1). The results presented a linear increase of the alkaline phosphatase activity up to 60 s, indicating that small incubation times could be successfully used.

3.1.2. Enzyme retention on NTA resin

First, in order to verify the potential need for a pre-concentration step of the alkaline phosphatase enzyme for alkaline phosphatase activity (APA) determination in water samples, some samples were tested using the diethanolamine assay [28]. The absorbance was measured and no signal increase was observed during the recommended time interval indicating, as expected, that APA in waters could not be directly assessed, probably due to their extremely low values.

In this scenario, a strategy was devised to pre-concentrate the enzyme, through solid phase extraction. Since alkaline phosphatase (AP) is a metalloprotein requiring metal ions, namely Zn^{2+} and Mg^{2+}, in its active site, the NTA Superflow resin was charged with $Zn^{2+}$to retain the enzyme which would bind to the Zn^{2+}ions. To assess the efficiency of the AP enzyme binding to the charged NTA resin, several batch studies were carried out.

The diethanolamine assay was performed by adding a charged NTA resin suspension to a standard spectrophotometric cuvette; it was possible to observe the substrate degradation by the appearance of the coloured product. The colour intensity was higher in the NTA resin indicating a higher concentration of enzyme (fully functioning). This experiment showed that this method could be efficient to extract the enzyme. In order to minimize the used amount of solid phase material, a reusable approach was then tested, and the NTA resin packed into a micro column. Thus, the diethanolamine assay was adapted to be carried out with a NTA resin column (CR_{NTA}) by passing through the solutions using a syringe. The first solution to pass through the CR_{NTA} was the enzyme solution, followed by water to remove the excess (any enzyme not retained). Then, the diethanolamine buffer was passed through the CR_{NTA} followed by the substrate. The collected effluent presented a yellow colour indicating that the substrate had been degraded inside the column. This proved that the enzyme was in fact retained in the charged NTA resin and it was functional, catalyzing the substrate *p*NPP to the product *p*NP. Some water samples were then assessed but no absorbance increase was registered. In face of the obtained results, the sample volume of 0.1 mL, suggested in the diethanolamine assay, was replaced by a 2.5 mL of water sample. With that volume, it became possible to observe the coloured product formed by the enzyme catalytic activity. Therefore, this was the sample volume set for further studies.

3.2. Sequential injection system

The preliminary studies confirmed the efficiency of pre-concentration/immobilization of AP in the charged NTA resin packed in a column (CR_{NTA}). So, a sequential injection system for the APA assessment with automatic inline pre-concentration/immobilization of the enzyme was developed (Fig. 2).

3.2.1. Physical parameters

3.2.1.1. Reagents volumes. To set the preliminary conditions for the flow system, the batch diethanolamine assay was reproduced in the SI system with a 5 fold volume reduction and maintaining the reagents relative proportions. As previously reported by Mesquita and Rangel [29], the minimal volume to be used for a SI method (with a peristaltic pump propulsion system) with a good repeatability is about 20 μL. With the 5 fold volume reduction, the enzyme activity standard volume is reduced to 22 μL, and the volume of substrate (*p*NPP) to 63 μL, so these were the volumes set. The volume of the coloured product formed (catalysed substrate) was set to 66 μL. This volume was chosen to be slightly above the volume of substrate sent to the NTA resin column in order to ensure that all formed product and/or not catalysed substrate was removed from the column.

The volume of diethanolamine buffer (DB_{SI}) was not evaluated independently but was ensured that the NTA resin was buffered before and after the substrate catalysis. Therefore, the aspirated volumes from the column were always higher than the ones sent to the NTA resin to ensure that the resin was kept buffered.

3.2.1.2. Sample volume. The volume of water sample used for the batch procedure was 2.5 mL. Therefore, according to the 5 fold volume reduction approach, a volume of 500 μL should be used in the SI system. However, a sample volume study was performed using an enzyme standard of 0.044 unit mL^{-1} and volumes in the range 250–630 μL. Although no significant differences were observed in the absorbance values, the chosen volume of 500 μL presented a slight increase compared to lower volumes and no further increase was observed for higher volumes.

3.2.1.3. Enzyme volume. The previously set enzyme volume of 22 μL was effective for all the optimization studies enabling an enzyme assessment above 0.1 units cm^{-1}. However, for APA determination in water samples, lower concentrations were required. In order to wide up the quantification range to lower levels and to establish the same operational conditions for sample and standards, the enzyme standard volume was changed from a volume of 22 μL to a volume of 500 μL.

