Speciation of phosphorus oxoacids in natural and waste water samples[☆]

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ABSTRACT

Phosphorus is a key nutrient and in natural environments regulates trophic status and consequently water quality. Therefore monitoring of phosphorus content in natural and wastewater is essential. Although several phosphorus species can be found in the environment, the majority of the methods developed are for orthophosphate determination. High performance liquid chromatography (HPLC) coupled with inductively coupled plasma with atomic emission spectroscopy (ICP-AES) has been first used in this study for the speciation of the most common phosphorus oxoanions in aquatic environments: orthophosphate, phosphite, hypophosphite, pyrophosphate and tripolyphosphate. The chromatograms have been obtained by registering the phosphorous 213.618 nm emission intensity variation with time. The pH and the ionic strength of the mobile phase have been the most critical variables of the chromatographic separation. Moreover, methanol addition promotes the elution of the most retained species. Finally, by using ammonium nitrate and a gradient elution, increasing ionic strength and decreasing the pH, the separation has been achieved in 12 min. Limits of detection have been included within the $1–5\,mg\,L^{-1}$ range. The developed methodology has been tested with spiked tap water and effluent water of a wastewater treatment plant (WWTP) obtaining recoveries in the range of 91.5–114.1% for a $20\,mg\,P\,L^{-1}$ spike concentration.

1. Introduction

Phosphorus is an essential nutrient for many living organisms, it plays a vital role in cell physiology and biochemistry and sometimes it is the element limiting growth. Phosphorus is found in nature in various forms: mineral forms, organic forms (such as phospholipids, nucleic acids and proteins), gaseous forms, particulate or colloidal forms and dissolved inorganic forms such as pentavalent, trivalent or univalent dissolved species [1,2]. In this last group simple and condensed phosphorus oxoanions can be found.

Orthophosphate [P(V)] is the most stable and abundant form of phosphorus in the environment, which is easy to assimilate, for this reason its most important use is in the field of fertilizers. Other important uses are as food additives and detergents as well [3,4]. However, it is well known that the excessive presence of

phosphorus compounds in aquatic environments causes problems of eutrophication [5]. Moreover, a study establishes that the excess intake of condensed phosphorus oxoanions is related with the inhibition of the intestine to absorb calcium [3]. For these reasons, the study of the behaviour and degradation of phosphorus species in waste and treated waters can be an important way to control these environmental and health problems, as well as to choose the suitable waste water treatment in treatment plants.

Although there exist different phosphorus species, with different oxidation states [6], common environmental analyses are performed as orthophosphate or total phosphorus, through acid hydrolysis and digestion transforming condensed and organic phosphorus species to orthophosphate. In these cases, the determination is carried out by molecular spectrometry (UV) or by ion chromatography (IC) with conductivity detection [4,7–9]. There are also studies in which determination of phosphorus involves the use of techniques such as capillary electrophoresis (CE) with UV or conductivity detection [10], or high performance liquid chromatography (HPLC) with an evaporative light scattering detection [11]. The determination of total phosphorus has also been carried out with inductively coupled plasma with atomic emission spectroscopy (ICP-AES) in soil samples [12] or electrothermal vaporization-ICP-AES in plants [13].

In recent years, speciation has taken an important role in environmental studies. The necessity to know the chemical structure of

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a compound to determine its essentiality or toxicity has conducted to the development of speciation analytical methods. Moreover, the speciation of an element in natural samples gives information about its bioavailability and mobility. For example, Ivey et al. [14] explain that there exist some microorganisms able to metabolize phosphite and hypophosphite, so the speciation data of this two species together with orthophosphate would provide essential information on the cycling and bioavailability of phosphorus species in natural environments.

To perform phosphorus speciation the most used technique is IC with [3,14] or without suppressed conductivity detection [4], indirect UV or fluorescence [4], although they are non selective detectors and in some cases pre or post column derivatization is required. Separation by CE is another option to achieve phosphorus speciation. In this case, the most common used detectors are conductivity, UV and mass spectrometry (MS) [15]. When MS is the selected detector, some authors perform the separation by liquid chromatography (LC).

