



Improved sequential injection method for phosphate quantification within a wide dynamic range with in-line pre-concentration to monitor soil leachates



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ABSTRACT

Phosphate is a well-known contaminant and its content is an indicator of water quality, so it is important to have an efficient monitoring of the soil leaching process.

In this work, an automatic, low cost flow analysis method, capable of real-time monitoring of the soil leaching processes using spectrophotometric detection, based on the molybdenum blue reaction, was developed. The developed methodology for phosphate determination was based on the molybdenum blue reaction and includes an on-line solid phase extraction (SPE) step, involving an AG1-X8 anionic exchange sorbent.

The described SI method enabled phosphate determination within a wide range, 1–80 μM , with a detection limit of 0.52 μM . It was successfully applied to leachates from laboratory scale soil columns (LSSC) and one sample analysis was carried out in triplicate, in less than 10 minutes.

1. Introduction

The soil structure is a fundamental property of soil use and management and it has impact in the storage and movement of water, gas and both macronutrients and micronutrients [1–3]. Another important property in the quality and productivity of the soil is the organic matter content since it can influence other properties, such as water retention, soil porosity and stability. In this context, the soil fertilization main purpose is to improve soil productivity by increasing the nutrients availability (both macro and micronutrients) to plants [3–6].

One of the essential nutrients required for the growth and energy transportation of all organisms is phosphorus. However, this element is commonly a limiting nutrient in terrestrial and aquatic ecosystems [7]. Phosphorus-based fertilizers are often used in agricultural soils to increase the soil fertility and boost its production. However, when used excessively or inappropriately, these fertilizers can have several negative impacts in soil, water, and overall ecosystem [8,9]. Because it is present in several different forms, the quantification of phosphorus is usually accomplished through the determination of soluble reactive phosphates, such as orthophosphates [10]. Phosphate is a contaminant of natural water (ground and surface) [11] so its concentration in these types of water is very low (less than 0.1 mg/L [12]).

The increase of agriculture fertilizers usage and because the soil leaching process has a huge impact on the quality of surface and ground waters, monitoring of nutrients and contaminants, such as phosphate, which can be a nutrient and/or a contaminant, is essential in environmental and agricultural studies [13]. An effective monitoring process will enable to ensure water quality and minimize pollution of natural waters.

Most methods for phosphate determination in water are based on the molybdenum blue chemical reaction [7,10–12,14].

This colorimetric reaction is based on the formation of heteropoly acid, from phosphate and acidic molybdate, which is subsequently reduced to blue colored complex [7,14]. Because several reductants can be used (ascorbic acid, tin (II) chloride, among others), resulting in different reaction conditions, the optimal wavelength may vary depending on the conditions chosen for the determination [7,14].

Flow-based methods, namely sequential injection systems, coupled with spectrophotometric detection allow an efficient miniaturization of chemical assays benefiting of low volume samples, low reagent consumption and limited waste generation [15–17]. Alongside the pre-concentration of the analyte of interest, the introduction of a solid phase extraction (SPE), allows the removal of the matrix, the removal of some interferences, and reduction of the already low consumption of reagents

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Table 1

Reported works for phosphate determination using flow methods in the last 10 years; FIA, Flow injection analysis; HSI, hydrodynamic sequential injection; FA, flow analysis; MPFS, multipumping flow system; SIA, sequential injection analysis; LOD, limit of detection.

Method	Sample	Dynamic Range	LOD	Reference number
Micro HSI	Water	0.01–0.1 and 0.3–1.0 mg L ⁻¹ of PO ₄ ³⁻ P	0.005 mg L ⁻¹ of PO ₄ ³⁻ P	[21]
Micro HSI	Water	0.2–3.0 mg P L ⁻¹	0.18 mg L ⁻¹	[22]
FA	Seawater	0.1–10 μM	–	[23]
FIA	Nutrient solution	3–100 mg L ⁻¹	0.034 mg L ⁻¹	[24]
MPFS	Wastewater Freshwater	0.1–12 mg L ⁻¹	–	[25]
Micro HSI	Water	0.1–6 mg P L ⁻¹	0.1 mg L ⁻¹	[26]
MPFS	Fertilizers	6.6–20.0 mg L ⁻¹ P ₂ O ₅	0.1 mg L ⁻¹ P-PO ₄	[27]
FIA	Water	0.20–15.0 mg L ⁻¹	0.054 mg L ⁻¹	[28]
FIA	Not applied	up to 300 mg L ⁻¹	5.5 mg L ⁻¹	[29]
HSI	Wastewater	0.1–4.0 mg L ⁻¹	80 μg P L ⁻¹	[30]
FIA	Natural water	0.5–1000 μg L ⁻¹ P,	0.04 μg L ⁻¹ P	[31]
MP-FS	Natural water	up to 20 mg L ⁻¹	0.2 mg L ⁻¹	[32]
SIA	High salinity waters	14.8–73.9 μmol P L ⁻¹	0.3 μmol P L ⁻¹	[33]
SIA	Freshwaters	0.24–9.5 μM	0.07 μM	[20]

[18,19]. In the work of Mesquita et al from 2011 [20] it was evidenced the overall interest in combining sequential injection (SI) and phosphate determination with a list of the works (#16) addressing the subject. In the last ten years this approach has been kept a priority interest as shown in all the recent works devoted to phosphate determination using flow techniques (Table 1).

In Table 1 it is clear that the methods targeting natural waters need a very low detection limit [20,23,31,33] in the order of μmol/L. On the other hand, methods targeting nutrient solution and fertilizers need quantification in higher dynamic range [24,27] as consequence of high content in phosphate of supplement solutions. So, for targeting the leaching process, a combination of low detection limit and high dynamic range must be targeted. An efficient monitoring of the process will enable to anticipate ground water contamination.

In this context, the aim of this work was the development of an automatic, versatile and robust sequential injection (SI) method, capable of real-time monitoring of the soil leaching processes by phosphate monitoring. The idea was to perform the determination of phosphate in a wide quantification range by using the molybdenum blue chemical reaction with solid phase extraction (SPE) for in-line pre-concentration prior to spectrophotometric detection. The choice of the molybdenum blue reaction was based upon the overall acceptance use of this reaction to phosphate determination as shown in a recent extensive review [14]. The developed method was applied to leachates produced from a laboratory scale soil column (LSSC) of agricultural soil. The option for a LSSC with about 50 cm high was made aiming to include the entire column of surface soil. In previous works, intact soil cores had been used [34] but the maximum size attained was 25 cm, which represented mainly the organic layer on top of the surface soil layer. The choice of agricultural soil was made due to the pertinent issue of over fertilization used in agriculture. In order to obtain the leachates, rain simulations were carried out using collected rain water and rain water to which a commercial fertilizer was added [35–37]. The solutions used in the rain simulations were analyzed in the developed system before and after going through the LSSC column, in order to determine the phosphate concentration.

2. Material and methods

2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and boiled deionized water (resistivity < 0.1 mS/cm).

A 30 g/L ascorbic acid solution was prepared weekly by dissolving 3 g of the solid (C₆H₈O₆, Normapur) in 100 mL of water and stored in the refrigerator.

