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Escola Superior de Biotecnologia

SEQUENTIAL INJECTION SYSTEM FOR THE DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY IN NATURAL WATERS AND PLANT ROOTS WITH IN-LINE SOLID PHASE EXTRACTION

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to achieve the Master of Science level in Microbiology

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Under supervision of

António O. S. S. Rangel

Raquel B. R. Mesquita

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Abstract

The present work describes the development of a sequential injection system for the determination of alkaline phosphatase activity, after solid phase extraction/preconcentration and also the determination of phosphate in natural waters and plant roots.

The determination of alkaline phosphatase activity was based on the sepctrophotometric detection of a colored product (*p*-nitrophenol), resulting from the catalysis of a non colored substrate (*p*-nitrophenyl phosphate). Due to the low levels of alkaline phosphatase in waters, it was preconcentrated in a NTA Superflow resin charged with Zn^{2+} ions. This was possible due to the enzyme necessity for metals in its active site.

The phosphate determination was based on the spectrophotometric determination of a colored complex (molybdenum blue), resulting from the formation of the complex of orthophosphate with molybdate, followed by its reduction with ascorbic acid.

The proposed methodology allowed the determination of alkaline phosphatase activity within a range between 0.044 – 0.441 unit cm^{-3} and 19 – 280 μmol dm^{-3} *p*NP. A determination rate of 17 h^{-1} and detection limits of 0.025 unit cm^{-3} and 1.9 μmol dm^{-3} *p*NP were obtained.

Phosphate determination was also possible to perform within a range between 0.98 – 49.9 μmol dm^{-3} $H_2PO_4^-$, and with a detection limit of 0.29 μmol dm^{-3} $H_2PO_4^-$. A determination rate of 32 h^{-1} was obtained.

Resumo

No âmbito desta dissertação, desenvolveu-se uma metodologia de análise por injecção sequencial para a determinação da actividade da enzima fosfatase alcalina após pré-concentração e de fosfato em diferentes amostras de águas e raízes.

A determinação da actividade da enzima fosfatase alcalina foi realizada com base na detecção espectrofotométrica do produto formado (*p*-nitrofenol) após degradação enzimática do substrato *p*-nitrofenil fosfato. Devido aos seus baixos valores em águas, a enzima foi pré-concentrada numa resina (NTA Superflow) previamente carregada com iões Zn^{2+}. Esta imobilização foi conseguida através do facto de a enzima ser uma metaloproteína.

A determinação de fosfato foi realizada através da detecção espectrofotométrica do complexo azul de molibdénio. A reacção do azul de molibdénio ocorre pela formação de um complexo entre o fosfato inorgânico e o molibdato, seguida da redução pelo ácido ascórbico.

O sistema desenvolvido permitiu a determinação da actividade da enzima fosfatase alcalina num intervalo de concentrações compreendido entre 0.044 – 0.441 unit cm^{-3} e 19 – 280 µmol dm^{-3} *p*NP. Obtiveram-se os limites de detecção de 0.025 unit cm^{-3} e 1.9 µmol dm^{-3} *p*NP com um ritmo de determinação de 17 h^{-1}.

A determinação do anião fosfato foi também possível num intervalo de concentração de 0.98 – 49.9 µmol dm^{-3} $H_2PO_4^-$, com um limite de detecção de 0.29 µmol dm^{-3} $H_2PO_4^-$. O ritmo de determinação de fosfato foi de 32 h^{-1}.

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Index

Abbreviations

AP: Alkaline phosphatase

APA: Alkaline phosphatase activity

FIA: Flow injection analysis

IUPAC: International Union of Pure and Applied Chemistry

LED: Light-emitting diode

LOD: Limit of detection

LOQ: Limit of quantification

MCFIA: Multi commuted flow injection analysis

MPFS: Multi pumping flow systems

MRC: Multi-reflection cell

MSFIA: Multi syringe flow injection analysis

P: Phosphorus

*p*NPP: *p*-Nitrophenyl phosphate

*p*NP: *p*-Nitrophenol

SFA: Segmented flow analysis

SIA: Sequential injection analysis

SI-LOV: Sequential injection Lab-on-valve

SPE: Solid phase extraction

1.

Introduction

1.1. Phosphorus in the environment

Phosphorus (P) is an important nutrient required by every organism since it is part of nucleic acids (DNA and RNA) and phospholipids located in the membranes. It has an essential role in energy metabolism due to its presence in ATP, ADP, AMP, and PPi (pyrophosphate) (Koch et al., 2009; Sebastián et al., 2004). In soils, P is the most limiting nutrient that controls living processes in plants. It is required in large amounts and so it is considered a macronutrient.

P is present in natural waters and soils as two soluble sources, orthophosphates ($H_2PO_4^-$ and HPO_4^{2-}), which are named dissolved inorganic phosphate, and organic phosphorus compounds. The first one is readily available for assimilation by organisms such as bacteria, algae and plants, while the second one needs to be mineralized in order to be part of the soluble orthophosphate pool (Rees et al., 2009; Gambin et al., 1999). Orthophosphates are usually present at lower concentrations because of the limited solubility of various minerals containing phosphorus. The photosynthesis process also lowers the concentration because of the biological uptakes of phosphate. The occurrence of organic phosphates may be due to the breakdown of organic pesticides that contain this species in their composition (Gambin et al., 1999; Mhamdi et al., 2007). Organic phosphorus compounds (phosphate esters) include nucleic acids, phospholipids, inositol phosphates, phosphoamides, phosphoproteins, sugar phosphates, phosphorus containing pesticides and organic condensed phosphates (Yaqoob et al., 2005).

In agriculture, the utilization of fertilizers containing phosphate causes its increase in soils and consequently the increase of the amount that leaches to surface and interstitial waters. Elevated concentrations in waters can lead to eutrophication since phosphorus promotes a massive growth of algae and plants which will deplete the dissolved oxygen and kill fishes and other aquatic life.

Phosphorus levels in aquatic systems are influenced by mineralization of algae and the dissolution of phosphate minerals from anthropogenic point source changes of sewage and industrial effluents and from the inputs from agricultural land. In the end, total phosphorus concentrations in water varies from approximately 0.01 mg/L to 1.0 mg/L in polluted rivers (Yaqoob et al., 2005).

1.2. Alkaline phosphatase in the environment

1.2.1. Alkaline phosphatase in water

When the dissolved inorganic phosphate content in waters is depleted if compared to other nutrients, phytoplankton and bacteria have the ability to obtain P from dissolved organic phosphorus. This is due to the production of extracellular enzymes such as alkaline phosphatase (AP) that hydrolyze phosphate monoesters liberating inorganic phosphate and organic matter (Gambin et al., 1999; Ivančić et al., 2010; Boge et al., 2006). Alkaline phosphatase mineralizes organic phosphorus in order to recycle P and make it available for assimilation. In that order, AP is regulated by inorganic phosphate concentrations and internal P levels, which makes this enzyme an excellent indicator of P status (Chròst and Overbeck, 1987; Sebastián et al., 2004; Koch et al., 2009; Koike and Nagata, 1997; Labry et al., 2005).

1.2.2. Alkaline phosphatase in plants

As it has been previously mentioned, phosphorus is an essential nutrient. It is essential for plants and because of that it limits agricultural production. Many plants have developed several mechanisms to acquire phosphorus from soil, in order to adapt to P-deficiency. These mechanisms include modifications in root structure, formation of symbioses with mycorrhizae and production of root exsudates such as organic anions and enzymes (George et al., 2008). Since P is only available when present as orthophosphate, in P-depleted soils, plants and microbes have the ability to hydrolyze organic P compounds by the production of enzymes such as phosphatases. According to previous studies (Tarafdar and Jungk, 1987), this enzyme has been determined in the three components of the rhizosphere (soil, microorganisms and plant roots).

It has also been demonstrated that, when P is present at low levels, increased activity of phosphatase in plant roots and in rhizosphere is observed, along with a decline in organic phosphorus in soils. This gives evidence of a relationship between the enzyme phosphatase and soil and plant P-levels (Tarafdar and Jungk, 1987).

1.2.3. Alkaline phosphatase as a metalloprotein

Metals are usually present in nature as part of complexes with biomacromolecules. Complexes between metals and proteins (metalloproteins) have been studied and are known to perform a diversity of functions in biochemical processes such as catalytic, regulatory and others (Szpunar and Lobinski, 1999).

Alkaline phosphatase (EC 3.1.3.1), schematically represented in Fig. 1.1, is an homodimeric metalloprotein, containing one Mg^{2+} and two Zn^{2+} ions in the active site. Magnesium is an important structural stabilizer of the enzyme, while zinc ions are directly involved in catalysis. One of the two zinc ions is necessary for catalysis and is important in binding both the substrate and phosphate (Bortolato et al., 1999).

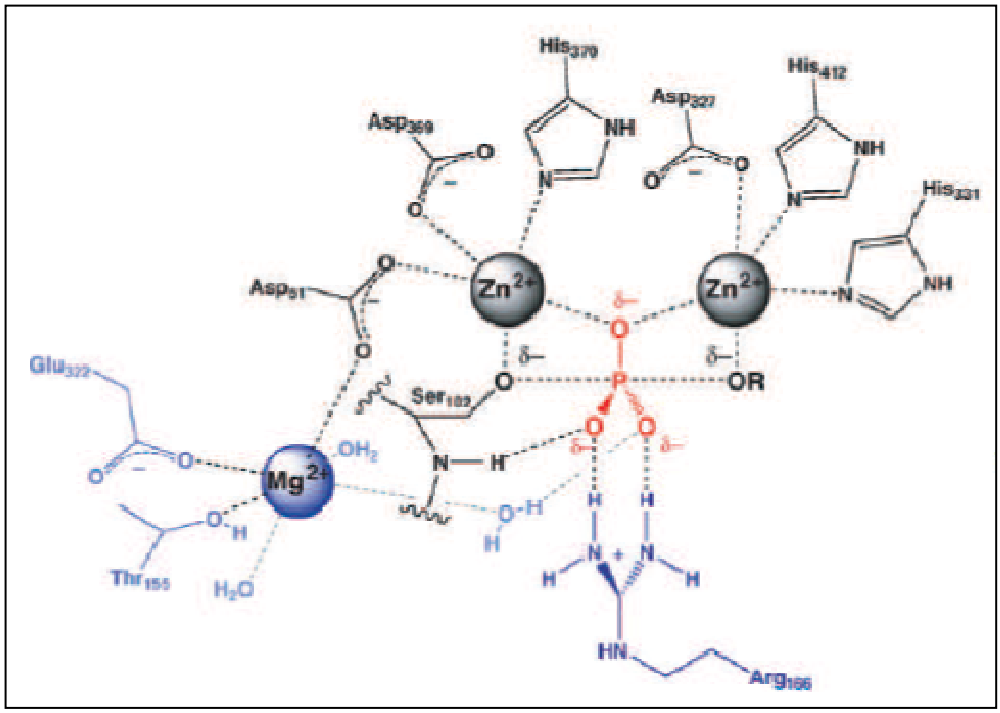


Figure 1.1. E. coli alkaline phosphatase active site. Functional groups unique to AP are shown in blue and conserved functional groups are shown in black (Zalatan et al., 2008).

1.2.4. Determination of alkaline phosphatase activity

The determination of alkaline phosphatase activity is typically achieved by incubating the enzyme with its substrate and measuring the final product. This procedure is usually time consuming due to long periods of incubation and requires sample pretreatment that is a laborious process.

In that way, flow analysis appears as an interesting alternative for routine analysis since it is possible to automate the analytical procedure. In this way, faster analysis is possible and also the determination is less susceptible to human errors.

1.3. Flow analysis

In the late fifties, a new concept of flow analysis emerged with the aim of automating chemical analysis. This concept has been exploited along the years and presents significant advantages for routine analysis. When compared to conventional batch analysis, flow systems allow a faster, real-time and automatic determination. It also diminishes sample and reagent consumption with low effluent production.

1.3.1. Brief history

Flow analysis started with segmented flow analysis (SFA), which consists in the use of an air flow to create air bubbles between reagent and sample plugs in order to promote the mixture (Skeggs, 1957). With this flow system, the reaction between sample and reagent (chemical equilibrium) is complete as well as the mixture between them (physical equilibrium). In the middle seventies, a new flow analysis concept, flow injection analysis (FIA) was proposed by Růžička and Hansen (1975) and is presented in Fig. 1.2.

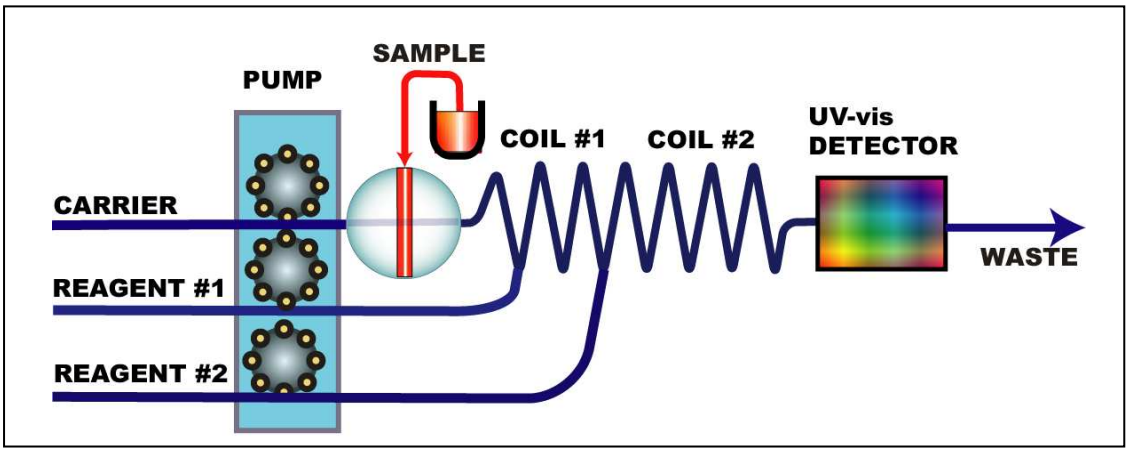


Figure 1.2. Manifold of a flow injection analysis system (Růžička, 2009).

This technique, compared to the previous one, is based on a non-segmented flow stream where the sample is injected into the flow stream by means of an injection valve. Chemical and physical equilibrium are not usually achieved, and because of that a transient signal is obtained. These characteristics in flow analysis have advantages such as speed of analysis (since it is not necessary to wait for the chemical and physical equilibrium), easy implementation and low cost. In spite of these advantages, the relatively high reagent consumption and effluent production are two main limitations of FIA.

1.3.2. Sequential injection systems

In 1990, sequential injection analysis (SIA), proposed by Růžička and Marshal (1990), emerged as an alternative to FIA in order to overcome its disadvantages. The common SIA manifold is presented in Fig. 1.3. This flow technique consists of the sequential aspiration of sample and reagents through a selection valve towards a holding coil, which by flow reversal are directed to the detector. In this concept, the mixture is obtained by a change in flow direction, while in FIA the mixture is promoted by the use of confluences.