3.2.2. Manifold configuration

Two configurations for connecting the CR_{NTA} to the selection valve were studied: in a lateral port of the selection valve (Fig. 2) or in the pathway to the detector.

The column in the way to the detector would result in a smaller dilution of the formed product, sent directly to the detector without being aspirated into the holding coil. However, under such set-up, the excess of enzyme standard and sample would also be sent to the detector. Furthermore the buffering of the resin (both prior and after the assay) and the subsequent washing between samples would be sent through the detector requiring additional and longer protocol steps. Moreover, the flow of the solutions always in the same direction could result in the resin compaction. With the CR_{NTA} in the lateral port, conditioning and washing the NTA resin would be facilitated, and solutions would flow in two directions. However, it would require aspirating the formed product before sending to the detector, resulting in a slightly higher dilution. So, the diethanolamine assay was reproduced in the SI system with both configurations to verify possible differences in sensitivity (Fig. 3).

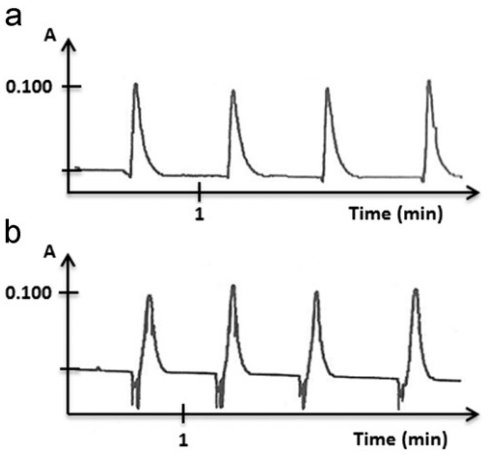

Fig. 3. Signal obtained for the APA assay in the SI system with two manifold configurations; (a), column positioned in a lateral port of the selection valve; (b), column positioned in the path to the detector.

For the same enzyme activity standard, a similar absorbance value was obtained for both configurations indicating similar sensitivity, and no effect of different dilution factors occurred. Together with the above mentioned advantages in conditioning and washing, the configuration with the column in the lateral port presented a lower schlieren signal (Fig. 3a), so it was the chosen configuration.

3.2.3. Elution study

The pre-concentration/immobilization approach for retaining the AP enzyme from the water samples required the elution of the packed resin between samples. To achieve such a requisite, an eluting buffer was used to remove the enzyme from the resin between samples and/or standards. In order to avoid interference in the determination, the enzyme was eluted in denaturing conditions at the end of the analytical cycle. The chosen elution buffer was "Buffer E" from the NTA handbook [9], and the denaturing effect was assessed with the conventional (batch) diethanolamine assay [28](ESI Fig. 2).

3.2.4. Interferences

According to Koncki et al. [17], some ions could inhibit alkaline phosphatase activity, and an interference study was performed for key ions present in water samples. The tested concentrations were based on the maximum levels allowed for natural waters. The percentage of interference was calculated by comparing the peak heights of two enzyme standards of 0.1 unit mL^{-1}: with and without. The studied ions (together with the respective reagents), the tested concentrations, and the interference percentages are shown in Table 2.

The calculated percentages of interference were lower than 5% meaning that no significant interference was expected from different natural water samples.

3.3. APA calculation methods

The developed SI method enabled the assessment of alkaline phosphatase activity (APA) using two alternative calculation methods: enzyme calibration curve, Enz_{CC} (ESI Fig. 3), and product calibration curve, NP_{CC} (ESI Fig. 4). The calculation procedures,

explained in detail in the materials and methods section, were used for APA determination in different water samples (ESI Table 1).

The results show similar values for APA obtained by the two calculation methods, relative deviations below 1%. The results of both methods were plotted and a linear relationship was established: $NP_{cc}=1.012\ (\pm 0.028)\ Enz_{cc}-0.001\ (\pm 0.003)$, being the values in parenthesis 95% confidence limits. These figures show that the estimated slope and intercept did not differ statistically from values 1 and 0, respectively. Therefore, there was no evidence for systematic differences between the two sets of results [30] so both calculation methods could be used for APA determination. Despite being an indirect calculation, the NP_{CC} calculation method allowed the determination of APA without a daily enzyme calibration curve. Due to the established linear relation between AP and *p*NP concentrations, only the product calibration curve was necessary to perform the APA determination. This feature enabled to decrease the consumption of the enzyme solution, making this approach less expensive. However, it was essential to evaluate the stability of the product standards.