The molybdate reagent was prepared weekly, according to Mesquita et al. (2012) [33], by dissolving 2.5 mg of potassium antimony (III)

oxide tartrate hemihydrate (K(SbO)C₄H₄O₆ · 0.5 H₂O, Merck), 0.4 g of ammonium molybdate tetrahydrate ((NH₄)₆Mo₇O₂₄ · 4H₂O, Honeywell Fluka) and 0.18 g of tartaric acid (C₄H₆O₆, Merck) in 10 mL of 2M sulfuric acid (H₂SO₄), diluting to 25 mL of water and stored in the refrigerator. The final concentrations in the molybdate reagent being: 0.1 g/L C₄H₄KO₇Sb; 16 g/L (NH₄)₆Mo₇O₂₄ · 4H₂O; 7.5 g/L C₄H₆O₆; and 0.78 M H₂SO₄.

The eluent solution consisted of a 0.50 M sodium chloride (NaCl, Merck) solution and was prepared by dissolving 1.5 g of the solid in 50 mL of water.

A 1.0 mM phosphate stock solution was prepared by dissolving 7 mg of potassium dihydrogen phosphate (KH₂PO₄, Panreac) in 50 mL of water. A 200 μM phosphate solution was prepared by diluting 10 mL of the stock solution to 50 mL of water and the working standards in a range of 0.5–80 μM were prepared by dilution of the previous solution. All phosphate solutions and standards were stored in a refrigerator.

A washing buffer solution of ammonia/ammonium was prepared by dissolving 0.85 g of ammonium chloride (Merck) in 4 mL of commercial ammonia solution (d=0.91, 25%; Merck) and diluted to 100.0 mL of water.

For the leaching process simulation, rainwater and a commercial fertilizer were used to perform rain simulations. The rainwater was collected during the wet season in the metropolitan Porto area: a plastic container was left overnight collecting rain which was then filtered (filter 0.2 μm) before use. The commercial fertilizer solution (KB® universal liquid fertilizer) was prepared according to the manufacturer instructions: 7 mL of the fertilizer diluted to 1 L of rainwater. The composition of the KB® fertilizer (EC fertilizer NPK 6-3-6 + Micro fertilizer) is: 6% Total nitrogen (N), 3% Phosphorus pentoxide (P₂O₅) soluble in water, 6% Potassium oxide soluble in water, and water soluble trace elements (0.01% Boron (B), 0.002% Copper chelated with EDTA, 0.03% Iron chelated with DTPA, 0.01% Manganese chelated with EDTA, 0.001% Molybdenum, 0.002% Zinc chelated with EDTA).

2.2. Apparatus

2.2.1. Flow injection manifold – pre-concentration preliminary studies

A Gilson Minipuls 3 peristaltic pump propelled the solutions with a PVC pumping tube connected to the homemade acrylic commutator [38] and confluence devices. The tubing connecting the different components was made of PTFE (Omnifit) with 0.8 mm i.d.

2.2.2. Sequential injection system

The carrier solution was propelled by a Gilson Minipuls 3 peristaltic pump with a PVC pumping tube connected to the central channel of a ten-port electrically actuated selection valve (Valco VICI Cheminert

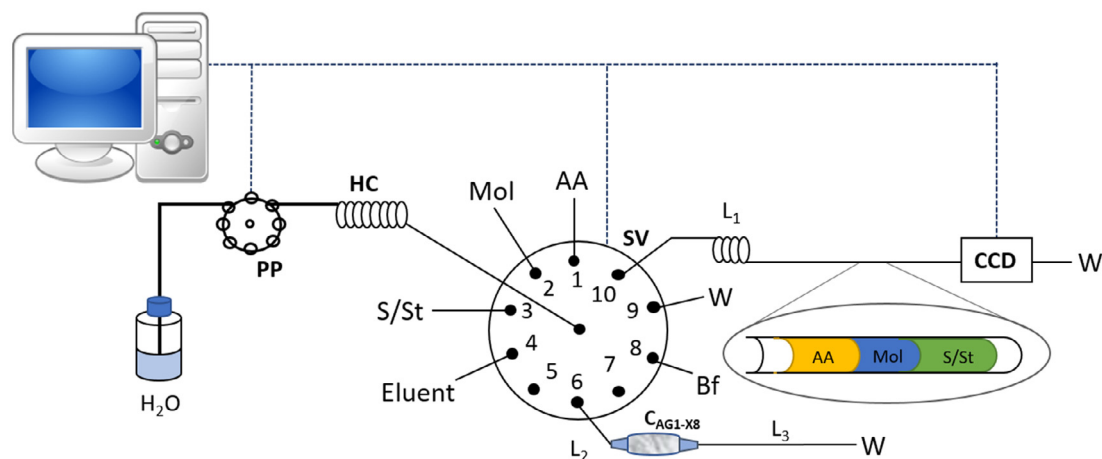


Fig. 1. Sequential injection manifold with in-line SPE for the determination of phosphate in natural waters and leachates; SV, selection valve; PP, peristaltic pump; HC, holding coil, 200 cm; AA, ascorbic Acid 30 g/L; MR, molybdate reagent 16 g/L; S/St, phosphate sample/standard; E, eluent solution, NaCl 0.5 M; B, washing buffer solution $\text{NH}_4^+/\text{NH}_3$; RC, reaction coil with 120 cm; CCD, spectrophotometer ($\lambda=710$ nm); L_i , connections with 3 cm and 20 cm length, respectively; $C_{\text{AG1-X8}}$, column packed with AG1-X8 anionic resin; W, waste.

Table 2

Sequential injection protocols for the determination of phosphate in the concentration ranges of 0.5–10 μM and 10–80 μM .

	Step	SV Position	Time (s)	Flow Rate ($\mu\text{L/s}$)	Volume (μL)	Description
In-line SPE - lower phosphate range	A	3	20	45	911	Aspiration of sample
	B	6	35	30	1060	Propel to column
	C	4	5	16	79	Aspiration of eluent
	D	6	6	16	95	Propel to column
	E	1	4	45	186	Aspiration of ascorbic acid solution
	F	2	3	16	48	Aspiration of molybdate reagent
	G	6	5	16	79	Aspiration of eluted sample
	H	10	7	58	423	Propel to reaction coil
	I	10	35	0	0	Pause in reaction coil
	J	10	30	58	1758	Propel to detector ($\lambda=710\text{nm}$)
	K	8	8	58	481	Aspiration of the washing buffer solution
	L	9	10	58	597	Propel to waste
	M	6	4	45	186	Propel water to column
Higher phosphate range	A	1	4	45	186	Aspiration of ascorbic acid solution
	B	2	3	16	48	Aspiration of molybdate reagent
	C	3	5	16	79	Aspiration of sample
	D	10	14	30	425	Propel to reactor
	E	10	45	0	0	Pause in reactor
	F	10	30	58	1758	Propel to detector ($\lambda=710\text{nm}$)

C25-3180EUHB) and the tubing connecting to the solutions and the detector was made of PTFE (Omnifit) with 0.8 mm i.d.

A computer (HP Pavilion zt3000) equipped with a National Instruments DAQcard-DIO interface card, running a homemade software, was used to control the selection valve (SV) position and the peristaltic pump direction and speed.

2.2.3. Detection system

An Ocean Optics USB 4000 charged coupled device detector (CCD), equipped with a pair of 300 μm fibre optic cable and a Micropack DH-2000 deuterium halogen light source, was used as the detection system. A Hellma 178.011-OS flow-cell with 10 mm light path and 30 μL inner volume was used as flow cell. Data acquisition signal was obtained at 710 nm and it was performed through the OceanOptics – Spectrasuite software running in a personal computer (HP L1706).