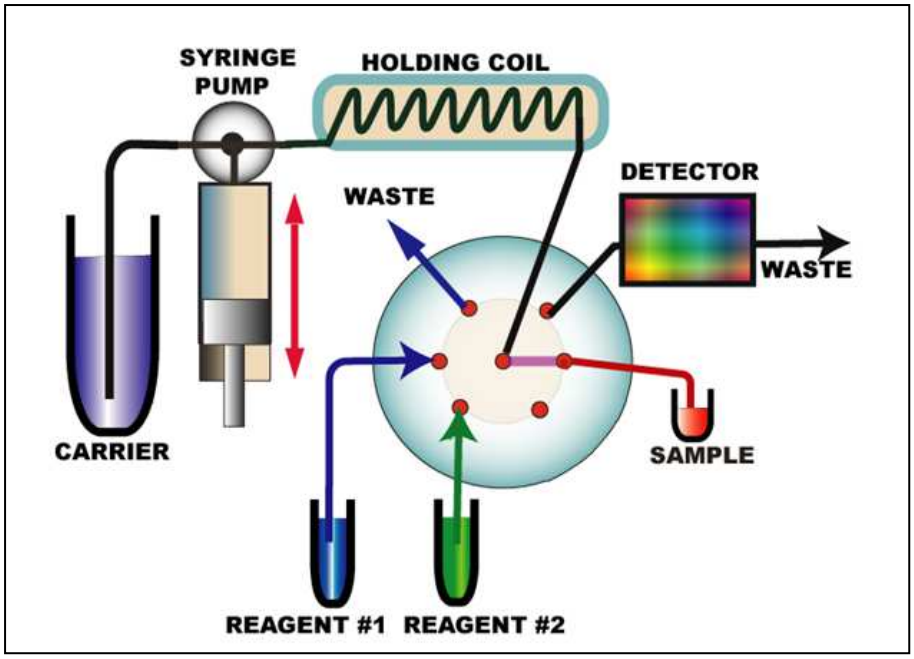


Figure 1.3. Manifold of a sequential injection analysis system (adapted from Růžička, 2009).

This technique overcomes some limitations presented by FIA allowing reagent saving and low effluent production. There is no need of physical reconfiguration for multiparametric determinations. In FIA, to perform several determinations it is usually necessary to change the physical configuration while in SIA it is possible to place different reagents on the port of the selection valve. Because of that, this system is also versatile since it is possible to perform several determinations using the same manifold. With SIA it is also possible to couple several devices that perform in-line sample pretreatments such as dialysis units (separation/dilution), resins (separation/preconcentration) and others (Mesquita and Rangel, 2009).

A new related technique was subsequently developed to further improve and optimize the SIA concept. The micro sequential injection lab-on-valve (SI-LOV) proposed by Růžička (2000) allowed to compact the SIA system. This new technique is based on the sequential injection concept but it incorporates the detection system in the selection valve which allows the use of volumes in the micro scale. This down scale makes possible to have even lower values of reagent and sample consumption and effluent production.

1.3.3. Other flow techniques

After flow injection analysis, several other techniques have been developed. These techniques, with the same concept of flow injection, vary mainly in terms of equipment. This different equipment brings new ways for inserting sample and reagents and for sending to the detector.

Multi commuted flow injection analysis (or sometimes named multi commutation), firstly described by Malcolme-Lawes et al. (1987), uses solenoid valves (with two positions) in place of the injection valve. These solenoid valves can introduce the reagent and sample independently (one valve each) or can introduce both sample and reagent with the same valve.

Multi syringe flow injection analysis (MSFIA) described by Cerdà et al. (1999) couples solenoid valves with syringe pumps, moved by a common piston. The equipment of propulsion in multi syringe increases the robustness of the methodology when compared to flow injection and multi commutation.

In multi pumping flow systems (MPFS), described by Lapa et al. (2002), solenoid micro pumps do both the propulsion and insertion of sample and reagents, with no need for valves. Since micro solenoid pumps perform the propulsion, a down scale of the system is achieved and because of that it is possible to have a portable system. In spite of that, due to its small size, the system has a lack of robustness. Also, the necessity of a solenoid pump for every reagent and sample makes necessary the existence of a manifold per determination.

1.3.3.1. Liquid-solid extraction: solid phase extraction

Solid phase extraction (SPE) is characterized by the separation of an analyte from its matrix due to its affinity to a solid support (Matsuoka and Yoshimura, 2010).

The application of SPE techniques to FIA, SIA, and SI-LOV allows to perform sample-pretreatment processes such as sample clean-up, analyte preconcentration, and removal of matrices and/or interfering substances. SPE together with flow analysis systems improves its simplicity, accuracy, reproducibility, being easier to automate. Flow analysis techniques coupled with SPE have many advantages when compared to similar conventional batch determinations such as higher sample throughput, reduced reagent consumption and waste production, reduced sample contamination, lower LOD and/ or LOQ, automation by programmed control, and hyphenation with many kinds of detectors. The application of SPE also eliminates the need for laborious and time consuming sample pretreatment (Motomizu and Sakai, 2008).

In this work, a Nitrilotriacetic Acid (NTA) Superflow resin was used. NTA is an aminopolycarboxylic acid that can sequester all metal ions when present in the fully deprotonated form (Anderegg, 1982). Because of that, this resin is known by its application in metals preconcentration (Lohan et al., 2005). Since AP is a metalloprotein that needs metal ions (Zn^{2+} and Mg^{2+}) in its active site, this resin was used for the enzyme immobilization by previously complex zinc ions to the NTA resin.

1.4. Flow analysis techniques and phosphorus environmental analysis

An option was made to describe phosphorus analysis previously to alkaline phosphatase activity analysis due to its importance and influence in the enzyme activity. Firstly, it is necessary to determine the water phosphate pool in order to relate with alkaline phosphatase activity.

Water analysis is very important, being complex for phosphorus determination since it is present in many different forms of different organic and inorganic compounds. As it was previously explained, orthophosphate, the dominant species, when present in excess, is responsible for water eutrophication. Because of that, orthophosphate monitoring is of great importance as an indicator of contamination, in order to understand the causes and to develop a better water management. Total phosphorus determination is also of great interest since it represents the maximum amount of bioavailable phosphorus (Spivakov et al., 1999).

Phosphorus in waters can be part of organic and inorganic compounds that can be present as dissolved, colloidal or particulate fraction and further characterization is presented in Fig. 1.4 (Worsfold et al., 2005).

Phosphorus can also be divided in filterable, when it passes through a 0.22 μm filter, and non filterable (particulate phosphorus), when it does not pass through a 0.22 μm filter (Spivakov et al., 1999).

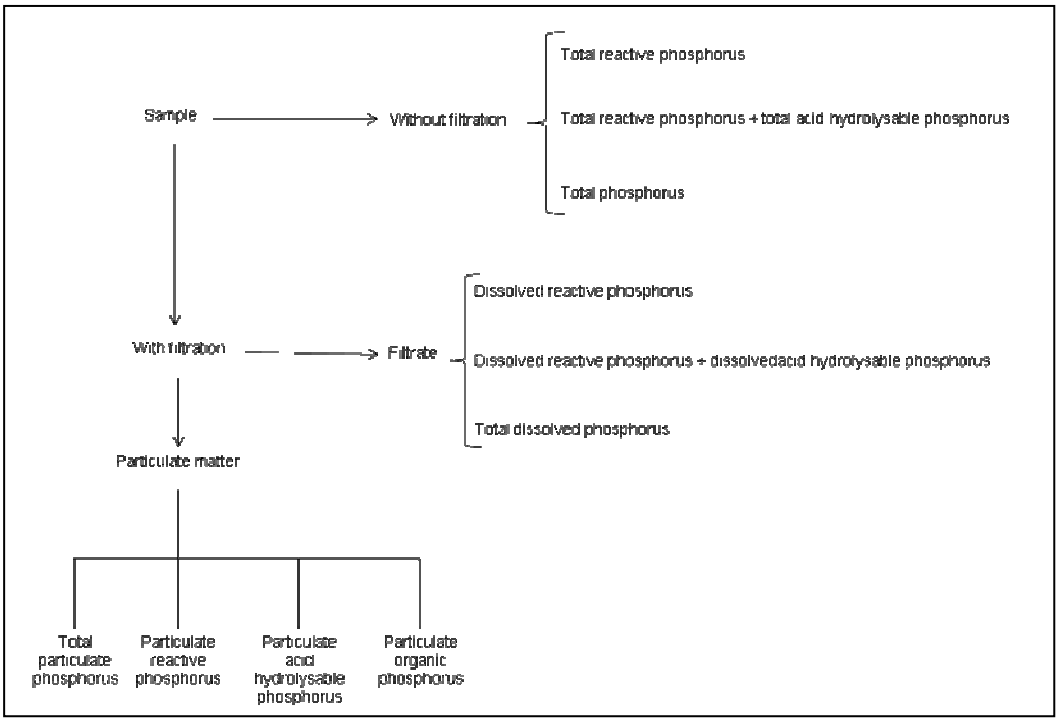


Figure 1.4. Analytical scheme for phosphorus species determination in water (adapted from Worsfold et al., 2005).

Many works have already been developed for phosphate determination in different samples using flow analysis. Furthermore, several reviews (Motomizu and Li, 2005; Morais et al., 2005; Maher and Woo, 1998) about orthophosphates determination in waters have been reported, clearly indicating the importance of phosphate monitoring in water samples. A review of the works developed for phosphate determination using flow analysis was done and is presented in Table 1.1. This review only considered the studies performed since year 2005 because the review from Motomizu and Li (2005) already describes the ones developed until that year.

Table 1.1. Flow systems applied to phosphate determination in different samples

Methodology	P fraction	Sample	Detection	Reagent(s)	Dynamic range	RSD	LOD	Determination rate	Reference
FIA	Phosphate	Freshwater	Chemiluminescence	Molybdate, lucinegin	Up to 500 µg dm^{-3}	1.3 – 3.2%	0.95 µg dm^{-3}	120	Rehman et al., 2010
FIA	Total phosphorus	Estuarine and marine waters	Spectrophotometric	Molybdate, tin(II) chloride	0 – 200 µg P dm^{-3}	4.6%	1 µg P dm^{-3}	115	Gentle et al., 2010
AMFA	Phosphate	River water	Spectrophotometric	Molybdate, malachite green	n. g.	n. g.	0.17 µmol dm^{-3}	n. g.	Uemura et al., 2010
MCFIA	Orthophosphate	River and tapwater	Photometric	Molybdate, tin(II) chloride	10 – 800 µg PO_4^{3-} dm^{-3}	2%	2.4 µg PO_4^{3-} dm^{-3}	20	Silva et al., 2009
FIA	Available phosphorus	Soils	Amperometric	Molybdate	0.1 – 10 mg dm^{-3}	0.5%	0.02 mg dm^{-3}	35	Jakmunee and Junsomboon, 2009
FIA	Reactive phosphate	Seawater	Photometric	Molybdate, tin(II) chloride	10 – 100 µg P dm^{-3}	0.66%	2 µg P dm^{-3}	n. g.	Ellis et al., 2009
Reverse FIA	Soluble reactive phosphorus	Seawater	Spectrophotometric	Molybdate, ascorbic acid	0 – 165 nmol dm^{-3}	1.54, 1.86%	0.5 nmol dm^{-3}	15	Ma et al., 2009
FIA	DOP, DRP, EHP	Porewater	Spectrophotometric	Molybdate, tin(II) chloride	n. g.	<3, <6%	0.2, 1.9 µg P dm^{-3}	n. g.	Monbet et al., 2009
FIA	Dissolved reactive phosphorus	Surface and groundwaters	Spectrophotometric	Molybdate, vanadate	Up to 500 µg dm^{-3}	2.2%	17 µg dm^{-3}	60	Neves et al., 2008
SIA	Soluble reactive phosphorus	Seawater	Spectrophotometric	Molybdate, ascorbic acid	3.4 – 1134 nmol dm^{-3}	2.50%	1.4 nmol dm^{-3}	6 - 10	Ma et al., 2008
FIA	Reactive phosphorus	Soil extracts	Spectrophotometric	Molybdate, ascorbic acid	Up to 2 mg dm^{-3}	1.9 – 4%	6 – 26 µg dm^{-3}	72	Hurtado et al., 2008
FIA	Phosphate	Natural and wastewater	Spectrophotometric	Molybdate, ascorbic acid	0 – 2 mg dm^{-3}	3.6%	0.15 mg dm^{-3}	n. g.	Tsoulfanidis et al., 2008

DOP, dissolved organic phosphorus; DRP, dissolved reactive phosphorus; EHP, enzymatically hydrolysable phosphorus.
n. g. – not given.

Table 1.1. Flow systems applied to phosphate determination in different samples (cont.)

Methodology	P fraction	Sample	Detection	Reagent(s)	Dynamic range	RSD	LOD	Determination rate	Reference
SIA	Phosphate	Coastal waters	Fluorimetric	Molybdate, rhodamine	n. g.	n. g.	0.05 µmol dm^{-3}	100	Frank and Schroeder, 2007
FIA	Orthophosphate	Seawater	Spectrophotometric	Molybdate, ascorbic acid	3.2 – 48.5 nmol dm^{-3}	4.52%	1.57 nmol dm^{-3}	n. g.	Liang et al., 2007
FIA	DOP, DRP, TDP, EHP	Porewater, sewage liquors	Spectrophotometric	Molybdate, tin(II) chloride	n. g.	<3%	0.2 µg P dm^{-3}	n. g.	Monbet et al., 2007
SIA	Reactive phosphate	Coastal waters	Fluorimetric	Molybdate, rhodamine	n. g.	n. g.	0.05 µmol dm^{-3}	270	Frank et al., 2006a
FIA	Soluble reactive phosphorus	Soil extracts	Spectrophotometric	Molybdate, tin(II) chloride	Up to 20 mg P dm^{-3}	n. g.	0.02 mg P dm^{-3}	n. g.	Buanuam et al., 2006
SIA	Reactive phosphate	River and marine waters	Fluorimetric	Molybdate, rhodamine	n. g.	n. g.	0.3 µmol dm^{-3}	120	Frank et al., 2006b
AIA	Phosphorus	Soils and sediments extracts	Spectrophotometric	Molybdate, tin(II) chloride	0.0 – 1.0 mg P dm^{-3}	0.16 – 0.36%	0.02, 0.04 mg P dm^{-3}	72	Amornthammarong et al., 2005
FIA	Phosphate	Freshwaters	Chemiluminescence	Luminol, H_2O_2	2 – 10x10^{-6} mol dm^{-3}	1.8 – 3.8%	2x10^{-7} mol dm^{-3}	60	Yaqoob et al., 2005
FIA	DRP, DOP	Natural waters	Spectrophotometric	Molybdate, tin(II) chloride	0.01 – 6 mg P dm^{-3}	0.3, 1.0%	0.01 mg P dm^{-3}	22	Tue-Ngeun et al., 2005
FIA	DRP	Fresh and marine waters	Amperometric	Molybdate	10 – 1000 µg P dm^{-3}	5.2, 5.9, 22, 1.0%	3.4, 0.18 µg P dm^{-3}	70	Udnan et al., 2005
MSFIA	Available phosphorus	Soil extracts	Spectrophotometric	Molybdate, ascorbic acid	0.75 – 15 mg dm^{-3}	<1.7%	0.30 mg dm^{-3}	15	Almeida et al., 2005

DOP, dissolved organic phosphorus; DRP, dissolved reactive phosphorus; TDP, total dissolved phosphorus, EHP, enzymatically hydrolysable phosphorus.
n. g. – not given.

As it is possible to observe in Table 1.1, most methods use spectrophotometric detection based on the reaction of orthophosphate with molybdate in acidic medium. After formation of the complex between phosphate and molybdate (molybdenum yellow) its reduction is performed by a reducing agent such as vanadate, ascorbic acid, stannous chloride, and malachite green resulting in a product that has its maximum absorption wavelength between 650 – 850 nm, depending on the agent used (Motomizu and Li, 2005; O'Toole et al., 2007).