3.3.1. Study of the pNP standards stability

Product standards in the linear dynamic range of 19–280 μmol L^{-1} *p*NP were prepared and used for two months. The calibration curve was performed in the day of the *p*NP standards preparation, as well as one and two months later. The sensitivity obtained with the calibration curve after 2 months (1.28 L mol^{-1}) was about half of the sensitivity obtained for the first calibration curve (2.36 L mol^{-1}). Nevertheless, after one month the decrease in sensitivity was only about 10%. Therefore, the *p*NP standards could be used for one month without appreciable decrease in sensitivity.

3.4. Features of the developed system

The features of the developed system are demonstrated in Table 3. The LOD and LOQ in enzyme activity units were calculated as three and ten times, respectively, the standard deviation of the mean intercept of two calibration curves, according to IUPAC recommendations [31,32]. The LOD and LOQ in product concentration were calculated as three and ten times the standard deviation of ten consecutive injections of deionised water, according to IUPAC recommendations [31,32].

The *p*NP calibration curve presented corresponds to the mean slope and intercept of three calibration curves in consecutive days with the respective standard deviation. The enzyme calibration curve corresponds to the mean slope and intercept of three calibration curves performed fortnightly with the respective standard deviation.

The determination rate was calculated based on the time spent per cycle. A complete analytical cycle took about 2.1 min. An analytical cycle is the sum of the time needed for each step plus the time necessary for the port selection in the selection valve.

With the developed methodology, the overall reagent consumption per determination was1.08 mg of *p*NPP, 60 mg of urea, 1.7 mg of potassium dihydrogen phosphate (KH_2PO_4), 2 mg of Tris–HCl, 3 μg of magnesium chloride hexahydrate ($MgCl_2.6H_2O$), and 3 μg of diethanolamine.

The effluent production per determination was 2.82 mL with a sample consumption of 501 μL, as mentioned above.

3.5. Application to different samples—dynamic water systems and roots

3.5.1. Recovery studies-different types of water samples

Since the initial enzyme concentrations in natural waters were below the detection limit, estuarine water samples were spiked with1.2 mL of enzyme stock solution (0.441 unit mL^{-1}) in 5 mL of sample, yielding a final AP activity of 0.100 unit mL^{-1}. The calculation of the recovery percentage was made according to IUPAC recommendation [33] and the results obtained are presented in Table 4.

The developed SI methodology provided recovery ratios with an average of 101% (standard deviation 8.3), and a statistical test (*t*-test) was used to evaluate if it did not significantly differ from 100% [30]. For a 95% significance level, the calculated *t*-value was 0.336 with a correspondent critical value 2.969, thus indicating the absence of multiplicative matrix interference.

Table 2
Potential interfering species and respective percentage of interference.

Tested ion	Prepared from the reagent	Concentration mg L^{-1}	Interference (%)
Cl^-	NaCl	70	−3.3
Ca^{2+}	$CaCO_3$	100	−0.3
Mg^{2+}	$MgN_2O_6\cdot 6H_2O$	50	1.8
K^+	K 1000 mg L^{-1}	12	0.8
Na^+	Na 1000 mg L^{-1}	50	1.6
Al^{3+}	Al 1000 mg L^{-1}	20	−4.4
Fe^{3+}	Fe 1000 mg L^{-1}	0.20	1.3

Table 4
Application of the sequential injection system for the alkaline phosphatase activity determination in spiked estuarine water samples and respective recovery studies.

Sample type	Sample ID	Added conc. (unit mL^{-1})	Found			Recovery (%)
			Conc. (unit mL^{-1})	SD	RSD (%)	
Estuarine water	E 1	0.100	0.116	0.002	1.4	116
River water	R 1	0.100	0.102	0.005	4.8	102
	R 2	0.100	0.105	0.002	1.6	105
	R 3	0.100	0.097	0.000	0.0	97
	R 4	0.100	0.097	0.006	6.7	97
Well water	W 1	0.100	0.090	0.006	7.2	90
Interstitial water	I 1	0.100	0.102	0.037	36.6	102

Table 3
Features of the developed SI method for alkaline phosphatase activity in water samples.

Dynamic range	Calibration curve[a]	LOD	LOQ	Determination rate (h^{-1})
18.5–280 μmol L^{-1} (*p*NP) 0.0441–0.441 unit mL^{-1} (APA)	$A=2.09\times10^{-3}\ (\pm 2\times10^{-5})$ μmol L^{-1} $pNP^+ 5.0\times10^{-2}\ (\pm 1.0\times10^{-2})$ $A=0.296\ (\pm 0.025)$ unit APA $mL^{-1}+0.069\ (\pm 0.033)$	1.9 μmol L^{-1} 0.008 unit mL^{-1}	4.5 μmol L^{-1} 0.026 unit mL^{-1}	17

[a] Values in parenthesis correspond to the standard deviation of the equation parameters $n=3$.