2.3. Sequential injection manifold and procedure

The sequential injection (SI) manifold with in-line solid phase extraction (SI-SPE) developed for the colorimetric determination of phosphate in natural waters and leachates, is presented in Fig. 1.

The wide phosphate quantification range of 0.5–80 μM attained with the developed method was accomplished in two separate procedures, with and without phosphate pre-concentration. For the range 0.5–10 μM (lower range) the solid phase extraction (SPE) of phosphate was employed, while for the range 10–80 μM (higher range), the determination was carried out without SPE. The sequence of steps, including the operation time and volume, for both determination ranges i.e. with and without SPE, are shown in Table 2.

For the concentration range of 0.5–10 μM , the protocol was initiated by aspirating the sample and propelled it through the AG1-X8 resin column (steps A and B) for performing phosphate retention and ensuring that the column was washed. Then, eluent was also aspirated (NaCl 0.5 M) and propelled to the column (steps C and D) ensuring that it remained inside the resin column for phosphate elution. Subsequently, the ascorbic acid solution was aspirated followed by the molybdate reagent and the eluted phosphate (steps E, F and G). These plugs were then propelled to the reaction coil (step H) where they stay for 35 seconds (step I) to promote the reaction. Afterwards, it continued to be propelled to the detector (step J) where the absorbance increase was registered.

The following steps were washing steps: to wash the holding coil the buffer solution was aspirated and sent to waste (steps K and L); and to

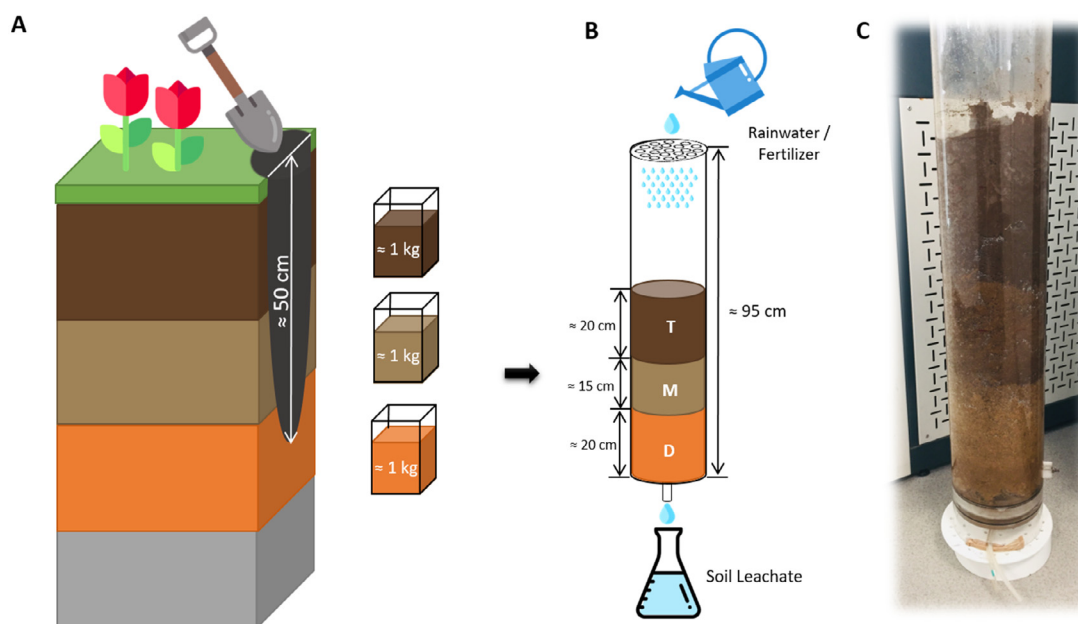


Fig. 2. Laboratory scale soil column set up with three layers: top (T), middle (M) and deep (D); A, schematic representation of set up procedure; B, schematic representation of the leachate production process; C, photograph of the LSSC.

wash the resin column carriers, deionized water, was propelled through (step M).

For the concentration range of 10–80 μM , first the ascorbic acid solution was aspirated followed by the molybdate reagent and the sample (steps A, B and C). Then, mixing of the aspirated plugs was promoted by propelling to the reaction coil (step D); a stop period of 45 seconds was made to promote the reaction (step E) before sending to the detector (step F).

At the end of the day's work, the entire detection system was washed in order to prevent the adsorption of the molybdenum blue on the coil's and flow cell's walls: washing $\text{NH}_4^+/\text{NH}_3$ buffer solution was aspirated to the holding coil and propelled through the reaction coil and flow cell.

2.4. Laboratory Scale Soil Column (LSSC) setup

To simulate the leaching process, a laboratory scale soil column (LSSC) of about 50 cm height was set up, with a faithful replication of the soil layers different characteristics at different depths, and rain simulations performed (Fig. 2).

The soil was collected in Northwestern Portugal, by separately saving the visually distinct layers (#3) while digging a hole with approximately 50 cm depth. For each layer of soil, of about 20 cm depth each, 1 kg of soil was collected (Fig. 2(A)) and then reassembled in an acrylic column to accurately recreate the soil structure: top layer (T), middle layer (M) and deeper layer (D). The established LSSC was then used for leachate production by "rain simulations" as reproduction of the leaching process.

The rain simulation process was carried out daily by dispensing 100–500 mL of rainwater or the commercial fertilizer on top of the LSSC column and collecting the leachate at the bottom (Fig. 2(B)). The daily simulations resulted in a residence time of about 10 min.

2.4.1. Soil characterization

The general characteristics of the collected soil, namely its texture classification, pH, water capacity retention and organic matter, were assessed (Table 3).

Composed samples of the three layers were made to assess the pH and the water retention capacity. As for the organic matter, it was assessed

for each layer independently and, as expected, the top layer presented higher organic content as it corresponds to the organic layer of the soil (about 20 cm depth). It was about 10% higher than for the other two layers: the T layer contained 713.8 mg of organic matter per gram of soil, the M layer 642.1 mg per gram of soil and the D layer 650.1 mg per gram of soil.

The top layer (T) showed to be composed of approximately 76% sand and 24% of clay and silt, while the two layers below (M and D) were mainly composed of sand, 94% and 99%, respectively. These values result in different soil texture categories were schematic represented (ESI Fig. 1).

As the column was composed mainly by sand/sandy soil layers, a fast infiltration rate [39] was expected, and in fact the measured infiltration rate was 150 mm/hour, which corresponds to a high infiltration rate.

2.5. Reference procedure and certified materials

To evaluate the accuracy of the developed SI-SPE method, results obtained were compared with the reference procedure 4500-P from Standard Methods for Examination of Water and Wastewater [40].

Additionally, three certified materials to establish reference water standards were used: VKI QC RW1 ampoules for nutrients analysis in recipient water; EnviroMAT Waste Water, High (EU-H-3); EnviroMAT Ground Water, High (ES-H-2).

3. Results and discussion

3.1. SPE preliminary studies – flow injection system

Since low values of phosphate in both rainwater and leachate (obtained from rainwater) were expected, a pre-concentration step was needed, and the idea was to incorporate it in the manifold for an in-line process.

To attain this pre-concentration, solid phase extraction (SPE) with an ionic resin packed column (ESI Fig. 2) was the chosen approach. In order to carry out a detailed study of the performance of different ionic resins, column sizes, and eluents, a flow injection (FI) manifold (Fig. 3) was assembled. Some of the conditions for the colorimetric reaction were

Table 3

Characterization of the collected surface soil to set up the laboratory scale soil column (LSSC); top layer (T), middle layer (M) and deep layer (D).