The analytical method based on the formation of the molybdenum yellow prior to its reduction is based on the following reactions:

$$H_3PO_4 + 12\ H_2Mo_{12}O_4 \rightarrow H_3P(Mo_{12}O_{40}) + 12\ H_2O$$

$$Mo(VI) \xrightarrow{\text{Reducing agent}} Mo(V)$$ (van Staden and Taljaard, 1998)

Other methods based on the chemiluminescence, fluorimetric and amperometric detection are also used.

Sixty percent of the works presented use flow injection systems for phosphate determination and water is the most analysed sample.

1.4.1. Schlieren effect in flow systems

Schlieren effect (SE) results from the deflection of the light beam which alters the intensity of the transmitted light beam. The SE is usually caused by the concentration gradient if refractive indices (RI) of carrier, sample and reagents are significantly different. The interface between these solutions can produce optical lenses and so a signal resulting of light deflection is registered, schlieren effect. If this signal is concomitant with the one to be measured at the same wavelength, erratic results are obtained, with more evident effects at low analyte concentrations. This problem is of great importance in SIA systems since no confluence points are used to improve the mixing (Dias et al., 2006).

In FIA and SIA photometric detection, the schlieren effect can affect the accuracy, precision and limit of detection. Two types of schlieren effect that can occur in flow analysis systems have been previously identified by Dias et al. (2006). The first effect, which is reproducible, occurs when RI of an injected liquid differs from that of the recipient liquid. Depending on the relative magnitudes of the sample and carrier refractive indices, this has the effect of dispersing or focusing light rays from the source either towards or away from the detector, giving rise to artifact or schlieren peaks. The second effect is due to variations in the refractive indices of elements of sample and carrier/reagents that present differences in temperature, viscosity and concentration. This effect can also be influenced by pump pulsation and incomplete mixing of the plugs.

In order to prevent or minimize the schlieren effect, detector optics have been designed that are capable of tolerating changes in the refractive index. Alternatively, carrier and reagent streams can be matched by adjusting viscosity, salinity as well as improving the mixing between plugs. In spite of that,

for samples with a highly variable matrix like estuarine waters, this improvement is not enough. New ways of reducing schlieren effect have to be used. A more efficient alternative is to direct the light transversely across the stream of the flowing liquid. The use of dual-wavelength detection is also an alternative. This approach is done by using a detection wavelength at the maximum absorbance of the chromophore and a second where only the schlieren signal occurs. This allows to select the correct wavelength according to optimum schlieren compensation capabilities.

Liquid-core waveguide cells allow the use of increased optical pathlength since light is propagated by total internal refraction. These type of cells tend to exhibit high sensitivity due to the increased physical length. After that, multi-reflection cells described by Ellis et al. (2009) are a good compromise between enhanced sensitivity and schlieren effect tolerance. This is due to the introduction of light across the flow. The light is introduced across the flow, and the angle of incidence of the light beams introduced in this manner is much more acute (Frenzel and Mckelvie, 2008).

In this work a multi-reflection cell was used to minimize the Schlieren effect in the phosphate determination.

1.5. Flow analysis techniques and alkaline phosphatase activity determination

The use of enzymatic reactions in flow analysis systems has been employed in order to facilitate routine biochemical analyses and applications in biocatalysis. The application of FIA and SIA to perform enzymatic assays is an excellent option since these techniques allow a low sample and reagent consumption and so reduced analysis costs, ease of operation and fast analysis are possible, which are all requirements for bioassays and usually difficult to obtain with traditional techniques (Silvestre et al., 2011).

Several articles already described the alkaline phosphatase activity (APA) determination (Koch et al., 2009; Hartwell et al., 2007; Lespilette et al., 2007; Koncki, et al., 2006; Ivančić et al., 2010; Boge et al., 2006; Koike and Nagata, 1997; Martinez and Azam, 1993; Hoppe, 1983; Perry, 1972). APA can be determined using substrates specific for this enzyme such as methyl-umbelliferyl phosphate, MUF-P, *p*-nitrophenyl phosphate, *p*NPP and monofluorophosphate, MFP, that originate products possible to measure with different detection methods (fluorescence, spectrophotometric, potentiometric and amperometric). The traditional techniques employed are laborious and time consuming since they include a step of incubation that varies a lot, ranging from a minimum of 30 minutes to a maximum of 28 hours (Koike and Nagata, 1997; Hartwell et al., 2007; Ivančić et al., 2010; Boge et al., 2006; Martinez and Azam, 1993; Hoppe, 1983; Perry, 1972; Estela and Cerdà, 2005; Tzanavaras and Themelis, 2002; Chichester et al., 2008).

Flow analysis appears as an interesting solution to traditional techniques for alkaline phosphatase activity determination. As it was explained, it is possible to decrease the reagent and sample consumption and the assay time.

The works developed until year 2011 for alkaline phosphatase activity determination using flow analysis techniques are presented on Table 1.2.

Table 1.2. Flow analysis systems applied to alkaline phosphatase activity determination

Methodology	*Substrate*	*Product of catalysis*	*Sample*	*Detection*	*Dynamic range*	*LOD*	*RSD*	Determination rate (h^{-1})	Incubation time (min)	References
FIA	MFP	Fluoride	Serum	Potentiometric	n. g.	n. g.	n. g.	n. g.	n. g.	Rozum and Koncki, 2008
FI-BI	*p*-NPP	*p*-NP	Blood	Spectrophotometric	1 – 10,000 U dm^{-3}	1 U dm^{-3}	6, 5%	n. g.	5	Hartwell et al., 2007
FIA	MFP	Fluoride	Human serum	Potentiometric	n. g.	30 U dm^{-3}	n. g.	5 - 6	n. g.	Ogończyk and Koncki, 2007
FIA	dfMUF-P	MUF	Seawater	Fluorescence	0 – 2,000 nmol dm^{-3}	0.49 – 0.77 nmol dm^{-3}	n. g.	n. g.	5, 20	Gaas and Ammerman, 2007
FIA	*p*-NPP	*p*-NP	Human urine samples	Amperometric	$7.7x10^{-14}$ – $7.7x10^{-12}$ mol dm^{-3}	$5x10^{-14}$ mol dm^{-3}	4.5%	n. g.	30	Fanjul-Bolado et al., 2006
FIA	MFP	Fluoride	n. g.	Potentiometric	n. g.	<10 µg dm^{-3}	n. g.	8	n. g.	Koncki et al., 2006
FIA	CDP	-	n. g.	Chemiluminescence	$6x10^{-12}$ – $8x10^{-5}$ mol dm^{-3}	$6x10^{-6}$ mol dm^{-3}	n. g.	n. g.	Overnight	Zhang and Cass, 2006
FIA	3-indoxyl phosphate	Indigo	n. g.	Amperometric	1 – 25 U dm^{-3}	1 U dm^{-3}	1 - 9%	n. g.	65	Díaz-González et al., 2002
FIA	*p*-NPP	*p*-NP	n. g.	Amperometric	$1.4x10^{-15}$ – $1.4x10^{-11}$ mol dm^{-3}	$1.4x10^{-15}$ mol dm^{-3}	1.8%	n. g.	60	Ruan and Li, 2001
FIA	HNP	DHN	Sandwich immunoassay	Amperometric	n. g.	60 nmol dm^{-3}	n. g.	n. g.	20	Másson et al., 1999
FIA	*p*-nitrophenyl dihydrogen phosphate	H_2O_2	Serum samples	Amperometric	0.010 – 3 mmol dm^{-3}	0.0050 mmol dm^{-3}	1.9%	n. g.	n. g.	Raba and Mottola, 1994
FIA	Phenyl phosphate	Phenol	Human serum	Electrochemical	40 – 500 U dm^{-3}	n. g.	5%	n. g.	6	Jackson et al., 1993

FI-BI, flow injection - bead injection; MFP, monofluorophosphate; *p*-NPP, *p*-nitrophenyl phosphate; dfMUF-P, 6,8-difluoro-4-methylumbelliferone-phosphate; CDP, disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.1]decan}-4-yl)-1-phenyl-phosphate; HNP, 4-hydroxy-naphthyl-1-phosphate; *p*-NP, *p*-nitrophenol; MUF, 6,8-difluoro-4-methylumbelliferone; DHN, dihydroxy naphthalene.
n. g. – not given.

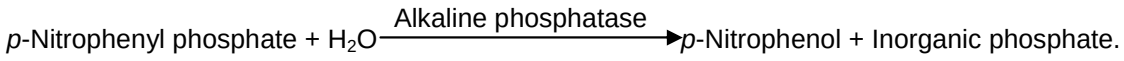
As it is possible to see in Table 1.2, all the works employed flow injection analysis. This is probably due to the easy operation of a FIA manifold.

Additionally, many of the works (Fanjul-Bolado et al., 2006; Díaz-González et al., 2002; Ruan and Li, 2001; Másson et al., 1999; Jackson et al., 1993) used flow analysis only for the final product measurement, and not for the whole process. In fact, the assay for APA determination, the incubation of the enzyme with its substrate, is done off-line, and the product formed is then determined by means of the flow system. In that way, in those works a long period of incubation is used and flow analysis does not bring many advantages for the APA conventional measurement.

In that way, SIA could be an interesting alternative to FIA for the enzyme activity determination since it is possible to downscale the enzymatic procedure and lower reagent and sample consumption and the assay time. Also, according to Mesquita and Rangel (2009), SIA presents a set of characteristics that are advantageous for water analysis.

It was also possible to see that few works use the spectrophotometric determination of the colored product *p*-nitrophenol. Most of them used the amperometric detection. In spite of that, spectrophotometry has been the most used for quantitative analysis of chemical components and it is interesting due to its simple and easy operation systems (Matsuoka and Yoshimura, 2010).

In this work the alkaline phosphatase activity was determined spectrophotometrically by measuring the colored product (*p*-nitrophenol), resultant from the catalysis of a non colored substrate (*p*-nitrophenyl phosphate) according to the following equation:



1.6. Objectives

The main objective of this thesis was to develop a sequential injection methodology for the determination of alkaline phosphatase activity in natural waters. Also the scope was extended to plant roots.

The activity of alkaline phosphatase was determined in different water samples with varied concentration ranges and matrices. The idea was to make this determination with no sample off-line prior treatments. Due to the AP low levels in waters and due to the complexity of sample matrices, a separation process would have to be implemented. Therefore, one of the objectives was to implement an in-line enzyme preconcentration system. This would be a significant contribution in this area, not extensively exploited in the literature. Another objective was to gain experience in the problematic of enzymatic analysis.

Another objective was to optimize a previous SIA methodology developed for the phosphate determination. In this context, a multi-reflective cell (MRC), especially designed to minimize the schlieren effect in high salinity samples, coupled to a light emission diode (LED) as light source was used as detection system.

The final purpose was to relate phosphate concentrations with alkaline phosphatase activity.

2.

Materials and Methods

2.1. Reagents and solutions

Solutions were prepared with analytical grade chemicals and boiled deionized water (specific conductance less than 0.1 μS cm^{-1}).

A 1.0 mmol dm^{-3} diethanolamine buffer was daily prepared: 0.0026 g of magnesium chloride hexahydrate was dissolved in water and, after dissolution, 2.4 cm^3 of diethanolamine was added and, following homogenization, water was added to 25 cm^3. The pH was adjusted to 9.8 using a 4 mol dm^{-3} HCl solution. This solution was diluted 5 times.

The substrate, *p*-nitrophenyl phosphate, DiTris salt (30 mmol dm^{-3}) was also daily prepared for alkaline phosphatase activity determination by dissolving 0.0277 g in 2 cm^3 of deionized water.

Alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa was purchased from Sigma Aldrich. Enzyme dilutions (179.3 and 0.441 unit cm^{-3}) were prepared in 10 mmol dm^{-3} Tris HCl, pH 8.0, 2.5 mmol dm^{-3} $MgCl_2.6H_2O$, and 0.15 mmol dm^{-3} $ZnCl_2$ solution. Glycerol at 50% was included for long term storage at 2 – 8 ºC. Alkaline phosphatase working solution was a 0.2 unit cm^{-3} prepared in diethanolamine buffer.

AP standards (0.022 – 0.441 unit cm^{-3}) were prepared by appropriate dilution of the 0.441 unit cm^{-3} enzyme solution.

For the elution of the enzyme in denaturing conditions, buffer E (Ni-NTA Superflow Cartridge Handbook, 2007) was prepared by dissolution of 24 g of urea, 0.69 g of KH_2PO_4, and 0.79 g of Tris-HCl, in deionized water. After that, water was added to complete a volume of 50 cm^3. The pH was adjusted to 4.5 using a 4 mol dm^{-3} HCl solution. The final concentrations were of 8 mol dm^{-3} urea, 100 mmol dm^{-3} of KH_2PO_4 and 100 mmol dm^{-3} of Tris-HCl.

Buffer NPI-250 (Ni-NTA Superflow Cartridge Handbook, 2007) was used for preliminary studies. This buffer was prepared by dissolving 0.136 g of KH_2PO_4, 0.351 g of NaCl and 0.340 g of imidazole in deionized water. The volume was completed to 20 cm^3 with water. The pH was adjusted to 8.0 using NaOH. The final concentrations obtained were of 50 mmol dm^{-3} KH_2PO_4, 300 mmol dm^{-3} of NaCl and 250 mmol dm^{-3} of imidazole.

A stock solution of 560 μmol dm^{-3} *p*-nitrophenol, *p*NP, was prepared by dissolving 0.0156 g of the solid (*p*-nitrophenol) in 200 cm^3 of deionized water. Working standards (19 – 280 μmol dm^{-3}) were prepared by proper dilution of the stock solution.

The molybdate reagent was daily prepared by dissolving 0.32 g of ammonium heptamolybdate-tetra-hydrate (16 g dm^{-3}), 0.0020 g of potassium antimony (III) oxide tartrate hemihydrate (0.1 g dm^{-3}) and 0.15 g of tartaric acid (7.5 g dm^{-3}) in 3.9 cm^3 of 4 mol dm^{-3} sulphuric acid (0.78 mol dm^{-3}) and deionized water. After homogenization, water was added to 20.0 cm^3.

The 30 g dm^{-3} ascorbic acid solution was prepared by dissolving 0.60 g of ascorbic acid in 20.0 cm^3 of deionized water.

A phosphate stock solution (71.6 mg dm^{-3}) was prepared by dissolving 0.050 g of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) in 500 cm^3 of deionized water and stored in a refrigerator. A phosphate solution (10 mg dm^{-3}) was prepared by appropriate dilution of the stock solution. Working standards (5.54 – 49.9 μmol dm^{-3}) were prepared by appropriate dilution of the 10 mg dm^{-3} solution and stored in the refrigerator when not in use.

In order to prevent the deposition of the molybdenum blue in the reactor and in the flow cell walls, a 1 mol dm^{-3} NH_4Cl/NH_3 washing solution was used (Lima et al., 1997; Morais et al., 2003). This solution was prepared by dissolving 0.17 g of ammonium chloride in 0.75 cm^3 of commercial ammonia solution (d = 0.91, 25%). After homogenization, deionized water was added to 20.0 cm^3.

A 0.03 mmol dm^{-3} bromothymol blue (BTB) dye in a 0.01 mol dm^{-3} sodium tetraborate solution was used in preliminary studies.