In recent years the use of ICP-AES or inductively coupled plasma with mass spectrometry (ICP-MS) detection system has become more common. ICP detectors present high selectivity for phosphorus containing compounds, high linearity so it is not necessary to dilute the most concentrate samples and high compatibility with the most used mobile phases in LC. When ICP detectors are used the separation can be carried out by classical IC [16–18], LC [5,19–22] and in case of some species by gas chromatography (GC) [23,24]. An example is the work of De Brabandere et al. [5] who used LC tandem mass spectrometry (MS/MS) for identification of the species and the quantification was carried out by LC-ICP-AES.

Until now, phosphorus speciation has been focused in types of phosphorus species; speciation of simple oxoanions (orthophosphate, phosphite and hypophosphite) in water samples [1,14,16], or speciation of condensate phosphates in food samples [3]. Other types of species determined are phosphorus organic compounds (inositol phosphates, nucleotides or metabolites) in food [22], in aquatic sediments [5] or in biological samples [19]. Organophosphorated herbicides like glyphosate, glufosinate and AMPA have been also determinated in water samples by LC coupled to ICP-MS [18,21], as well as, these species together with orthophosphate in soil extracts [17]. However, there is a lack on speciation studies of simple oxoanions together with condensed oxoanions.

The main goal of this work is to develop a method for phosphorus speciation in natural and waste water samples, including simple and condensate oxoanions. To achieve this objective we have selected five species of phosphorus: orthophosphate (+V), phosphite (+III), hypophosphite (+I), pyrophosphate (+V) and tripolyphosphate (+V) which are the most commonly found in these environments. The method has been developed using a HPLC system with an anion exchange column coupled to an ICP-AES as a detector.

2. Materials and methods

2.1. Standards and reagents

The standards and reagents used in this study were prepared with doubly deionized water (milliQ) obtained from Millipore water purification systems (18.2 $M\Omega\,cm^{-1}$, Millipore, Bedford, MA, USA).

The 1000 mg P L^{-1} stock standard solution of orthophosphate, hypophosphite, phosphite, pyrophosphate and tripolyphosphate were prepared by dissolving appropriate amounts of sodium dihydrogen phosphate anhydrous obtained from Panreac Química (Barcelona, Spain), sodium hypophosphite monohydrate obtained from Fluka Chemie (Buchs, Switzerland), sodium phosphite

dibasic pentahydrate purchased from Sigma–Aldrich (Steinheim, Germany), sodium pyrophosphate tetrabasic decahydrate from Fluka Chemie (Buchs, Switzerland) and pentasodium tripolyphosphate hexahydrate from Sigma–Aldrich (Steinheim, Germany), respectively, in milliQ water. All stock standard solutions were stored in amber glass bottles at 4 °C. The storage conditions were found elsewhere [1]. The stability of stock standard solutions (data not shown) based on storage time was studied for periods of up to 15 days. Diode array spectrophotometer was used to register the absorption spectra of phosphite, pyrophosphate and tripolyphosphate; phosphate and hypophosphite do not have a maximum absorbance above 190 nm. Results showed that the stock standard solutions were stable for periods up to 15 days. Based on these results and previous studies [1] stock solutions were prepared every 7–15 days.

The working standard solutions were prepared every two days by dilution in water acidified with nitric acid at pH 2.

Ammonium nitrate was prepared with the appropriate amount of nitric acid Suprapure® provided from Merck (Darmstadt, Germany) and ammonia obtained from Panreac Química (Barcelona, Spain). The pH was adjusted with nitric acid and ammonia. Methanol HPLC-grade was purchased from Carlo Erba (Milan, Italy). Acetic–acetate, formic–formiate and citric–citrate buffers were prepared dissolving appropriate amounts of acetic, formic and citric acid, respectively, and with the addition of ammonia to obtain the buffer solution. Acetic acid was obtained from Fluka Chemie (Buchs, Switzerland), formic acid from Sigma–Aldrich (Steinheim, Germany) and citric acid anhydrous from Panreac Química (Barcelona, Spain). The mobile phases were prepared in milliQ water and were filtered through a disposable 0.20 μm cellulose nitrate membrane filter purchased from Whatman (Dassel, Germany). This solution was degassed in an ultrasonic bath prior to use.