Layer ID	pH ^a	Retention capacity ^a (g H ₂ O/100 g soil)	Organic matter (mg / g)	Granularity (%)		Soil texture Category
				Sand	Clay & Silt	
T	4.34	39.6	713.8 ± 0.3	75.7	24.2	Sandy clay loam
M			642.1 ± 10.7	93.5	6.5	Sand
D			650.1 ± 14.5	99.2	0.8	Sand

^a for these parameters a composed sample made with the 3 layers of soil was used.

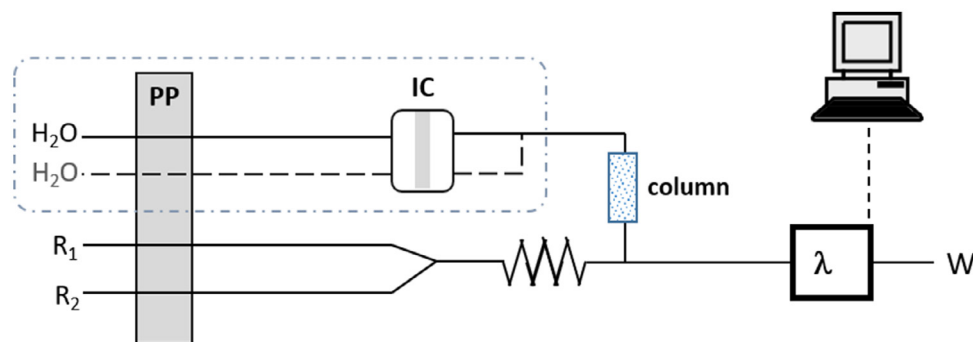


Fig. 3. Flow injection manifold used for the resin column studies; IC, injection commutator with one or two loops and carrier flow streams (dashed square); PP, peristaltic pump; R₁, ascorbic acid 7 g/L; R₂, ammonium molybdate 6 g/L; λ, detector; W, waste.

based upon previous work [20], namely the choice of the proportions in the molybdate reagent, and also the use of ascorbic acid as the reducing agent. Other flow parameters had to be re-evaluated.

The FI system was used due to its intrinsic simplicity and consisted of a peristaltic pump (PP in Fig. 3) to propel three flow streams, the carrier, the molybdate reagent and ascorbic acid (H₂O, R₁ and R₂ in Fig. 3). An injection commutator (IC in Fig. 3) was employed in order to enable the use of one or two sample/standard loops (ESI Fig. 3) requiring one or two carrier streams (dashed square in Fig. 3).

The first study to be carried out was the choice of the resin and three commercially available anionic resins, AG1-X8, Dowex 50 × 8 and Dowex Marathon 11, were tested using one loop approach (ESI Fig. 3A). After assembling the columns (ESI Fig. 2) the phosphate standard (20 mg/L) was loaded at the IC loop (500 μL), passing through the column and the signal register (Fig. 3). When using the resin AG1-X8, unlike the other two resin, no signal was observed inferring that the phosphate had been completely retained, so this resin was chosen.

Then, to remove the retained phosphate from the column, different eluents, NaOH, NaNO₃ and NaCl, were tested. The AG1-X8 column was loaded with phosphate standard and then the IC loop was loaded with the tested eluents.

Using NaOH as eluent, no signal was observed, indicating that it failed to remove the phosphate from the column; when NaNO₃ was used as eluent there was a continuous increase of the baseline and high oscillation of the signal. The NaCl was the chosen eluent since it successfully removed phosphate from the column producing a registered signal without causing interference in the color reaction.

Afterwards, the influence of the eluent volume was studied and, for this study, the two loops approach (ESI Fig. 3(A) and (B)) was employed using two carrier streams (dashed square in Fig. 3). Calibration curves were established and the eluent (NaCl) volumes in the range 50–100 μL were tested (ESI Fig. 4(A)). As the sensitivity (calibration curve slope) increased up to 75 μL of NaCl without a significant increase of the intercept, it was the volume chosen.

The influence of the column size was also tested, setting the minimal to 1 cm, as previously reported for reproducible results [41], three different column sizes, 1, 1.5 and 2 cm were assembled. Calibration curves were established and the sensitivity decreased with the increase of the column size (–17% and –28% for 1.5 and 2 cm, respectively) and so, a 1 cm length column was chosen.

3.2. Colorimetric reaction parameters – sequential injection manifold

In order for the methodology to attain a wide quantification range, it was important to combine, in the same flow-based manifold, the possibility of performing a pre-concentration approach, and a direct (no pre-concentration) measurement.

In this context, sequential injection (SI) was considered a better choice, as it would enable to comprise both approaches in a single manifold, resorting to its computer-controlled capacity.

Having set, in Section 3.1, the anionic resin, the size of the column, the eluent and the eluent volume, the following studies were performed in the SI system described in Section 2.2. For the reaction conditions studies, namely reagents concentrations, solutions volumes, and reaction extension, calibration curves were traced using the direct determination without the pre-concentration step. The sample volume was set to 75 μL, the same previously set for the eluent.

3.2.1. Ascorbic acid

The ascorbic acid was the first reagent to be studied and both the influence of its concentration and its volume was assessed. Calibration curves within the range 10–80 μM were established for ascorbic acid concentrations of 10, 20 and 30 g/L (ESI Fig. 4(B)). The concentration that resulted in a higher sensitivity (calibration curve slope) was 30 g/L of ascorbic acid, which was the concentration used in the study of the influence of the volume of ascorbic acid. Calibration curves with volumes of 150, 185 and 230 μL of ascorbic acid 30 g/L were established (ESI Fig. 4(C)) and there was no statistically significant difference between sensitivities obtained with the volumes of 150 and 185 μL (variation <10%). However, to assure a reagent excess, and supported by a better correlation factor, 186 μL was chosen as the optimal volume of ascorbic acid. The highest volume tested resulted in the lowest sensitivity recorded so it was discarded.

3.2.2. Molybdate reagent

The molybdate reagent preparation was based upon previously reported studies by Mesquita et al. [33]. In order to find the best volume of the molybdate reagent for the developed SI-SPE method, three volumes (50, 80, 95 μL) were tested by establishing calibration curves (ESI Fig. 4(D)). There was no statistically significant difference (variation

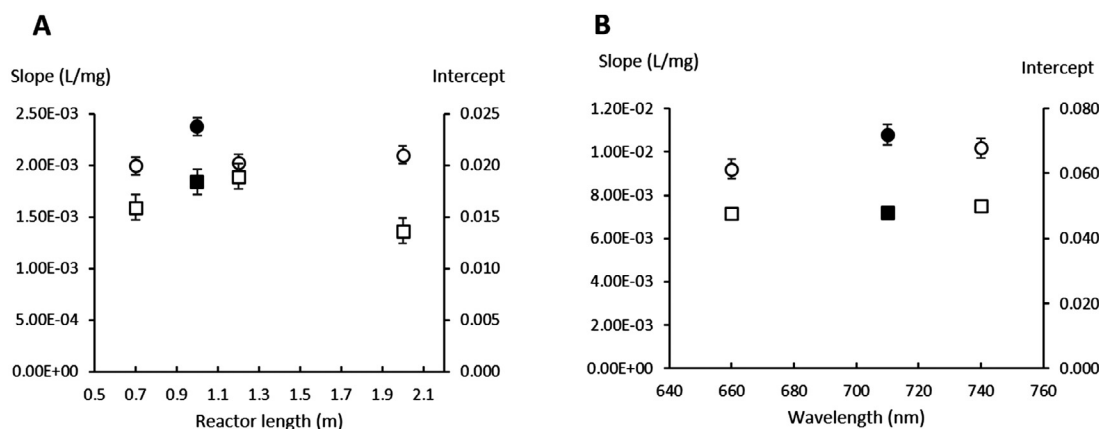


Fig. 4. Study of the influence in calibration curve slope (○) and intercept (□) of the: (A) reactor length; and (B) wavelength of detection; the points in black represent the chosen parameters.