2.2. Sample collection and preparation

Different water samples were collected and analyzed for the alkaline phosphatase activity and inorganic phosphate determination: pore waters, estuarine waters, river waters, well waters and sea waters. Water samples were directly introduced in the system.

Root samples (Fig. 2.1) were obtained from different plants for the alkaline phosphatase activity determination.

The root sample preparation was similar to the one described by George et al. (2008). Plant roots were ground in liquid nitrogen and, previously to its use, triturated (Fig. 2.2) and suspended in 5 volumes of homogenizing buffer (15 mmol dm^{-3} MES pH 5.5, 5 mmol dm^{-3} cysteine). The suspension obtained was centrifuged (14,000 rpm, 15 min). The supernatant was used for APA measurements.

MES (2-(*N*-morpholino)ethanesulfonic acid) was used in the buffer since it is usually applied as a buffering agent in biochemistry.

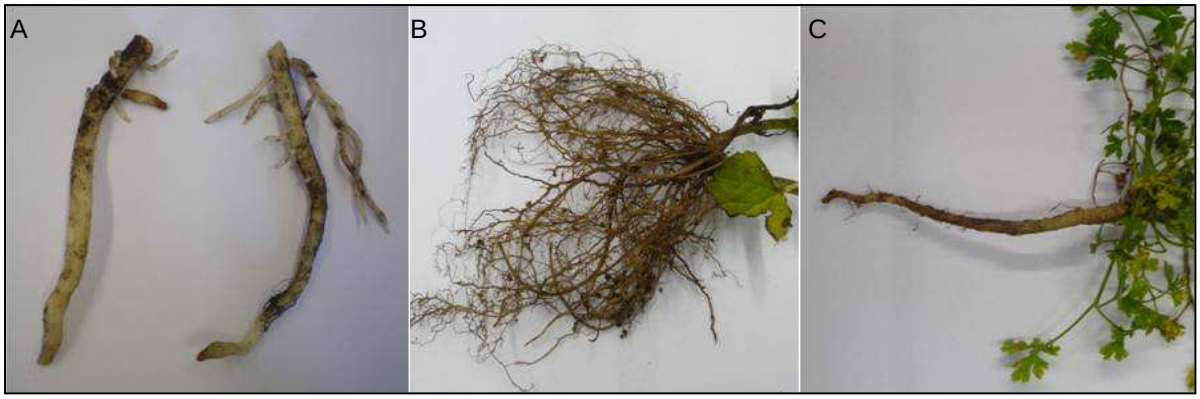


Figure 2.1. Photography of plant roots. Plant source: A) *Agapanthus africanus*, B) *Petroselinum crispum*, C) *Melissa officinalis.*

Figure 2.2. Photography of plant roots after being triturated. Plant source: A) *Agapanthus africanus*, B) *Petroselinum crispum*, C) *Melissa officinalis.*

2.3. Apparatus

Solutions were propelled by a Gilson Minipuls 3 peristaltic pump with a PVC pumping tube, connected to the central channel of an eight-port electrically actuated selection valve (Valco VICI 51652-E8). All tubing connecting the different components of the sequential injection system were made of Teflon from Omnifit, with 0.8 mm id.

A Hitachi 100-40 UV-VIS spectrophotometer with a Hellma 178.711-QS flow cell (10 mm light path, 30 mm^3 inner volume) was used as detection system (λ at 405 nm) for the determination of alkaline phosphatase activity. For phosphate determination, an especially designed multi-reflective flow cell (MRC) (Ellis et al., 2009), equipped with a red LED (λ max at 660 nm) light source connected to a 12 V power supply regulated to 5 V using a multimeter was used. This cell was kindly supplied by Professor Ian McKelvie (Melbourne University, Australia). The output voltage was set to zero V while the LED was on and using deionized water. Analytical signals were recorded using a Kipp & Zonnen BD 111 chart recorder.

A personal computer (Samsung SD 700) equipped with a PCL818L interface card, running with a homemade software written in Quickbasic 4.5, controlled the selection valve position and the pump rotation sense and speed.

2.4. Preparation of the NTA column

An acrylic column (3 mm i.d. and 20 mm length) used for bead packing was coupled to the SIA system and is represented in Fig. 2.3.

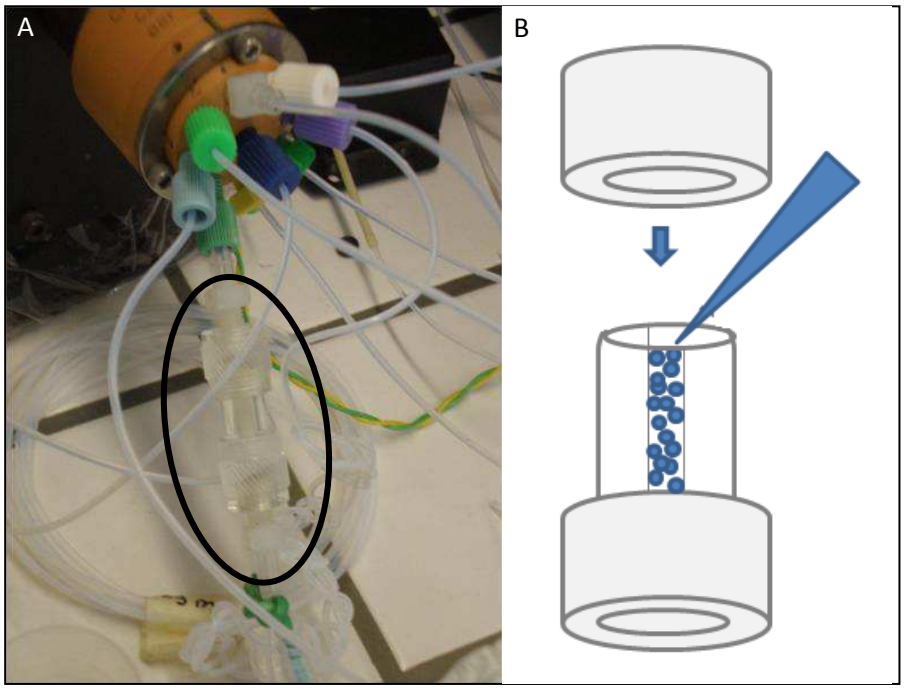


Figure 2.3. Photography (A) and schematic representation (B) of the acrylic column used for bead packing.

Nitrilotriacetic Acid Superflow resin (highly cross-linked 6% agarose, 60 – 160 mm of bead diameter, 50% suspension in 30% ethanol, 30510, Qiagen) was introduced in the acrylic column by means of a Gilson micropipette.

The resin was daily charged with 2% Zn^{2+} (obtained from the solid $ZnCl_2$), according to QIAGEN product information.

2.5. Sequential injection manifold and procedures

The sequential injection manifold used for the colorimetric determination of alkaline phosphatase activity and phosphate is depicted in Fig. 2.4 and the correspondent photography is shown in Fig. 2.5.

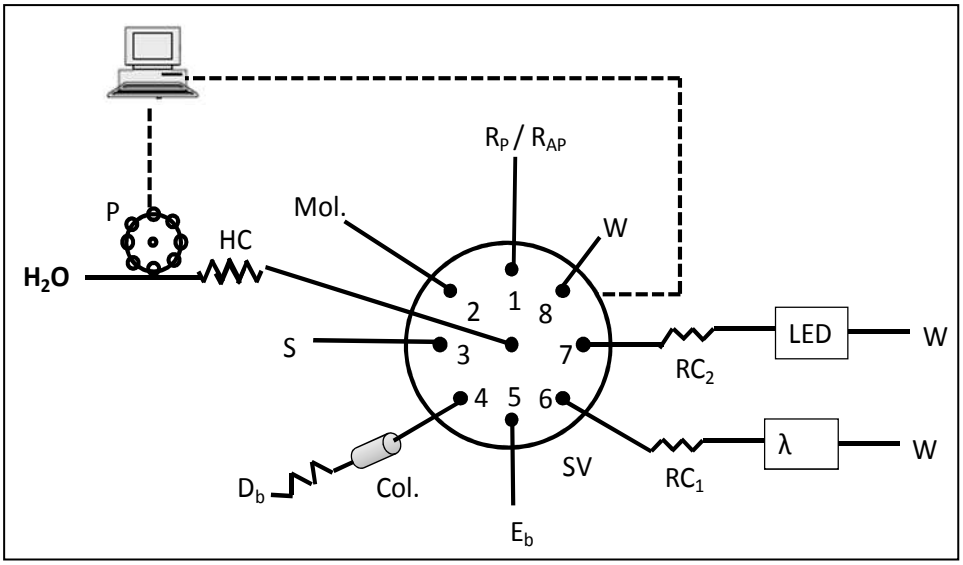


Figure 2.4. Sequential injection manifold for the spectrophotometric determination of alkaline phosphatase activity and phosphate: P, peristaltic pump; SV, eight-port selection valve; HC, 4.25 m holding coil; S, sample or standard; Col., column with NTA Superflow resin charged with Zn^{2+} ions; R_{AP}, *p*NPP or *p*NP; E_b, eluting buffer; D_b, diethanolamine buffer; RC_1, 0.95 m reaction coil; λ, spectrophotometer (405 nm); R_P, ascorbic acid solution; Mol., molybdate reagent; RC_2, 1.82 m knitted reaction coil; LED, light-emitting diode at 660 nm; W, waste.

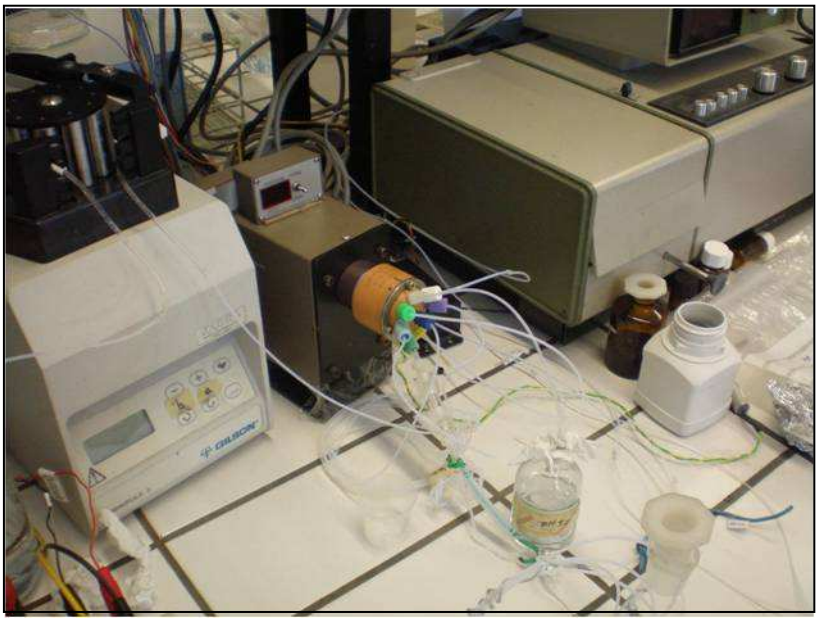


Figure 2.5. Photography of the sequential injection manifold.

A sequential injection manifold was used for preliminary studies in alkaline phosphatase activity determination and is depicted in Fig. 2.6.

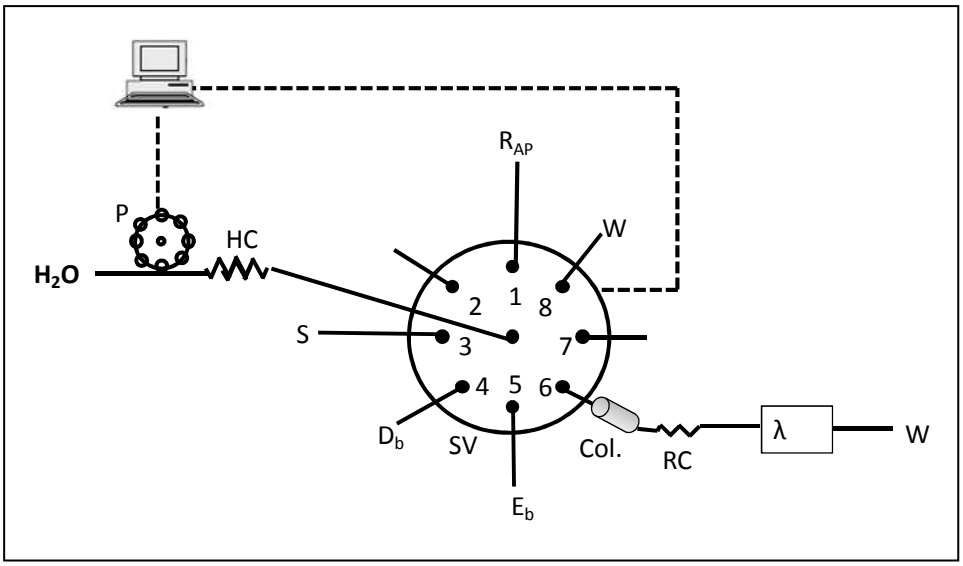


Figure 2.6. Sequential injection manifold for preliminary studies in the spectrophotometric determination of alkaline phosphatase activity: P, peristaltic pump; SV, eight-port selection valve; HC, 4.25 m holding coil; S, sample or standard; Col., column with NTA Superflow resin charged with Zn^{2+} ions; R_{AP}, *p*NPP or *p*NP; E_b, eluting buffer; D_b, diethanolamine buffer; RC, 0.95 m reaction coil; λ, spectrophotometer (405 nm); W, waste.

The sequence of the steps and respective time and volumes for the determination of alkaline phosphatase activity is presented in Table 2.1 and the one for the determination of phosphate is given in Table 2.2.

Table 2.1. Sequential injection protocol for the determination of alkaline phosphatase activity

Step	SV position	Time (s)	Pump speed	Pump direction	Volume (mm^3)	Description
A	3	8	40	a	501	Aspiration of enzyme standard[a]/ water[b]/ sample[c]
B	4	16	20	b	501	Propelling to column
C	4	20	20	a	627	Aspiration for removal of excess (enzyme that did not adsorb)
D	1	2.5	20	a	78	Aspiration of substrate *p*NPP[a,c]/ standard *p*NP[b]
E	4	2	20	b	63	Propelling to column for enzyme catalysis
F	8	12	40	b	752	Propelling to waste to wash the holding coil
G	4	2.1	20	a	66	Aspiration of the product *p*NP
H	6	45	40	b	2820	Propelling to the detector (λ = 405 nm)
I	5	2	40	a	125	Aspiration of eluting buffer
J	4	4	20	b	125	Propelling to column to remove the enzyme adsorbed
L	4	7	20	a	219	Aspiration to wash the column
M	8	4	40	b	251	Washing the holding coil

[a] only aspirated to perform the enzyme calibration curve;
[b] only aspirated to perform *p*NP calibration curve;
[c] only aspirated to determine APA in samples.

First the sample was aspirated and propelled to the column (steps A and B), where the AP is immobilized on the NTA Superflow resin charged with zinc ions. After that, the column was washed in order to remove the enzyme that was not retained, followed by the aspiration of substrate *p*NPP (steps C and D). In step E, the *p*NPP is propelled to the column to incubate with the immobilized enzyme. The holding coil is washed and the product formed was aspirated from the column and propelled to the detector (steps F, G and H). With the purpose to elute the enzyme from the resin between samples, an eluting buffer is aspirated and sent to the column (steps I and J). The solution is aspirated from the column, which remains filled with diethanolamine buffer, and propelled to waste (steps L and M).