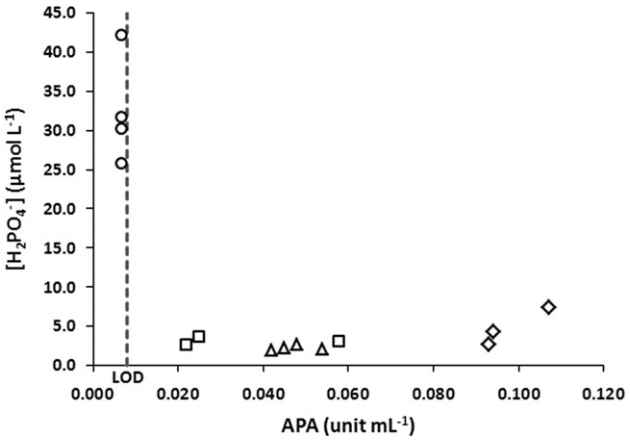

Fig. 4. Relationship between the concentration of phosphate ion and the alkaline phosphate activity in different water samples: ◇, interstitial water; □, estuarine water; △, river water; ○, well water; dashed line indicates the APA limit of detection (LOD).

3.5.2. Dynamic water systems characterization: Phosphate/alkaline phosphatase relationship

After the validation of the developed SI system for APA determination, the possibility of a relationship between the phosphate level and the APA was tested. Thus, a combined determination of APA and phosphate was carried out for different water samples (ESI Table 2). Several analysed water samples, namely river and estuarine water, presented APA values below the LOD, and were not included (ESI Table 2). An exception was made for well water. For this type of water, all samples presented APA values lower than LOD but it was interesting to observe the relationship with the phosphate concentration. The phosphate determination was previously reported by Mesquita et al. [27] and the necessary reagents were accommodated in the same manifold. The results obtained were plotted (Fig. 4) to assess the possible relationship between the two parameters.

The observation of the plotted results shows evidence of a possible relationship between the type of sample with the concentrations of phosphate and the APA value. In fact, all well water samples, with the APA values below LOD, presented high concentration of phosphate. Accordingly, low values of APA meant that as phosphate was available at higher concentrations, no degradation of organic compounds was needed. In samples with low phosphate concentration, APA values were higher, which could mean that phosphate was liberated from organic compounds in order to increase its availability.

3.5.3. Plant root samples

Due to the environmental relationship between interstitial water, plant roots and microorganisms present in the rhizosphere, determination of APA in plant roots samples was also carried on as mentioned above. After the preparation of the root samples, APA was assessed in different types of plant roots with the developed SI methodology (ESI Table 3). The results obtained showed, as expected, that the APA values in plant roots were significantly higher than in natural water samples. With the developed SI method, alkaline phosphatase activity was effectively determined in all root plant samples.

4. Conclusions

The developed SI method allowed the determination of alkaline phosphatase activity in different types of water samples and plant roots. The determination was accomplished by connecting a NTA resin column to a sequential injection system in order to perform enzyme pre-concentration. When compared to the batch diethanolamine assay, the incubation time presents a 25 fold reduction from 5 min to only 0.2 min with an overall determination rate of 17 h^{-1}. Furthermore, comparing with conventional determinations, the developed SI methodology represents not only a time consuming reduction but also a less laborious alternative. Additionally, the use of a flow analysis approach results in an important reagent consumption, a very important feature when enzymatic analysis is involved.

As far as we know, the described work is the first to use sequential injection analysis technique for the alkaline phosphatase activity determination. All previously reported flow methods for APA determination (ESI Table 4) used flow injection analysis (FIA), and among them only one aimed for water samples (sea water) [15]. When compared to the FIA methods, the described SI methodology presents a considerable decrease in the incubation time and a much higher determination rate together with an important reagent saving.

The use of NTA resin charged with Zn^{2+} ions proved to be an effective solution for pre-concentration of AP enzyme from natural water samples enabling the determination for low values without increasing incubation time. The enzyme was indirectly retained by binding to the Zn^{2+} ions needed in its active site for catalysis. The use of NTA resin for AP pre-concentration in a flow system was also described for first time, as far as we know.

Finally, the described SI methodology for APA determination could be combined with a previously reported SI methodology for phosphate determination [27] in the same manifold enabling to establish a relationship between APA and inorganic phosphate water samples.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.06.071.

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