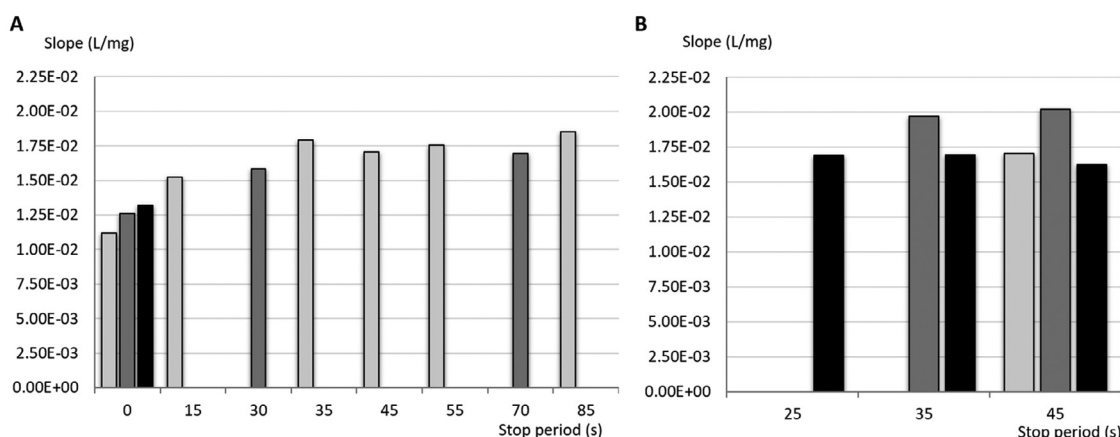


Fig. 5. Study of the influence of different flow rates (F) and stop periods in the calibration curve slope of the phosphate determination; light gray bars, $F = 58 \mu\text{L/s}$; dark gray bars, $F = 30 \mu\text{L/s}$; black bars $F = 16 \mu\text{L/s}$; (A) same F before (F_b) and after (F_a) the stop period; (B) different F_b with a F_a of $58 \mu\text{L/s}$.

<10 %) between the three tested volumes, the volume of $48 \mu\text{L}$ was chosen for reagent saving.

3.2.3. Reactor and detection wavelength

Considering the slow reduction kinetics associated to the molybdenum blue reaction [14], different reaction coils lengths were tested to improve reaction extension. Values between 0.7 and 2 m were tested, and calibration curves were set (Fig. 4(A)). The reaction coil with 1 m resulted in a calibration curve with the highest sensitivity and therefore was the chosen length to continue the optimization studies.

The spectra was made for different reaction times (ESI Fig. 5) and the maximum absorption was observed in the range 720–740 nm. However, due to the used of 660 nm in a previous work [20,33], three wavelengths were tested, 660, 720, 740 nm (Fig. 4(B)).

Calibration curves were set for all the wavelengths and the wavelength that allowed a better sensitivity (highest slope) was the 710 nm. Therefore, the following studies were performed with the detection at 710 nm.

As previously mentioned the molybdenum blue reaction has a slow reduction kinetics [14]; so, aiming to improve the reaction extension, the flow rate of sending to the detector was studied. Different flow rates (F) result in different reaction times and consequently reaction rate. This study of the flow rate was combined with the study of having a stop period prior to detection.

When there was a stop period, two flow rates had to be accountable: flow rate before the stop period (F_b) and flow rate after the stop period (F_a). Three flow rates 58, 30 and $16 \mu\text{L/s}$, and several stop periods, 0–

85 s were tested (Fig. 5). Calibration curves were established for the different conditions and the slopes were compared.

When there was no stop period, (Fig. 5(A)) the sensitivity increased with the decreased of the flow rate with $16 \mu\text{L/s}$ attaining the highest sensitivity (black bar in Fig. 5(A)).

However, this would result in an increase of the determination time, decreasing the determination rate (22 determinations per hour). The idea was to include the stop period in order to increase the sensitivity without increasing too much the determination time.

Stop times were tested for 58 and $30 \mu\text{L/s}$ flow rates maintaining the same flow rate before and after the stop period, $F_b = F_a$ (Fig. 5(A)). It was observed that stop periods over 35 s did not significantly improve the sensitivity. Having established to include a stop period with two flow rates to be considered, before (F_b) and after (F_a) the stop period, it was tested to have different F_b maintaining the F_a at $58 \mu\text{L/s}$ (Fig. 5(B)).

This approach enabled to definitely chose a stop period of 45 s, setting the determination rate at 35 determinations per hour.

All these studies were made for the phosphate quantification range of 10–80 μM , attained without pre-concentration. The chosen parameters will be employed for the lower range to be achieved with the solid phase extraction approach for pre-concentration.

3.3. Phosphate determination with pre-concentration – SI-SPE

Solid phase extraction (SPE) is often used to perform a pre-concentration step for low concentrations assessment. As above-mentioned (Section 3.1), to attain the expected low concentration of

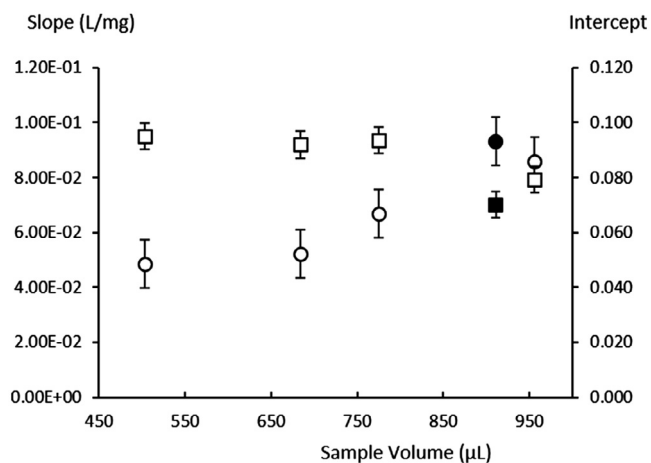


Fig. 6. Study of the influence of the sample volume in the calibration curve slope (○) and intercept (□); the point in black represent the chosen parameters.

Table 4

Assessment of potential interference from major ions; phosphate standards of 1mg/L (With SPE); and 0.5 mg/L (Without SPE).

Potential interfering ion	Tested values	Percentage of Interference	
		With SPE	Without SPE
NO ₃ ⁻	50 mg/L	-1%	-1%
NO ₂ ⁻	1 mg/L	2%	4%
Cl ⁻	1g/L	3%	5%
SO ₄ ²⁻	1g/L	-4%	9%
SiO ₃ ⁻	15 mg/L	1%	8%
BrO ₃ ⁻	1 mg/L	0%	-
Br ⁻	1 mg/L	-2%	-
S ²⁻	1 µg/L	-2%	-

phosphate in rainwater and leachates obtained from rainwater a pre-concentration step using SPE was planned for the developed methodology.