Table 2.2. Sequential injection protocol for the determination of phosphate

Step	SV position	Time (s)	Pump speed	Pump direction	Volume (mm^3)	Description
A	1	3	40	a	188	Aspiration of ascorbic acid solution
B	2	2	40	a	125	Aspiration of molybdate reagent
C	3	8	40	a	501	Aspiration of standard/ sample
D	7	70	40	b	3760	Propel to detector (λ = 660 nm)

First the ascorbic acid solution and the molybdate reagent are aspirated (steps A and B), followed by the aspiration of sample (step C). The mixing is promoted by the reversion of the flow when propelling the plugs towards the detector. After aspiration of all solutions, the mixture is propelled to the detector (step D).

To prevent the deposition of the molybdenum blue in the reaction coil and flow cell walls, a 1 mol dm^{-3} NH_4Cl/NH_3 washing solution was used at the end of a day work (Lima et al., 1997; Morais et al., 2003). The solution was manually aspirated and propelled to the detector to wash the reactor coil and flow cell.

2.6. Conventional batch determinations

2.6.1. Alkaline phosphatase activity

Alkaline phosphatase activity was measured using the substrate *p*-nitrophenyl phosphate (*p*NPP) according to the method proposed by Sigma, “Diethanolamine assay”. The reagents (buffer and *p*NPP) were pipetted to a cuvette and mixed by inversion. Alkaline phosphatase enzyme solution, 0.1 – 0.2 unit cm^{-3}, was added and mixed with reagents. Immediately after, the increase in absorbance at 405 nm was recorded for approximately 5 minutes.

2.6.2. Reference method for phosphate determination

To evaluate the accuracy of the system for phosphate determination, the results obtained with the proposed SIA system were compared with those obtained by a reference method (APHA 4500-P E).

2.7. Calculation of the enzyme activity

The developed SIA system enabled the determination of alkaline phosphatase activity with two methods: direct, through the enzyme calibration curve or indirect, through a product (*p*-nitrophenol) calibration curve with subsequent interpolation in the correlation between the *p*-nitrophenol and enzyme standards.

2.7.1. Direct - enzyme calibration curve

Alkaline phosphatase standards (0.022 – 0.441 unit cm^{-3}) were prepared and the final absorbance was measured after incubation of the enzyme standard with the substrate (*p*-nitrophenyl phosphate). The calibration curve based on the established relationship A = f([AP]) was performed and samples were assessed directly by interpolating the absorbance value in the calibration curve.

2.7.2. Indirect - product calibration curve

The *p*NP standards (19 – 280 µmol dm^{-3}) were prepared and a linear relationship was established (A = f ([*p*NP])). After that, the absorbance of the enzyme standards was measured and interpolated in the previously established linear relationship. With the concentration of *p*NP resulted from the interpolation, a new linear relationship was established (*p*NP = f([AP])).

The samples were assessed as follows: the absorbance value obtained was interpolated in the product calibration curve to determine *p*NP concentration. After that, the *p*NP concentration obtained was interpolated in the second calibration curve to determine AP concentration.

2.8. Ammonium, nitrate and nitrite determinations in water samples

Ammonium, nitrate and nitrite determinations were performed for further characterization of the water samples, according to Segundo et al. (2011) and Mesquita et al. (2009).

3.

Results and Discussion

3.1. Introduction

As it was previously mentioned, alkaline phosphatase activity is dependent on phosphate levels, which makes this enzyme a good indicator of P-status. So, when phosphate availability is low, APA increases in order to breakdown organic compounds that include phosphate in their composition. By degrading these compounds, the release of inorganic phosphate is done which increases its bioavailability. In that way, it is important to first determine phosphate and consequently correlate its concentration with alkaline phosphatase activity.

3.2. Phosphate determination

The spectrophotometric determination of phosphate was already developed and previously described by Mesquita et al. (2011). In that scenario, the SIA manifold was assembled and some of the parameters were restudied, viewing a possible further economy of sample and reagents.

The optimization of the colorimetric reaction was carried out using the MRC and a LED light source (660 nm). Preliminary studies were carried using a BTB solution.

3.2.1. Optimization of the volume of reagent and sample using BTB

The first parameter to be studied was the aspirated volumes. These preliminary studies were carried out using a bromothymol blue model solution (0.03 mmol dm^{-3}). Using a borax solution (0.01 mol dm^{-3}) as carrier, the bromothymol blue solution was introduced sequentially in each port involved and the resulted peak profiles were recorded. This study was done by observing the overlap of signals that mimic the mixture that occurs when the plugs are sent to the detector. The volumes were chosen in order to promote the best mixture between sample and reagents.

Volume optimization was performed for simulating two different aspiration sequences (“sample - molybdate reagent - ascorbic acid solution” and “ascorbic acid solution - molybdate reagent – sample”).

The sample volume was studied ranging from 501 to 752 mm^3. For the molybdate reagent, the range of study was 70.5 – 157 mm^3. The ascorbic acid volume was also tested between 94 – 282 mm^3. For the aspiration sequence “sample – molybdate reagent – ascorbic acid solution”, the volumes that allowed a best mixture between the plugs, and for that reason were chosen, were 752, 157 and 219 mm^3 for the sample, molybdate reagent and ascorbic acid solution, respectively (Fig. 3.1A).

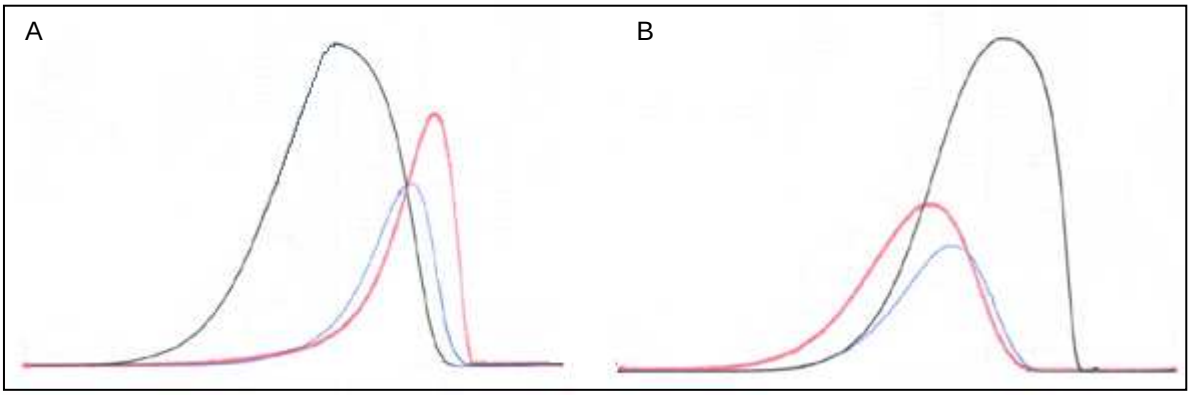


Figure 3.1. Signal output for a sequential injection run using BTB as reagents, sample and carrier (black line, sample; blue line, molybdate reagent; red line, ascorbic acid solution). A, aspiration sequence of "sample – molybdate reagent – ascorbic acid solution"; B, aspiration sequence of "ascorbic acid solution – molybdate reagent – sample".

For the inverted aspiration sequence, the volumes chosen were 188, 125 and 627 mm^3 for the ascorbic acid solution, molybdate reagent and sample, respectively (Fig. 3.1B) since they produced a better overlap between signals and so a better mixture between sample and reagents.

3.2.2. Aspiration sequence

After the aspirated volumes were tested and set, the aspiration sequence was studied using the reagents of the colorimetric reaction. Two calibration curves were performed with the two different aspiration sequences in order to determine which presented better sensitivity. In that way, the aspiration sequence of "ascorbic acid solution – molybdate reagent – sample" was chosen, since it presented a better sensitivity when compared to the aspiration order of "sample – molybdate reagent – ascorbic acid solution".

3.2.3. Chemical parameters

With the optimized volumes and aspiration sequence, a study of the concentration of both reagent solutions was studied.

First, the molybdate reagent was optimized. Ammonium heptamolybdate-tetra-hydrate and sulfuric acid concentrations were studied simultaneously keeping the ratio 1:60 constant according to previous studies (Going and Eisenreich, 1974; Zhang et al., 1999; van Staden and Taljaard, 1998; Huang and Zhang, 2008) as it is presented in Fig. 3.2. The values of concentration varied between 8 – 20 g dm^{-3} and 0.39 – 0.97 mol dm^{-3} for molybdate and sulfuric acid, respectively.

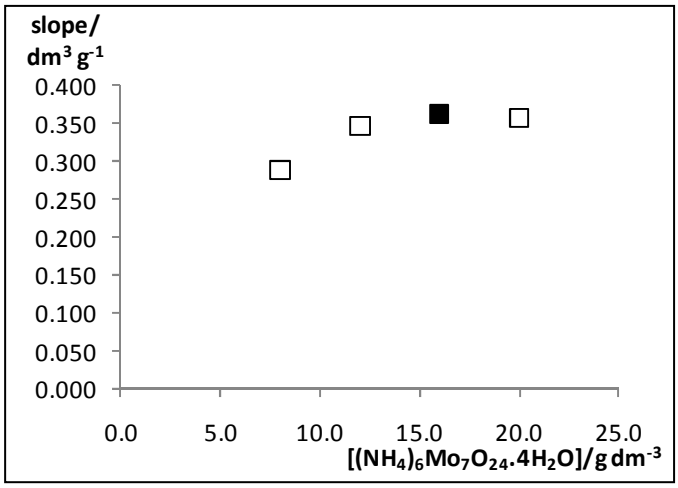


Figure 3.2. Study of the influence of the concentration of the reagent, ammonium heptamolybdate-tetra-hydrate, $(NH_4)_6Mo_7O_{24}.4H_2O$, (□). The point in black represents the chosen concentration.

Since a better sensitivity was obtained, the values of concentration chosen were of 16 g dm^{-3} and 0.78 mol dm^{-3} for molybdate and sulfuric acid, respectively.

After setting the ammonium heptamolybdate-tetra-hydrate and sulfuric acid concentrations, the content of potassium antimony (III) oxide tartrate hemihydrate was studied (Fig. 3.3).

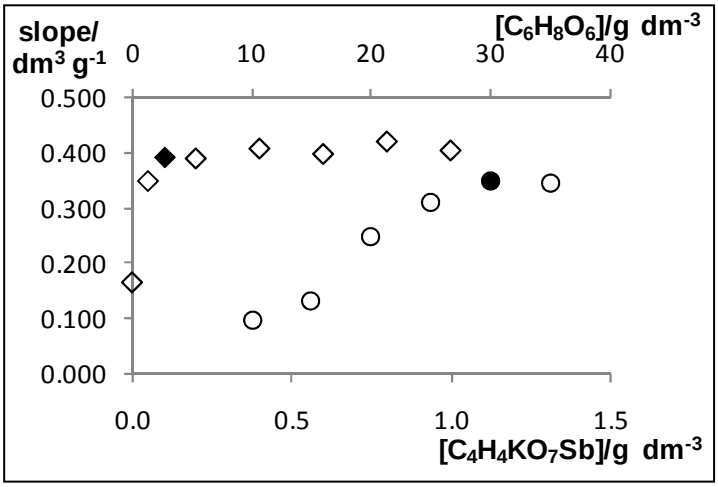


Figure 3.3. Study of the influence of the concentration of the reagents, potassium antimony (III) oxide tartrate hemihydrate, $C_4H_4KO_7Sb$, (◇) and ascorbic acid, $C_6H_8O_6$, (○). The points in black represent the chosen concentrations.

The antimony is introduced in the molybdate reagent as a catalyst (Going and Eisenreich, 1974; Wu and Růžička, 2001). In that way, it was included in the molybdate reagent as an attempt to improve the sensitivity of the reaction between phosphate and molybdate. As the sensitivity increased up to 0.1 g dm^{-3}, this was the value chosen.

Following the molybdate reagent optimization, the content of the ascorbic acid solution was also studied (Fig. 3.3). The purpose of ascorbic acid is to reduce the molybdenum yellow to molybdenum

blue (Wu and Růžička, 2001); therefore, it is important that this reagent is present in excess in order to assure the total reduction of the molybdenum yellow. Because of that, the concentration of 30 g dm^{-3} was chosen and also for the reason that the sensitivity increased until that value.

3.2.4. Sample volume

A new study was performed in order to diminish the sample volume. The sample volumes studied were 501, 627, 752 and 877 mm^3. The volume of 501 mm^3 was chosen since it allowed to spend less volume of sample without compromising the sensitivity and linearity.

3.2.5. Temperature

According to previous studies (Wu and Růžička, 2001), the reduction of molybdenum yellow to the blue heteropoly compound, which is the rate-determining step, can be accelerated not only by adding the catalyst antimony, but also by increasing the temperature. Because of that, the effect of temperature was studied in order to obtain a faster reaction and a better sensitivity. In spite of that, no significant changes were observed when the temperature was increased from room temperature (approximately 20 ºC) to 30 and 35 ºC.

3.3. Alkaline phosphatase determination

3.3.1. Preliminary studies

3.3.1.1. Conventional batch determinations

Preliminary studies were carried out with a stock solution of AP, 179.3 unit cm^{-3}, that was prepared from the stock solution of alkaline phosphatase from bovine intestinal. A 0.441 unit cm^{-3} solution was prepared by appropriate dilution and used to prepare the working solution (0.02 unit cm^{-3}) for batch experiments.

The procedure described in section 2.6.1. was followed. The increase in absorbance was measured during 5 minutes, for the enzyme working solution and can be observed in Fig. 3.4; the determination was performed in consecutive days.

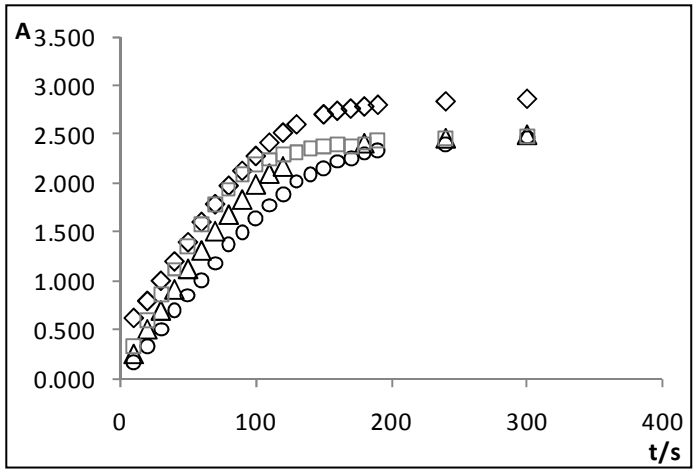


Figure 3.4. Absorbance records carried out for the colored product *p*NP to determine APA due to the enzymatic hydrolysis of the substrate *p*NPP. The □ represents the enzymatic assay performed in day 10.12.10, ◇ an enzymatic assay in day 11.12.10, △ an enzymatic assay in day 12.12.10 and ○ an enzymatic assay in day 13.12.10.

As it can be observed, alkaline phosphatase has its maximum linear rate until 60 seconds. Activity values ranging from 1.02 – 1.50 min^{-1} were obtained for the assays performed in consecutive days.

This method was applied to some water samples and no signal increase was observed. Therefore, this method did not present enough sensitivity for this purpose. As expected, a preconcentration step would be required.

3.3.1.2. Immobilization on NTA Superflow beads

Since alkaline phosphatase is present in waters at low values, an option was made to implement a preconcentration step. For that purpose, a NTA Superflow resin was used. This resin, as it has

already been said, is known by its application in metals preconcentration (Lohan et al., 2005). Since AP is a metalloprotein that needs metal ions (Zn^{2+} and Mg^{2+}) in its active site, the resin was used for the enzyme immobilization by previously forming a complex between zinc ions and NTA resin surface. Firstly, some batch studies were performed in order to see if AP was effectively retained in the NTA Superflow beads.

3.3.1.2.1. Bead suspension

The first study was done using the resin in a suspension. NTA Superflow beads were introduced in a cuvette and the diethanolamine assay was performed. It was possible to observe that there was a deposition of beads at the bottom of the cell. In spite of that, degradation of the substrate still occurred along with the formation of the colored product. In fact, a yellow coloration was observed at the bottom of the cuvette where the beads were located. In that way, it was possible to conclude that the enzyme was retained to the beads and catalyzed the substrate degradation, originating the colored product that remained close to the beads.

3.3.1.2.2. Bead column

Two options could be made about how to use the NTA Superflow resin in the flow system. Beads could be discharged after each measurement or reused. These two options have its advantages and disadvantages. By discharging the resin, new beads could be used between samples which can eliminate some possible interferences (Vidigal et al., 2011). In spite of that, this is a more expensive option. The reuse of beads allows to diminish its waste and makes this method less expensive. Because of that, the reutilization of beads was chosen and so the resin was introduced in an acrylic column. The diethanolamine assay was performed using the resin packed and the solutions were introduced in the column by means of a syringe. Firstly, the enzyme was introduced and the column was washed with water to remove the excess that was not retained. After that, the diethanolamine buffer was introduced followed by the substrate. The collected effluent presented a yellow color which means that the substrate was degraded when inside the column. This showed that the enzyme remained attached to the beads, and catalyzed the degradation of the substrate *p*NPP to the product *p*NP. With this study, it was possible to confirm the previously suspicions, that the enzyme was indeed retained in the NTA Superflow resin.

3.3.2. Sequential injection determination

3.3.2.1. Manifold configuration

The previous studies confirmed the possibility of preconcentration of AP. In that way, an attempt to perform this procedure in a SIA system was done.

Two configurations of the SIA system were studied in order to choose the best position for the column containing the NTA Superflow resin. The schematic representations of these two manifold

configurations are presented in section 2.5 and consisted in positioning the column in the path to the detector and in a lateral port of the selection valve. The diethanolamine assay was carried out in the SIA system in order to compare the two positions and the obtained signal (Fig. 3.5).

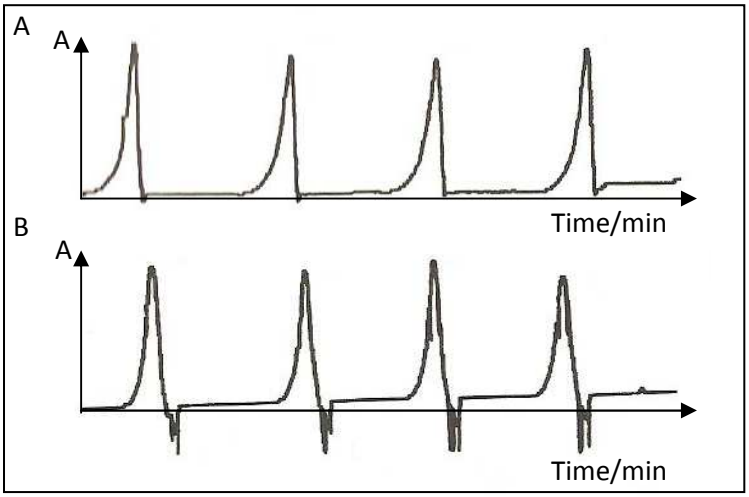


Figure 3.5. Signal obtained for the AP assay with two manifold configurations. A, column positioned in a lateral port of the selection valve; B, column positioned in the path to the detector.

The column positioned in a lateral port was chosen since it presented a less influence of the Schlieren signal as it is presented in Figure 3.5A.

3.3.3. Elution study

As previously explained, the packing of the resin in a column was chosen as a preconcentration approach. In that way, an eluting buffer had to be used to remove the enzyme from the beads between samples/standards. Therefore two elution approaches were tested: elution prior to determination and elution after determination.

3.3.3.1. Elution prior to determination

The buffer used for this approach was the NPI-250 buffer that allowed the elution of the enzyme in native conditions. In that way, the enzyme would be eluted from the resin previously to its determination.

Firstly, to determine if the NPI-250 buffer influenced APA, a comparative study was made between the activity of AP when prepared in both diethanolamine buffer and NPI-250 buffer. For that purpose, the conventional batch procedure (section 2.6.1.) was performed. The increase in absorbance was recorded for five minutes and the results obtained for the assays performed are presented in Fig. 3.6.

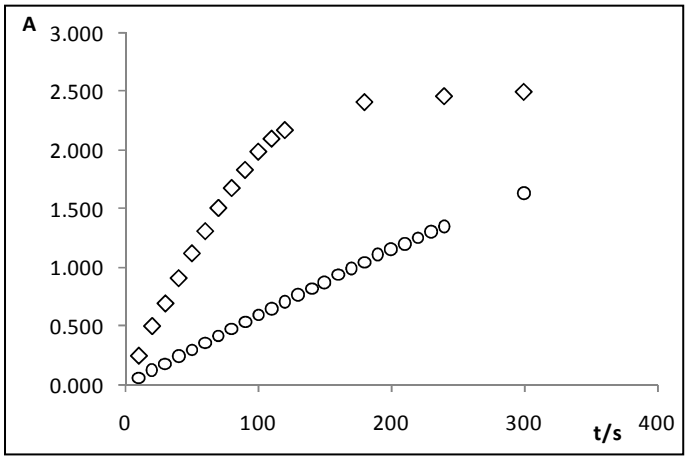


Figure 3.6. Study of the influence of the NPI-250 buffer in APA. The ◇ represents an enzymatic assay using the enzyme prepared in diethanolamine buffer and the ○ represents an equal enzymatic assay using the enzyme prepared in NPI-250 buffer.

As it is possible to observe, alkaline phosphatase activity decreased significantly when prepared in the NPI-250 buffer. Because of that, the prior determination approach was discarded.

3.3.3.2. Elution after determination

For this approach the eluting buffer (buffer E) eluted the enzyme in denaturing conditions. In this way, enzyme elution was done after the activity determination was performed which corresponds to the final of the analytical cycle. In this way, the buffer had no influence in APA. The determination of the final product formed after incubation of the substrate with the immobilized enzyme was performed. Since it was possible to determine APA without decreasing its activity and to wash the column, between samples, with desorption of the enzyme, the approach of eluting after determination was chosen.

3.3.4. Study of physical and chemical parameters

3.3.4.1 Reagent volumes

Using the diethanolamine assay (section 2.6.1), a study was performed to test the influence of reagents volume in alkaline phosphatase activity measurement. The diethanolamine assay (2700 mm^3 of buffer, 300 mm^3 of *p*NPP and 100 mm^3 of enzyme) was performed and compared with assays in which the volumes were changed (1350 mm^3 of buffer, 150 mm^3 of *p*NPP and 50 mm^3 of enzyme; 1350 mm^3 of buffer, 150 mm^3 of *p*NPP and 25 mm^3 of enzyme; 1350 mm^3 of buffer, 75 mm^3 of *p*NPP and 50 mm^3 of enzyme and 1350 mm^3 of buffer, 75 mm^3 of *p*NPP and 25 mm^3 of enzyme). The results obtained are presented in Fig. 3.7.

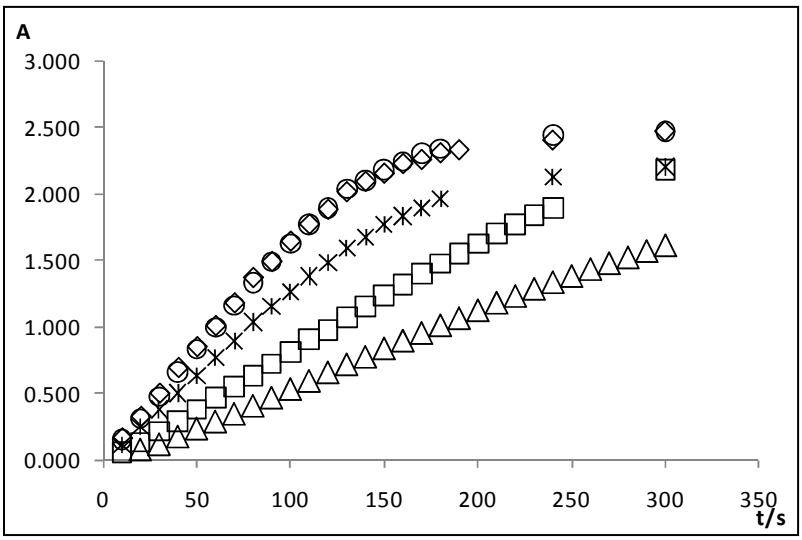


Figure 3.7. Study of the influence of reagents volume in the determination of alkaline phosphatase activity. The ◇ represents the conventional batch assay with volumes of: 2700 mm^3 of buffer, 300 mm^3 of *p*NPP, 100 mm^3 of enzyme, ✳ represents volumes of: 1350 mm^3 of buffer, 150 mm^3 of *p*NPP, 50 mm^3 of enzyme, ○ represents volumes of: 1350 mm^3 of buffer, 75 mm^3 of *p*NPP, 50 mm^3 of enzyme, △ represents volumes of: 1350 mm^3 of buffer, 150 mm^3 of *p*NPP, 25 mm^3 of enzyme and □ represents volumes of: 1350 mm^3 of buffer, 75 mm^3 of *p*NPP, 25 mm^3 of enzyme.

It was possible to observe that with a decrease in the enzyme volume, APA decreased and that by decreasing the total volume of the assay a small influence in APA was obtained. With a decrease in buffer and enzyme volumes to half and in substrate volume to a quart, alkaline phosphatase activity determination was performed and no significant interference was observed. In that way, for the reproduction of the diethanolamine assay in the SIA system, the *p*NPP volume was decreased from a volume of 300 to 78 mm^3.

No further studies in the reagent volumes were performed since the purpose was to change, the less possible, the APA assay when performed in the SIA system.

3.3.4.2. Sample volume

The influence of sample volume in APA determination was studied. Since the goal was to preconcentrate the enzyme, higher volumes of sample had to be used to increase the immobilization factor. So higher volumes of enzyme, when compared to the conventional batch procedure, were studied using an enzyme standard of 0.044 unit cm^{-3}. Values of 251, 376, 501 and 627 mm^3 were experimented. The aim was to see if the immobilization process was improved and so an increase in the product formation would be obtained due to an increase in the substrate degradation. The results obtained are presented in Fig. 3.8.

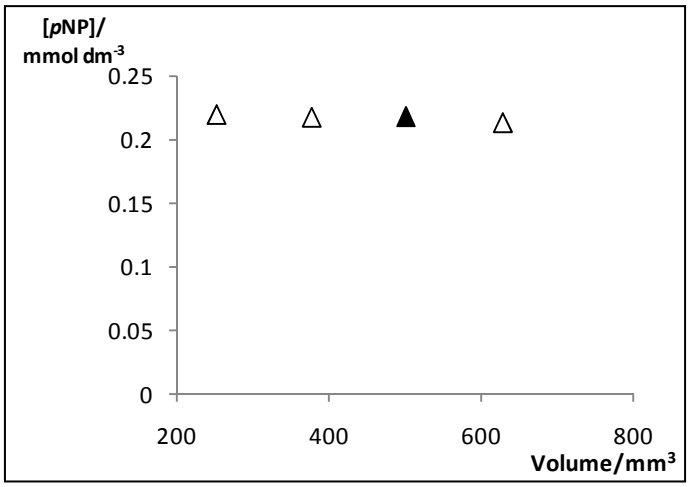


Figure 3.8. Study of the influence of the sample volume in the immobilization process and in APA determination. The point in black represents the chosen volume.

As it is possible to observe, no significant differences were obtained for the studied volumes. Because of that, a volume of 501 mm^3 was chosen since with a sample volume of 627 mm^3 no improvement in sensitivity was observed and with lower values, the step of preconcentration could be compromised.

3.3.4.3. Reagents concentration

A study of the influence of substrate concentration in alkaline phosphatase activity determination was performed. Values of 150 (used in the diethanolamine assay), 111, 72 and 30 mmol dm^{-3} were tested and the obtained results are presented in Fig. 3.9.

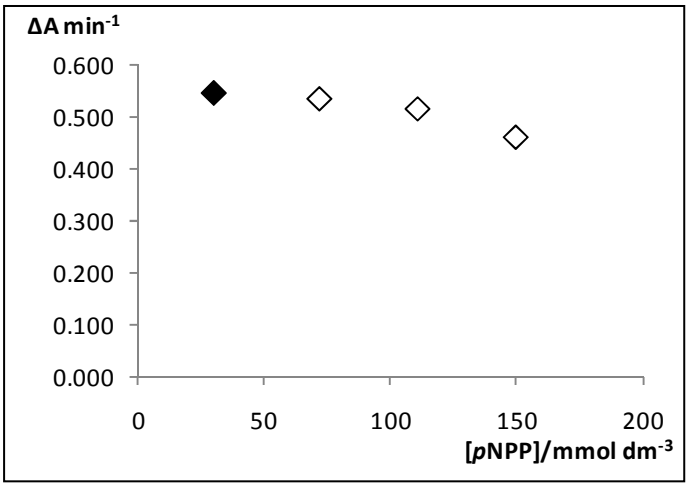


Figure 3.9. Study of the influence of the *p*NPP concentration in the APA determination. The point in black represents the chosen concentration.

As it is shown in Fig. 3.9, the product formation increased with the decrease in substrate concentration. Actually, the lowest value of enzyme activity was obtained for the concentration value

of 150 mmol dm^{-3}, that is the one used in the conventional batch procedure (diethanolamine assay). No significant differences were obtained for the concentration values of 111, 72 and 30 mmol dm^{-3}. In that way, and in order to decrease substrate consumption, a value of 30 mmol dm^{-3} of *p*NPP was chosen to perform the alkaline phosphatase activity assay.

Since *p*NPP concentration was decreased 5 times from a concentration of 150 to 30 mmol dm^{-3}, diethanolamine buffer concentration was also decreased 5 times in order to maintain the proportion with the substrate according to the diethanolamine assay. Thus, a 0.2 mmol dm^{-3} diethanolamine buffer was used.

Overall this study demonstrated that an excess of substrate was achieved.

3.3.4.4. Incubation time

The incubation time between the enzyme and its substrate was studied. A time of 0.2 minutes was taken as the minimal value for the incubation step. It was possible to conclude that there was no need for an incubation time of 5 minutes used in the diethanolamine batch assay. With a value of 0.2 minutes it was already possible to determine alkaline phosphatase activity in enzyme standard solutions (0.022 – 0.441 unit cm^{-3}) obtaining a linear calibration curve.

3.3.5. Interferences

According to Koncki et al. (2006), some ions inhibit alkaline phosphatase activity. For that reason, some possible interferences in APA from water samples were studied. The percentage of interference was calculated by comparing the peak heights of two enzyme standards (0.1 unit cm^{-3}), one with the interfering specie and the other one without it. The studied interfering ions, the tested concentrations and the interference percentages are shown in Table 3.1.