According to the preliminary studies, the SPE conditions were set: a column of approximately 1 cm length packed with AG1-X8 anionic exchange resin and 75 µL of NaCl 0.5 M as eluent. In this context, the remaining parameter to be assessed was the sample volume to go through the column for phosphate pre-concentration. Several sample/standard volumes were tested, ranging from 500 to 950 µL, establishing calibration curves in the phosphate concentration range of 0.5–10 µM (Fig. 6). The sensitivity (calibration curve slope) increase up to 910 µL, accompanied by a decrease of the intercept, so it was the chosen volume.

The developed SI-SPE method (Fig. 2) with the two protocol sequences to comprise both approaches, with and without pre-concentration, effectively enables the phosphate quantification ranging from 0.5 to 80 µM.

3.4. Interferences

Aiming for application to natural waters (rainwater) and soil leachates (obtained from rain simulations with rainwater and fertilizer) an assessment of potential interferences was carried out. Major ions potentially present in natural waters were tested. In order to study the potential interference of those ions, mixed standards with the target analyte, phosphate, and the potential interfering ion were prepared.

Then, the absorbance values of the mixed standard (A_{ms}) were compared to the absorbance value of a phosphate standard (A_{ps}). The percentage of interference calculated as according to the equation: % interference = $(A_{ms} - A_{ps})/A_{ps}$ (Table 4). No interferences were observed since the calculated interference percentage was < 5% for the approach with SPE and < 10% for the approach without SPE.

3.5. Features of the developed SI system

The characteristics of the developed SI-SPE method for the determination of phosphate in a wide quantification range were summarized in Table 5, namely the dynamic ranges and the limit of detection.

The limit of detection (LOD) was calculated as corresponding to the concentration of three times the standard deviation of ten consecutive injection of deionised water, according to IUPAC recommendation [42].

The effluent volume was calculated by the sum of all the volumes propelled to waste per cycle. The sample consumption volume was much higher for the lower range of concentrations, with the in-line SPE, in order to attain an effective pre-concentration. Considering three replicas per sample, the overall sample consumption was about 2.7 mL and 240 µL for the determination with and without SPE, respectively. The determination rate was calculated as the sum of all the times spent in an entire analytical cycle, including the washing steps of the determination with SPE.

The overall reagent consumption per determination were 5.6 mg of C₆H₈O₆; 4.8 µg of C₄H₄KO₇Sb; 768 µg of (NH₄)₆Mo₇O₂₄·4H₂O; 360 µg of C₄H₆O₆; 3.7 mg of H₂SO₄; with additional consumption, for the lower range of concentrations with the in-line SPE, of: 4.1 mg of NH₄Cl; 4.4 mg of NH₃ and 2.3 mg of NaCl.

3.6. Validation and application

3.6.1. Accuracy assessment

To evaluate the accuracy of the developed sequential injection method with (SI-SPE) and without SPE (SI), certified water samples (CS) were analysed and the results obtained compared to the certified value (CV) (Table 6). Different solutions were obtained by dilution of three reference materials and analysed by the proposed method: six solutions (CS_{rw_1} to CS_{rw_6}) from the natural water QC-RW1; two solutions (CS_{ww_1} and CS_{ww_2}) from the wastewater EU-H-3 CRM; and the ground water ES-H-2 (CS_{gw}).

Additionally, samples of soil leachates (LS), obtained from rain simulations with rainwater (LS_{rw}) and fertilizer (LS_f), were analysed with the developed methodologies (SI/SI-SPE) and the results compared to the reference method (RM) (Table 6).

The comparison between the two sets of results was made by calculating the relative deviation percentage (RD%) as: $RD\% = ([PO_4^{3-}]_{SI/SI-SPE} - [PO_4^{3-}]_{CV/RM}) / [PO_4^{3-}]_{CV/RM}$. Most of the calculated relative deviation percentage were <10%, indicating no significant different between the two set of results.

A linear relation between the two sets of results was established for each approach with (ESI Fig. 6(A)) and without SPE (ESI Fig. 6(B)). The equations obtained were: $[PO_4^{3-}]_{SI-SPE} = 0.961 (\pm 0.058) \times [PO_4^{3-}]_{CV/RM} - 0.094 (\pm 0.32)$; $R = 0.998$, for the lower dynamic phosphate concentration range and $[PO_4^{3-}]_{SI} = 0.931 (\pm 0.128) \times [PO_4^{3-}]_{CV} - 2.70 (\pm 6.45)$; $R = 0.997$ for the higher dynamic phosphate concentration range, where the values in brackets represent the confidence interval at 95% confidence level. These values indicating that there is no statistical difference between the two sets of results [43] as the slope and the intercept do not statistically differ from 1 and zero, respectively.

3.6.2. Application to the leachates from the LSSC

Several rain simulations (#15), with both rainwater and fertilizer solution were made to produce leachates which were analysed using the developed method with (SI-SPE) or without (SI) the pre-concentration column, for lower and higher dynamic range, respectively (ESI Table 1). All the leachates were analysed with no sample pre-treatment and run with the SI approach first (due to a higher throughput), then, if the registered value was below the calibration curve values (10–80 µM), it was run with the SI-SPE approach.

As expected, the amount of phosphate in the leachate increased with repeated rain simulation with fertilizers. These results indicate the

Table 5

Features of the developed sequential injection system for phosphate determination; LOD, limit of detection.

Approach	Dynamic range (μM)	Calibration curve* $A = S \times [\text{PO}_4^{3-}] \mu\text{M} + b$	LOD (μM)	Effluent volume** (mL)	Sample consumption** (μL)	Determination rate (h ⁻¹)
With SPE	0.54–10	$A = 1.48 \times 10^{-2} (\pm 7 \times 10^{-4}) [\text{PO}_4^{3-}] + 5.3 \times 10^{-2} (\pm 7 \times 10^{-3})$ $R^2 = 0.9992 (\pm 3 \times 10^{-4})$	0.52	4.1	912	35
Without SPE	10–80	$A = 3.88 \times 10^{-3} (\pm 1 \times 10^{-5}) [\text{PO}_4^{3-}] + 2.2 \times 10^{-2} (\pm 5 \times 10^{-3})$ $R^2 = 0.998 (\pm 3 \times 10^{-3})$	–	2.2	80	20

* $n=3$; ** per determination.**Table 6**Application of the developed SI system to phosphate determination in certified water samples (CS₁) and soil leachates samples (LS₁); comparison of the results obtained with the developed method (SI/SI-SPE) and the certified value (CV) or the reference method (RM), respectively; RD, relative deviation.