Table 3.1. Potential interfering species and respective percentage of interference

Tested ion	Prepared from the reagent	Concentration/ mg dm^{-3}	% Interference
Cl^-	NaCl	70.0	-3.3
Ca^{2+}	$CaCO_3$	100	-0.3
Ag^+	$AgNO_3$	0.010	-2.4
Mg^{2+}	$MgN_2O_6.6H_2O$	50.0	1.8
K^+	K 1000 mg dm^{-3}	12.0	0.8
Na^+	Na 1000 mg dm^{-3}	50.0	1.6
Al^{3+}	Al 1000 mg dm^{-3}	20.0	-4.4
Pb^{2+}	Pb 1000 mg dm^{-3}	20.0	-8.1
Fe^{3+}	Fe 1000 mg dm^{-3}	0.200	1.3
H^+	HNO_3	1575	1.8

As it is shown in Table 3.1, no significant interferences were observed for the ions tested. This means that no interference was caused in the binding of the enzyme to zinc ions.

3.3.6. Study of the *p*NP standards stability

A study was made in order to assess the stability of the *p*NP standards. Calibration curves were performed monthly for two months with the same standards in the linear dynamic range of 19 – 280 µmol dm^{-3}. Firstly, a calibration curve was performed in the day of the *p*NP standards preparation. After that four calibration curves were performed one month and two months after the standards preparation. The sensitivity obtained for the calibration curves performed are presented in Fig. 3.10.

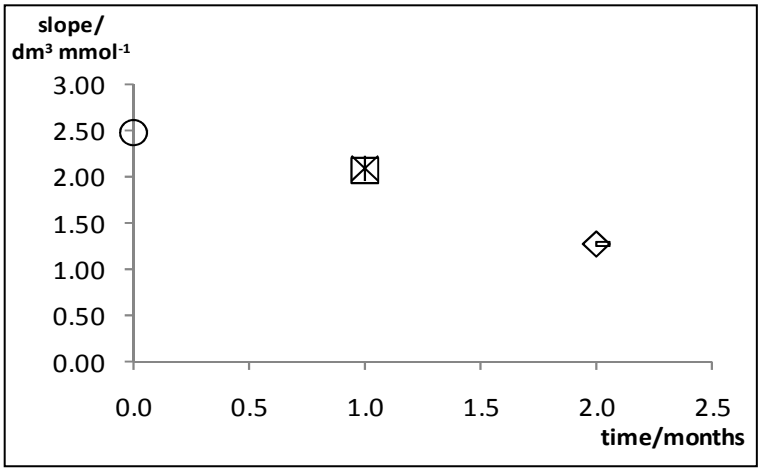


Figure 3.10. Study of the stability of *p*NP standards. The ○ represents the slope of a calibration curve performed in the day of standards preparation, ◇ and □ represent the slope of two calibration curves performed one month after standard preparation and ✳ and ▫ represent the slope of two calibration curves performed two months after standard preparation.

It was possible to conclude that *p*NP standards had to be prepared monthly since the sensitivity decreased significantly after two months from the preparation. Therefore, a sensitivity of approximately between 2.1 – 2.5 dm^3 $mmol^{-1}$ had to be obtained.

3.3.7. APA assessment

3.3.7.1. Direct method

Alkaline phosphatase activity in this work, as it has already been said, was determined by means of two different methods (direct or indirect). The direct method was determining APA in samples directly by using an enzyme calibration curve (Fig. 3.11). A sensitivity in the range of 0.27 – 0.44 cm^3 $unit^{-1}$ had to be obtained in order to accurately determine APA.

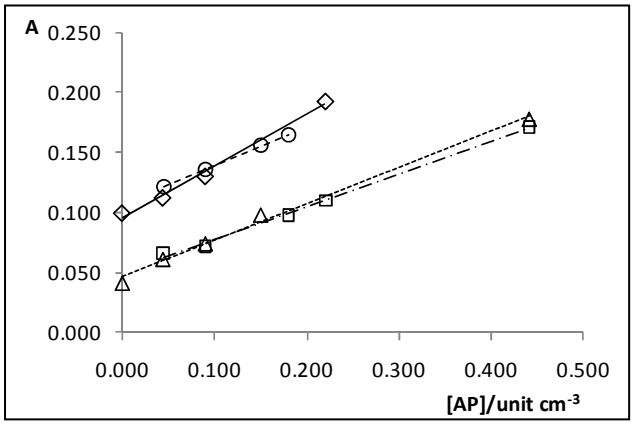


Figure 3.11. Alkaline phosphatase calibration curves. The ◇ represents a calibration curve in day 02.03.11, □ a calibration curve in day 09.03.11, △ a calibration curve in day 11.03.11 and ○ a calibration curve in day 16.03.11

3.3.7.2. Indirect method

For the indirect method, product calibration curves were performed (Fig. 3.12A). After that, a relationship between enzyme standards and the corresponding formed product was performed (Fig. 3.12B).

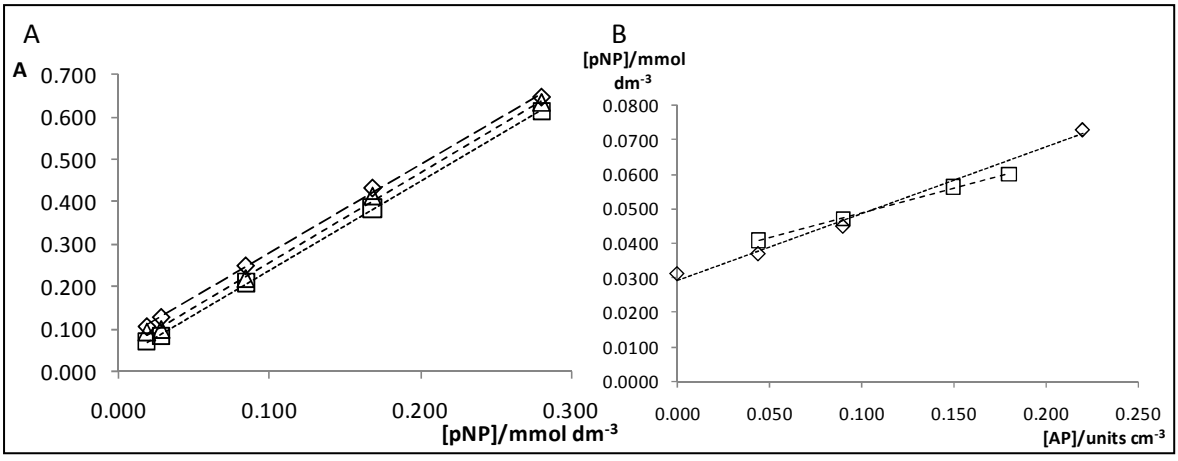


Figure 3.12. Determination of APA by determining the final product concentration. A, product calibration curve. The ◇ represents a calibration curve in day 140211, the □ a calibration curve in day 150211 and the △ a calibration curve in day 160211; B, relation between AP standards and the corresponding product concentration formed after incubation of the enzyme with substrate. The □ represents a relation obtained for an enzyme calibration performed in day 160311 and the ◇ represents a relation obtained for an enzyme calibration performed in day 020311.

In spite of being more complex when compared to the direct method, the indirect approach allows the determination of APA with no need to perform an enzyme calibration curve every day. Since a linear relation between AP and *p*NP concentrations was established, it was only necessary to perform

the product calibration curve to determine APA. This possibility allowed to decrease the consumption of the enzyme solution which makes this approach less expensive. In that way, for the relation *p*NP = f([AP]), a sensitivity with a slope value between 0.14 x 10^{-3} – 0.21 x 10^{-3} mmol $unit^{-1}$ had to be obtained in order to correctly determine values of APA.

The two methods previously described for APA determination were used to determine the enzyme activity in spiked water samples and the results obtained are compared in Table 3.2.

Table 3.2. Alkaline phosphatase activity determination by the two methods

	Direct method	Indirect method	
Sample type	[AP]/unit cm^{-3} ± SD	[AP]/unit cm^{-3} ± SD	%RD
Well water	0.090 ± 0.006	0.090 ± 0.007	0.1
Pore water	0.092 ± 0.003	0.088 ± 0.008	-4.1
River water	0.091 ± 0.002	0.091 ± 0.003	0.1
	0.074 ± 0.010	0.074 ± 0.012	0.1
	0.088 ± 0.003	0.088 ± 0.004	0.1
	0.118 ± 0.004	0.118 ± 0.005	0.0
	0.102 ± 0.005	0.102 ± 0.005	0.0
	0.104 ± 0.002	0.105 ± 0.002	0.0
	0.096 ± 0.000	0.097 ± 0.001	0.1
	0.096 ± 0.006	0.097 ± 0.006	0.1
	0.080 ± 0.007	0.080 ± 0.009	0.1
	0.078 ± 0.002	0.078 ± 0.005	0.1
	0.085 ± 0.003	0.085 ± 0.005	0.1

Equal values were obtained for alkaline phosphatase activity when determined by the two methods, with relative deviations between -4.1 and 0.1. This results show that both methods can be used in the determination of alkaline phosphatase activity.

3.4. Features of the developed system

Significant features of the developed system are demonstrated in Table 3.3. The LOD and LOQ for phosphate and *p*NP determination were calculated as three and ten times the standard deviation of ten consecutive injections of deionized water, according to IUPAC recommendations (1976; 1995). The LOD and LOQ for enzyme activity determination were calculated as three and ten times, respectively, the standard deviation of the mean intercept of three calibration curves, according to IUPAC recommendations (1976; 1995).

The phosphate calibration curve corresponds to the mean slope and intercept of six calibration curves in consecutive days with the respective standard deviations. The *p*NP calibration curve corresponds to the mean slope and intercept of three calibration curves in consecutive days with the respective standard deviation and the enzyme calibration curve corresponds to the mean slope and intercept of three calibration curves done in two weeks with the respective standard deviation.

The determination rate was calculated based on the time spent per cycle. A complete analytical cycle for alkaline phosphatase activity determination took about 2.1 min. For phosphate determination an analytical cycle took about 1.4 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.

Table 3.3. Features of the developed SIA system for both determinations

Parameter	Dynamic range	Calibration curve[a]	LOD	LOQ	Determination rate (h^{-1})
Phosphate	0.98 – 49.9 µmol dm^{-3}	H[b] = 0.091 (±0.008) µmol $H_2PO_4^-$ dm^{-3} - 0.089 (±0.057)	0.29 µmol dm^{-3}	0.98 µmol dm^{-3}	32
AP activity	19 – 280 µmol dm^{-3} *p*NP	A = 0.00209 (±0.00002) µmol dm^{-3} *p*NP + 0.04957 (±0.01034)	1.9 µmol dm^{-3}	4.5 µmol dm^{-3}	17
	0.044 – 0.441 unit cm^{-3}	A = 0.296 (±0.025) unit AP cm^{-3} + 0.069 (±0.033)	0.025 unit cm^{-3}	0.082 unit cm^{-3}	

[a] Values between brackets correspond to the standard deviation of the equation parameters

[b] Peak height (cm)

The repeatability of the system for P determination was determined by calculation of the relative standard deviation obtained by the mean of ten consecutive injections of different water samples. RSD % ([$H_2PO_4^-$] µmol dm^{-3} ± SD), wastewater 0.85 (10.9±0.1); wastewater treated 1.82 (20.5±0.4); interstitial water 1.21 (9.12±0.11).

With the developed methodology, the overall reagent consumption per APA determination was 1.08 mg of *p*NPP, 60 mg of urea, 1.71 mg of potassium dihydrogen phosphate (KH_2PO_4), 1.97 mg of Tris-HCl, 0.0029 mg of magnesium chloride hexahydrate ($MgCl_2.6H_2O$) and 0.0030 mg of diethanolamine.

The overall reagent consumption per phosphate determination was 2.00 mg of ammonium heptamolybdate-tetra-hydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$), 0.0125 mg of potassium antimony (III) oxide tartrate hemihydrate ($C_4H_4KO_7Sb$), 0.940 mg of tartaric acid ($C_4H_6O_6$), 5.64 mg of ascorbic acid ($C_6H_8O_6$) and 9.59 mg of H_2SO_4 for phosphate determination.

The effluent production per determination is 2.82 cm^3 for alkaline phosphatase activity and 3.76 cm^3 for phosphate and the sample consumption for both determinations is of 501 mm^3.

3.5. Application to water and root samples

3.5.1. Validation of phosphate determination

To determine the accuracy of phosphate determination, a certified reference river water (QC RW 1, VKI-9-3-0702) was analyzed. The certified water was prepared in different water samples (river, sea and estuarine waters) in order to have a final phosphorus concentration of 98.7 μg dm^{-3}. The relative deviations obtained for the different water samples are presented in Table 3.4.

Table 3.4. Application of the developed sequential injection method to the phosphate determination in a certified reference water prepared in different water samples and comparison with tabulated value

Sample type	Sample ID	Tabulated value/ μg P dm^{-3}	SIA μg P dm^{-3} ± SD	SIA RD (%)
River water (Douro river)	R1	98.7	101 ± 2	2.3
Sea water	P1		94.7 ± 3.1	-4.1
	P10		94.2 ± 3.4	-4.6
Estuarine water (Cávado river)	C3		94.8 ± 2.7	-4.0

Relative deviations of 2.3; -4.1; -4.6; and -4.0 were obtained for the certified water prepared in different water samples which indicates that there is no significant interference from the different water matrices. This confirms what has been said about salinity interference. In spite of its high salinity values, sea water was used and the relative standard deviation obtained varied between –4.6 and -4.1 which means that there was no significant interference.

Two well water samples (sample ID, P7 and P11) were assessed with the reference procedure. For the P7 sample, the reference procedure obtained 5.61 mg dm^{-3} (RSD = 5.2±4.9%) and with the SI method 5.58 (±0.12) mg dm^{-3} was obtained resulting in a relative deviation of -0.5%. As for the P11 sample the reference procedure obtained 9.40 mg dm^{-3} (RSD = 5.2±4.9%) and with the SI method 9.56 (±0.07) mg dm^{-3} was obtained resulting in a relative deviation of 1.7%.

3.5.2. Recovery studies for AP determination in different types of water

Since the initial enzyme concentrations in natural waters were below the detection limit, estuarine water samples were spiked with volumes of 0.1 cm^3 of enzyme stock solution (0.441 unit cm^{-3}) to a final volume of 10 cm^3 of sample. The recovery results obtained are presented in Table 3.5. The calculation of the recovery percentage was made according to IUPAC (2002), and using the so-called indirect method.

Table 3.5. Application of the sequential injection system for the alkaline phosphatase activity determination in spiked estuarine water samples and respective recovery studies

Sample ID	Added Conc./unit cm^{-3}	Found			Recovery (%)
		Conc./unit cm^{-3}	SD	RSD %	
XI - DOU FEV10	0.100	0.116	0.002	1.40	116
III - FEI1 FEV10	0.100	0.102	0.005	4.75	102
VI- PTE FEV10	0.100	0.105	0.002	1.55	105
IX - CLUB FEV10	0.100	0.097	0.000	0.00	97
VI - INH2 FEV10	0.100	0.097	0.006	6.72	97
P11 3.2010	0.100	0.090	0.006	7.23	90
DOU3 PW 030907	0.100	0.102	0.037	36.6	102

The SIA methodology provided recovery ratios with an average of 101% (standard deviation 8.3) and a statistical test (t-test) was used to evaluate if that mean recovery value did not significantly differ from 100% (Miller and Miller, 1993). Results showed that for a 95% significance level the recovery values did not differ from 100% as the calculated t-value was 0.336 with a correspondent critical value 2.969, thus indicating the absence of multiplicative matrix interference.

3.5.3. Sequential determination

After validation of the developed methodology for the alkaline phosphatase activity and phosphate determination, APA and phosphate were determined in different water samples. The values obtained are summarized in Table 3.6. Figure 3.13A shows the influence of phosphate concentration in alkaline phosphatase activity for different water samples. Nitrate and nitrite were determined in the water samples for further characterization. Figure 3.13B presents the obtained relation between APA and the ions nitrate and nitrite.

Table 3.6. Application of the sequential injection system for the alkaline phosphatase activity and phosphate determination in different water samples

Sample type	Sample ID	[AP] unit cm^{-3} ± SD	$[H_2PO_4^-]$ µmol dm^{-3} ± SD	$[NO_2^-]$ µmol dm^{-3} ± SD	$[NO_3^-]$ µmol dm^{-3} ± SD	$[NH_4^+]$ µmol dm^{-3} ± SD
Pore water (Douro river)	DOU3 PW 030907	<0.025	2.06 ± 0.14	<0.11	65.9 ± 0.3	<5.2
	INHA IV	0.093 ± 0.014	2.66 ± 0.29	0.64 ± 0.03	185 ± 4	<5 .5
Pore water (Cávado river)	CAV2 PW 040908	<0.025	2.47 ± 0.12	3.39 ± 0.05	65.8 ± 2. 3	<5.2
	CAV3 PW 040909	<0.025	2.56 ± 0.04	0.09 ± 0.04	36.5 ± 0. 6	<5.2
	CAV2 PW 151107	<0.025	2.57 ± 0.13	0.48 ± 0.08	32.8 ± 1. 1	<5.2
Pore water (Ave river)	Ave 1	<0.025	4.96 ± 0.09	1.64 ± 0.14	297 ± 4	<5.2
	Ave 2	<0.025	19.7 ± 0.3	6.40 ± 0.08	315 ± 2	<5.2
	Ave 1 PW 310309	0.094 ± 0.004	4.31 ± 0.09	0.10 ± 0.07	80.4 ± 2.0	<5.2
	Ave2 PW 310309	0.107 ± 0.005	7.44 ± 0.51	2.92 ± 0.24	29.6 ± 0.6	<5.2
Pore water (Lima river)	Lima PW	<0.025	3.26 ± 0.12	11.1 ± 0.2	860 ± 6	<5.2
Estuarine water (Ave river)	Ave1 310309	0.022 ± 0.006	2.53 ± 0.07	0.21 ± 0.02	87.6 ± 1.2	<5.2
	Ave2 310309	0.025 ± 0.004	3.53 ± 0.04	4.92 ± 0.04	328 ± 4	<5.2
	Ave3 310309	0.058 ± 0.011	3.00 ± 0.04	4.06 ± 0.06	309 ± 0	<5.2
River water (Lima river)	LIMA 1 180309	0.054 ± 0.013	2.02 ± 0.05	1.06 ± 0.02	30. 3 ± 1.7	<5.2
	LIMA 2 180309	0.042 ± 0.003	1.97 ± 0.13	3.23 ± 0.16	18. 7 ± 0.8	<5.2
	LIMA3 180309	<0.025	2.28 ± 0.24	-	-	<5.2
River water (Cávado river)	CAV 3 rio 040907	0.048 ± 0.004	2.74 ± 0.08	2.66 ± 0.11	64.6 ± 0.3	15 ± 2
	CAV 2 rio 040907	0.045 ± 0.012	2.27 ± 0.08	3.93 ± 0.02	55.3 ± 0.6	<5.2
River water (Douro river)	II - REF	<0.025	1.11 ± 0.07	4.59 ± 0.03	247 ± 2	-
	III - FEI	<0.025	2.03 ± 0.03	6.91 ± 0.10	213 ± 2	-
	IV - FEI 2	<0.025	1.93 ± 0.15	7.89 ± 0.05	260 ± 6	-
	V - INH 1	<0.025	2.12 ± 0.03	4.06 ± 0.74	159 ± 3	-
	VI - INH2	<0.025	1.90 ± 0.09	5.17 ± 0.17	180 ± 7	-
	VII - PTE	<0.025	1.78 ± 0.15	5.76 ± 0.56	198 ± 2	-
	VIII - INH4	<0.025	1.98 ± 0.03	5.80 ± 0.53	172 ± 3	-
	IX - CLUB	<0.025	1.63 ± 0.03	5.58 ± 0.27	139 ± 2	-
	X - INH6	<0.025	1.84 ± 0.19	5.26 ± 0.14	134 ± 2	-
	XI- DOU	<0.025	1.79 ± 0.07	8.86 ± 1.32	119 ± 3	-
Well	P4 161209	<0.025	31.6 ± 0.8	-	-	<5.2
	P6 161209	<0.025	42.2 ± 0.3	43.9 ± 5.1	2072 ± 9	<5.2
	P7 161209	<0.025	30.1 ± 1.0	-	-	<5.2
	P10 3.2010	<0.025	25.7 ± 0.5	85.7 ± 5.2	2312 ± 37	<5.2
	P11 3.2010	<0.025	42.1 ± 0.3	79.2 ± 3.1	3088 ± 58	<5.2

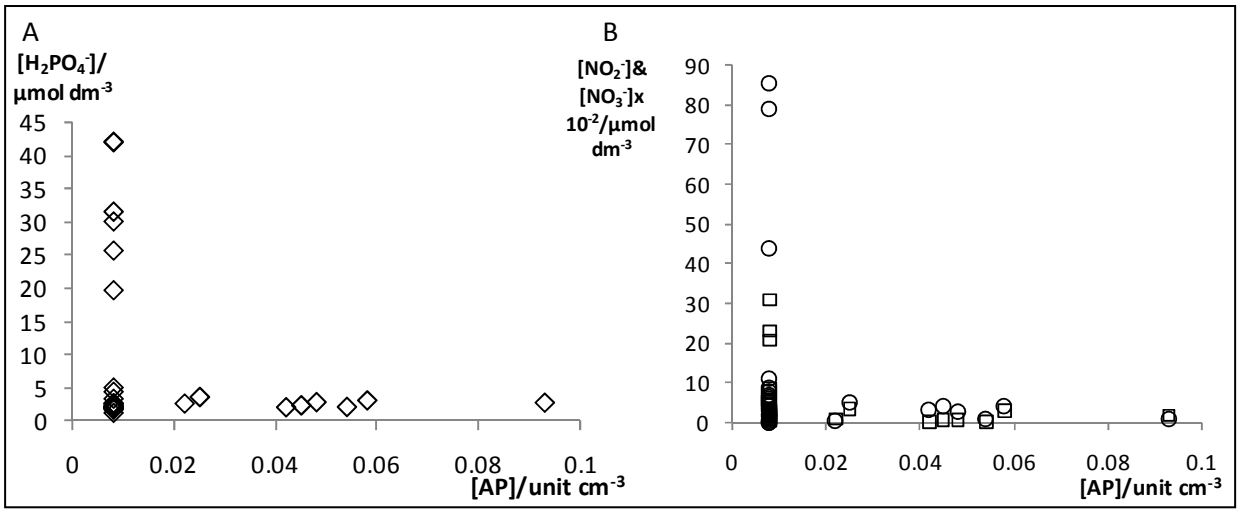


Figure 3.13. Influence of the concentration of ions in APA in different water samples. A, relation between alkaline phosphatase activity and phosphate concentration; B, relation between alkaline phosphatase activity and nitrate (square) and nitrite (circle) ions concentration.

As expected, alkaline phosphatase activity was lower than the limit of detection for several water samples since it is present in waters at relatively low concentrations.

It is possible to observe that for high levels of phosphate concentration, APA is lower while for the lowest levels of phosphate, generally, APA increases. In spite of that, for some samples this does not happens. This can be due to the fact that alkaline phosphatase activity is influenced by several factors and not only by P levels. According to Koch et al. (2009) APA is also influenced by carbon levels. It is as well known that enzyme activity is influenced by pH, temperature and by the immobilization process.

3.5.4. Alkaline phosphatase activity determination in root samples

AP is also active in root plants or in microorganisms that are present in the rhizosphere. Because of that, several root samples were obtained and the protocol described in section 2.2 was followed.

After root plant treatment (please see section 2.2.), APA was assessed in different types of plants with the developed SIA methodology. The values obtained are presented in Table 3.7 along with plant source that is presented as the plant species or family name. Also, some weed plants were obtained.

Table 3.7. Alkaline phosphatise activity in root samples

Plant source	[AP]/unit cm^3 ± SD
Zantedeschia aethiopica	0.244 ± 0.008
	0.369 ± 0.019
Canna flacida	0.203 ± 0.015
	0.424 ± 0.003
Agapanthus africanus	0.137 ± 0.008
	0.193 ± 0.014
Canna indica	0.108 ± 0.008
Trifolium	0.314 ± 0.003
	0.231 ± 0.011
Mentha spicata	0.511 ± 0.013
Plantago lanceolata	0.190 ± 0.016
Geranium robertianum	0.079 ± 0.008
Melissa officinalis	0.189 ± 0.005
Allium cepa	0.793 ± 0.013
Petroselinum crispum	0.241 ± 0.032
Weeds	0.394 ± 0.010
	0.359 ± 0.002
	0.828 ± 0.005
	1.53 ± 0.04
	2.17 ± 0.05
	0.714 ± 0.065

With the developed SIA system, alkaline phosphatase activity was determined in all root plant samples obtained. Comparing the results obtained for water and root samples, it is possible to observe that APA was higher in root plants than in water samples.

4.

General conclusions

4.1. Conclusions

The developed SIA methodology allowed the determination of alkaline phosphatase activity in water and root samples and inorganic phosphate in different types of water samples.

The enzyme activity determination was accomplished using a separation/preconcentration device coupled to the sequential injection system.

It was possible to separate the enzyme from its matrices by taking advantage of its metalloprotein characteristic. As far as we know, this is the first time that this enzyme was immobilized using the zinc ion as the mediator between the protein and the resin.

In this work, NTA Superflow resin was used due to its capacity to bind metals. In that way, zinc was retained to the resin and used to immobilized AP that has a need for this metal in its active site.

Very few works report the alkaline phosphatase activity determination using flow analysis. Most of them report a routine procedure that is laborious and time consuming. Also, the works that employ flow analysis use the FIA technique. Therefore, this was the first time (as far as we know) that sequential injection analysis was applied for APA determination. This technique allowed a time saving when compared to other routine analysis and, since this technique is controlled by a computer, human errors were avoided. It was also possible to couple a separation device and perform a sample pretreatment in-line which made APA determination possible in an automatic and miniaturized way.

APA determination was possible with an incubation time of 0.2 minutes and with a determination rate of 17 det. h^{-1}. When compared to previous works that present incubation times with a minimum of 5 minutes, the system developed presented a lower time of incubation and because of that, APA determination was faster. Due to this characteristic, the developed system can give a real time analysis since it is not necessary to wait overnight for the product formation.

The developed SIA methodology also allowed the determination of inorganic phosphate in different water samples by means of the molybdenum blue chemistry. This determination was done without the necessity of system reconfiguration which is an advantage of the SIA systems. APA and phosphate determination were possible with the same SIA manifold only by changing the reagents on the ports of the selection valve.

When compared to previously reported colorimetric flow systems for the phosphate determination, the developed methodology allowed lower effluent production.

Due to the multi-parametric determination, the system developed allows the determination of APA and P in the same sample in 3.5 minutes. In that way, a relation between alkaline phosphatase activity and inorganic phosphate in natural waters is possible to perform. This methodology could also be used for the determination of water P-status since APA is a good indicator of P levels.

When compared to the SIGMA's diethanolamine assay, the system developed allowed a reagent saving. The SIA system developed is a robust and reliable alternative for the spectrophotometric determination of APA and phosphate.

4.2. **Future Work**

As future work, it would be very interesting to miniaturize and compact the SIA system developed. This can be achieved by using a micro sequential lab-on-valve (SI-LOV) equipment (Fig. 4.1A). The selection valve, propulsion device and detector, are gather in the same box. This type of systems have advantages such as the use of volumes in the µL range.

This technique also presents advantages in reagent and sample saving due to the incorporation of the detection system in the selection valve. Because of this down scale, SI-LOV is a perfect tool for enzymatic and bead injection assays (Fig. 4.1B). With BI, beads replace the reagent and the assay is carried on the surface.

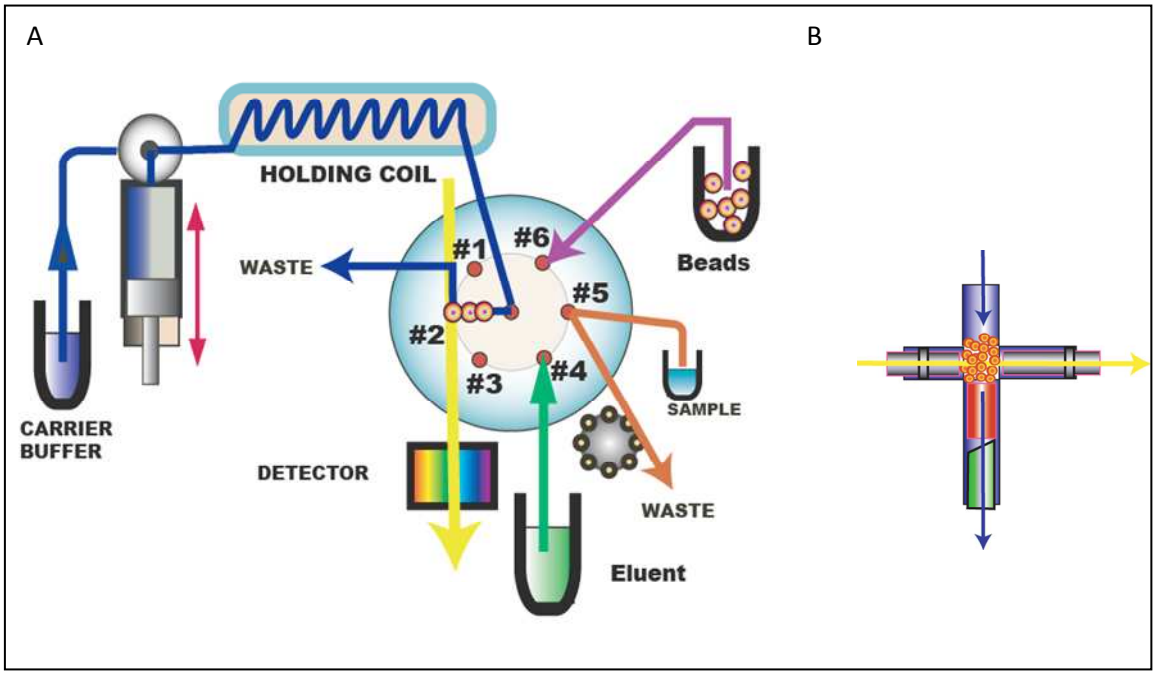


Figure 4.1. A, SI-LOV manifold; B, flow cell (Růžička, 2009).

With SI-LOV, APA determination can be performed with lower sample and reagent consumption when compared to the SIA system developed in this work. It is not necessary to couple separation devices since the preconcentration and detection steps can be performed in the flow cell. SI-LOV also allows enzyme rate measurements since it is possible to stop the flow in the detector. In that way, measurements of absorbance through time can be performed.

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