Approach	Sample ID	$[\text{PO}_4^{3-}]_{\text{CV}} (\mu\text{M})$	$[\text{PO}_4^{3-}]_{\text{SI}} (\mu\text{M})$	RD%
without SPE – higher concentration range	CS_rw_1	80.7 ± 1.6	79.3 ± 0.8	–2%
	CS_rw_2	40.4 ± 0.8	40.0 ± 0.4	–1%
	CS_rw_3	60.5 ± 1.2	56.5 ± 1.8	–7%
	CS_rw_4	13.7 ± 0.3	14.6 ± 2.9	7%
	CS_ww_1	25.3 ± 0.5	28.4 ± 0.2	12%
Approach	Sample ID	$[\text{PO}_4^{3-}]_{\text{CV/RM}} (\mu\text{M})$	$[\text{PO}_4^{3-}]_{\text{SI-SPE}} (\mu\text{M})$	RD%
With SPE – lower concentration range	CS_rw_5	9.70 ± 0.19	9.27 ± 0.9	–4%
	CS_rw_6	6.10 ± 0.12	6.07 ± 0.17	0%
	CS_ww_2	7.60 ± 0.15	7.32 ± 0.18	–4%
	CS_gw	0.72 ± 0.01	0.78 ± 0.06	8%
	LS_rw_1	0.74 ± 0.03	0.67 ± 0.01	–10%
	LS_f	4.52 ± 0.03	4.80 ± 0.29	6%
	LS_rw_2	1.44 ± 0.00	1.40 ± 0.31	–4%

potential contamination of ground waters when excess fertilization is employed.

4. Conclusions

In this work, an automatic, sequential injection method combining determination with and without solid phase extraction was developed for the quantification of phosphate within a wide concentration range in natural waters and soil leachates samples. In order to achieve the expected lower concentrations, the developed solid phase extraction approach, SI-SPE, proved to be highly effective. The chosen resin for the SPE, AG1-X8, showed the best performance in the flow in-line mode.

Being phosphate a well-known contaminant, its content is an indicator of water quality and it is of vital importance its monitoring in real-time. This becomes particularly relevant by the increase/abuse use of fertilizers in agricultural activities, which can lead to a phosphate excess in ground waters. The developed method enabled a phosphate determination in a concentration range of 0.5–80 μM, with detection limit of 0.5 μM.

Although there are some methods with lower quantification range and detection limit [20,21,31,33] the described methodology presents a wider dynamic range, specially targeted for monitoring the leaching process. Nevertheless, some of the mentioned works have only slightly better values [21,33], another is based on flow injection analysis technique [31] and the work of Mesquita et al [20] uses a non-commercially available specially designed multi-reflective flow cell.

Still, since phosphate concentration in waters should be below 0.1 mg/L [12] (1 μM), the developed sequential injection method is highly effective to monitor contamination due to excess levels in natural water samples including ground waters as simulated leachates.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2020.100015.

References

- [1] M. Naveed, P. Moldrup, H.Jö. Vogel, M. Lamandé, D. Wildenschild, M. Tuller, L.W. de Jonge, Impact of long-term fertilization practice on soil structure evolution, *Geoderma* 217–218 (2014) 181–189, doi:10.1016/j.geoderma.2013.12.001.
- [2] H. Zhou, H. Fang, S.J. Mooney, X. Peng, Effects of long-term inorganic and organic fertilizations on the soil micro and macro structures of rice paddies, *Geoderma* 266 (2016) 66–74, doi:10.1016/j.geoderma.2015.12.007.
- [3] S. Schlüter, U. Weller, H.J. Vogel, Soil-structure development including seasonal dynamics in a long-term fertilization experiment, *J. Plant Nutr. Soil Sci.* 174 (2011) 395–403, doi:10.1002/jpln.201000103.
- [4] A. Pernes-Debuysse, D. Tessier, Soil physical properties affected by long-term fertilization, *Eur. J. Soil Sci.* 55 (2004) 505–512, doi:10.1111/j.1365-2389.2004.00614.x.
- [5] T. Fan, B.A. Stewart, W. Yong, L. Junjie, Z. Guangye, Long-term fertilization effects on grain yield, water-use efficiency and soil fertility in the dryland of Loess Plateau in China, *Agric. Ecosyst. Environ.* 106 (2005) 313–329, doi:10.1016/j.agee.2004.09.003.
- [6] S. Zhang, X. Yang, M. Wiss, H. Grip, L. Lövdahl, Changes in physical properties of a loess soil in China following two long-term fertilization regimes, *Geoderma* 136 (2006) 579–587, doi:10.1016/j.geoderma.2006.04.015.
- [7] P. Worsfold, I. McKelvie, P. Monbet, Determination of phosphorus in natural waters: A historical review, *Anal. Chim. Acta* 918 (2016) 8–20, doi:10.1016/j.aca.2016.02.047.
- [8] A. Salehi, S. Fallah, A.A. Sourki, Organic and inorganic fertilizer effect on soil CO₂ flux, microbial biomass, and growth of *Nigella sativa* L., *Int. Agrophys.* 31 (2017) 103–116, doi:10.1515/intag-2016-0032.

- [9] M.K. Abbasi, A. Khizar, Microbial biomass carbon and nitrogen transformations in a loam soil amended with organic-inorganic N sources and their effect on growth and N-uptake in maize, *Ecol. Eng.* 39 (2012) 123–132, doi:10.1016/j.ecoleng.2011.12.027.
- [10] G. Duffy, I. Maguire, B. Heery, C. Nwankire, J. Ducrée, F. Regan, PhosphaSense: a fully integrated, portable lab-on-a-disc device for phosphate determination in water, *Sens. Actuators B Chem.* 246 (2017) 1085–1091, doi:10.1016/j.snb.2016.12.040.
- [11] A.T. Law Al, S.B. Adelolu, Progress and recent advances in phosphate sensors: a review, *Talanta* 114 (2013) 191–203, doi:10.1016/j.talanta.2013.03.031.
- [12] R.C. Rodríguez-Díaz, M.P. Aguilar-Caballeros, F. Rincón, A. Gómez-Hens, Determination of soluble phosphates in water samples using ytterbium(III) and dynamic measurements of light scattering intensity at long wavelength, *Talanta* 69 (2006) 1130–1135, doi:10.1016/j.talanta.2005.12.013.
- [13] A.B. Cundy, L. Hopkinson, R.L.D. Whitby, Use of iron-based technologies in contaminated land and groundwater remediation: a review, *Sci. Total Environ* 400 (2008) 42–51, doi:10.1016/j.scitotenv.2008.07.002.
- [14] E.A. Nagul, I.D. McKelvie, P. Worsfold, S.D. Kolev, The molybdenum blue reaction for the determination of orthophosphate revisited: opening the black box, *Anal. Chim. Acta* 890 (2015) 60–82, doi:10.1016/j.aca.2015.07.030.
- [15] N. Kaewwonglom, J. Jakmunee, Sequential injection system with multi-parameter analysis capability for water quality measurement, *Talanta* 144 (2015) 755–762, doi:10.1016/j.talanta.2015.07.005.
- [16] M. Miró, E.H. Hansen, Recent advances and perspectives in analytical methodologies for monitoring the bioavailability of trace metals in environmental solid substrates, *Microchim. Acta* 154 (2006) 3–13, doi:10.1007/s00604-006-0493-1.
- [17] M.I.G.S. Almeida, M.A. Segundo, J.L.F.C. Lima, A.O.S.S. Rangel, Potentiometric multi-syringe flow injection system for determination of exchangeable potassium in soils with in-line extraction, *Microchem. J.* 83 (2006) 75–80, doi:10.1016/j.microc.2006.02.004.
- [18] T.C.F. Ribas, I.V. Tóth, A.O.S.S. Rangel, A solid phase extraction flow injection spectrophotometric method for the zinc determination in plants, *Microchem. J.* 130 (2017) 366–370, doi:10.1016/j.microc.2016.10.016.
- [19] V. Camel, Solid phase extraction of trace elements, *Spectrochim. Acta B At. Spectrosc.* 58 (2003) 1177–1233, doi:10.1016/S0584-8547(03)00072-7.
- [20] R.B.R. Mesquita, M.T.S.O.B. Ferreira, I.V. Tóth, A.A. Bordalo, I.D. McKelvie, A.O.S.S. Rangel, Development of a flow method for the determination of phosphate in estuarine and freshwaters – comparison of flow cells in spectrophotometric sequential injection analysis, *Anal. Chim. Acta* 701 (2011) 15–22, doi:10.1016/j.aca.2011.06.002.
- [21] W. Khongpet, P. Yanu, S. Pencharee, C. Puangpila, S. Krattap Hartwell, S. Lapanantnoppakhun, Y. Yodthongdee, A. Paukpol, J. Jakmunee, A compact multi-parameter detection system based on hydrodynamic sequential injection for sensitive determination of phosphate, nitrite, and nitrate in water samples, *Anal. Methods* 12 (2020) 855–864, doi:10.1039/C9AY02327E.
- [22] W. Khongpet, S. Pencharee, C. Puangpila, S.K. Hartwell, S. Lapanantnoppakhun, J. Jakmunee, A compact hydrodynamic sequential injection system for consecutive on-line determination of phosphate and ammonium, *Microchem. J.* 147 (2019) 403–410, doi:10.1016/j.microc.2019.03.040.
- [23] S. Sateanchok, N. Pankratova, M. Cuartero, T. Cherubini, K. Grudpan, E. Bakker, In-line seawater phosphate detection with ion-exchange membrane reagent delivery, *ACS Sens.* 3 (2018) 2455–2462, doi:10.1021/acssensors.8b01096.
- [24] S. Karadağ, E.M. Görüşük, E. Çetinkaya, S. Deveci, K.B. Dönmez, E. Uncuoğlu, M. Doğu, Development of an automated flow injection analysis system for determination of phosphate in nutrient solutions, *J. Sci. Food Agric.* 98 (2018) 3926–3934, doi:10.1002/jsfa.8911.
- [25] S. Koronkiewicz, M. Trifescu, L. Smoczynski, H. Ratnaweera, S. Kalinowski, A novel automatic flow method with direct-injection photometric detector for determination of dissolved reactive phosphorus in wastewater and freshwater samples, *Environ. Monit. Assess.* 190 (2018) 133, doi:10.1007/s10661-018-6511-z.
- [26] W. Khongpet, S. Pencharee, C. Puangpila, S. Krattap Hartwell, S. Lapanantnoppakhun, J. Jakmunee, Exploiting an automated microfluidic hydrodynamic sequential injection system for determination of phosphate, *Talanta* 177 (2018) 77–85, doi:10.1016/j.talanta.2017.09.018.
- [27] C.C. Crispino, M.Y. Kamogawa, J.R. Ferreira, E.A.G. Zagatto, Sulphate radical generation through interaction of peroxydisulphate with Co(II) for in-line sample preparation aiming at spectrophotometric flow-based determination of phosphate and phosphite in fertilizers, *Talanta* 158 (2016) 270–275, doi:10.1016/j.talanta.2016.05.072.
- [28] J. Kozak, K. Latocha, J. Kochana, M. Wiecek, P. Kościelniak, Simultaneous spectrophotometric flow injection determination of phosphate and silicate, *Talanta* 133 (2015) 150–154, doi:10.1016/j.talanta.2014.07.057.
- [29] M. Fiedoruk, E. Mieczkowska, R. Koncki, L. Tymecki, A bimodal optoelectronic flow-through detector for phosphate determination, *Talanta* 128 (2014) 211–214, doi:10.1016/j.talanta.2014.04.086.
- [30] S. Somnam, S. Motomizu, K. Grudpan, J. Jakmunee, Hydrodynamic sequential injection with stopped-flow procedure for consecutive determination of phosphate and silicate in wastewater, *Chiang Mai J. Sci.* 41 (2014) 606–617.
- [31] E.A. Nagul, C. Fontàs, I.D. McKelvie, R.W. Cattrall, S.D. Kolev, The use of a polymer inclusion membrane for separation and preconcentration of orthophosphate in flow analysis, *Anal. Chim. Acta* 803 (2013) 82–90, doi:10.1016/j.aca.2013.07.052.
- [32] M.F.T. Ribeiro, C.M.C.M. Couto, P.M.M. Conceição, J.L.M. Santos, An automated multi-pumping pulsed flow system with spectrophotometric detection for the determination of phosphate in natural waters, *Anal. Lett.* 46 (2013) 1769–1778, doi:10.1080/00032719.2012.703273.
- [33] R.B.R. Mesquita, I.C. Santos, A.A. Bordalo, A.O.S.S. Rangel, Sequential injection system exploring the standard addition method for phosphate determination in high salinity samples: Interstitial, transitional and coastal waters, *Anal. Methods* 4 (2012) 1452–1457, doi:10.1039/c2ay05792a.
- [34] L.S. Mesquita, R.B.R. Mesquita, A. Leite, T. Moniz, M. Rangel, A.O.S.S. Rangel, Integrated Flow-based system displaying an in-line mini soil column to monitor iron species in soils leachates, *Commun. Soil Sci. Plant Anal* 51 (2020) 1089–1100, doi:10.1080/00103624.2020.1751186.
- [35] L. Jean-Soro, F. Borda, J.C. Bollinger, Column leaching of chromium and nickel from a contaminated soil using EDTA and citric acid, *Environ. Pollut* 164 (2012) 175–181, doi:10.1016/j.envpol.2012.01.022.
- [36] I.B. Bame, J.C. Hughes, L.W. Titshall, C.A. Buckley, Leachate characteristics as influenced by application of anaerobic baffled reactor effluent to three soils: a soil column study, *Chemosphere* 93 (2013) 2171–2179, doi:10.1016/j.chemosphere.2013.07.080.
- [37] R. Balint Nimirciag, E. Buratto, F. Ajmone-Marsan, Leaching of trace metals from soil under alternating oxic-anoxic conditions: a column study, *E3S Web Conf* (2013) 1, doi:10.1051/e3sconf/20130119005.
- [38] H. Bergamin F., E.A.G. Zagatto, F.J. Krug, B.F. Reis, Merging zones in flow injection analysis, *Anal. Chim. Acta* 101 (1978) 17–23, doi:10.1016/S0003-2670(01)83835-6.
- [39] C. Brouwer, A. Goffeau, M. Heibloem, Irrigation water management: training manual no. 1 – introduction to irrigation, Rome, Italy, 1985.
- [40] AWWA APHA, WEF, *Standard Methods for the Examination of Water and Wastewater*, 20th ed, Washington, DC, 1998.
- [41] R.B.R. Mesquita, S.M.V. Fernandes, A.O.S.S. Rangel, A flow system for the spectrophotometric determination of lead in different types of waters using ion-exchange for pre-concentration and elimination of interferences, *Talanta* 62 (2004) 395–401, doi:10.1016/j.talanta.2003.08.009.
- [42] International union of pure and applied chemistry, nomenclature, symbols, units and their usage in spectrochemical analysis – II. Data interpretation, *Pure Appl. Chem* 45 (1976) 99–103, doi:10.1351/pac197645020099.
- [43] J.C. Miller, J.N. Miller, *Statistics for Analytical Chemistry*, 3rd ed, Ellis Horwood PTR Prentice Hall, New York, 1993.