2.2. Instrumentation

The HPLC system (P2000 Thermo Separation products) consisted of an online degasser, a HPLC binary pump and a Rheodyne Series 7725 manual injector valve with a 20 μL injection loop. The separation was done with a 5 μm 150 mm × 4.1 mm anion exchange PRP-X100 column (Hamilton) and a PRP-X100 guard column (Hamilton), placed before the column, to retain the impurities. The separation of five phosphorus species was carried out with a gradient concentration and pH, from 4 mM to 200 mM and from pH 2.2 to 1.8, obtaining the separation in ~12 min at a constant flow of 1.5 mL min^{-1}. The mobile phases used were 4 mM of NH$_4$NO$_3$ with 2% of methanol at a pH 2.2 (A) and 200 mM of NH$_4$NO$_3$ with 2% of methanol at a pH 1.8 (B). The gradient was as follows: 100% A from 0 to 6 min; 100% A to 100% B from 6 to 9 min; 100% B from 9 to 13 min; and 100% B to 100% A from 13 to 15 min. The total run time was 17 min.

The chromatographic system has been hyphenated to a Liberty series II ICP-AES (Varian). The end of the chromatographic column was directly connected to the ICP-AES nebulizer using a simple Teflon tube. The ICP-AES was equipped with either a Babington nebulizer coupled to a double-pass spray chamber or a concentric nebulizer together with a thermostated double-pass spray chamber and a photomultiplier tube detector. Table 1 shows the experimental conditions employed in the detection step. Some other parameters were optimized daily for highest sensitivity. Peaks integration has been carried out with WinFaas 1.0 [25].

2.3. Samples

Effluent waste water was collected from the wastewater treatment plant (WWTP) in Blanes (Girona), directly in 1 L amber glass

Table 1
ICP-AES experimental conditions.

Integration time (s)	0.50
Power (kW)	1.20
Plasma flow (L min^{-1})	13.5
Auxiliary flow (L min^{-1})	1.50
Wavelength (nm)	213.618

bottle, which had been thoroughly rinsed with the sample before collection. Samples were transported, refrigerated to the laboratory and were stored at 4 °C prior to use.

Spiked tap water and effluent waste water were filtered with a 0.45 μm cellulose nitrate membrane filter.

3. Results and discussion

3.1. ICP-AES detection

The first step in the method development is to have a well adjusted detection system.

3.1.1. Addition of methanol

Several studies determined that the addition of small amounts of methanol in the samples improved the nebulization. Moreover, there are several studies dealing with sensitivity increase of some atomic emission lines in ICP-AES due to the presence of carbon containing compounds [26,27]. The energy transfer from metastable carbon atoms was considered as a possible pathway of enhancement emission intensity. For this reason, a study on the effect of methanol addition on the ICP-AES response to the phosphorus detection has been done. To perform the study, different calibration curves with different amounts of methanol have been prepared. It is also reported that the addition of large quantities of organic solvent on plasma can cool it and reduce its sensitivity to [28]. The results are shown in Fig. 1. With the addition of methanol an increase in sensitivity of phosphorus detection was observed, as well as an increase in the signal/background ratio (SBR) (12, 16 and 16 for 0, 2 and 4% of methanol, respectively, obtained with a 10 mg P L^{-1} standard solution). The addition of 2% of MeOH is the chosen option because the sensitivity increased significantly but the plasma was not appreciably degraded, so a signal reduction was not observed. It has also to be taken into account that the addition of small amounts of organic modifiers on anionic exchange chromatographic system allows the elution of highly retained anions [3,29].

3.1.2. Sensitivity for the different phosphorus species

Previous studies have proved that different species with the same content of an element can contribute differently to
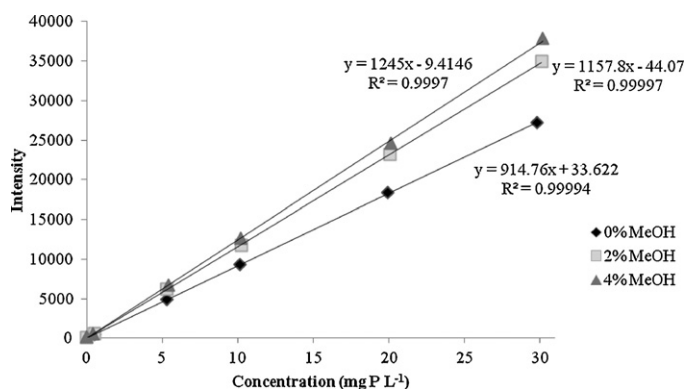

Fig. 1. Effect of the addition of methanol in phosphorus ICP-AES response.
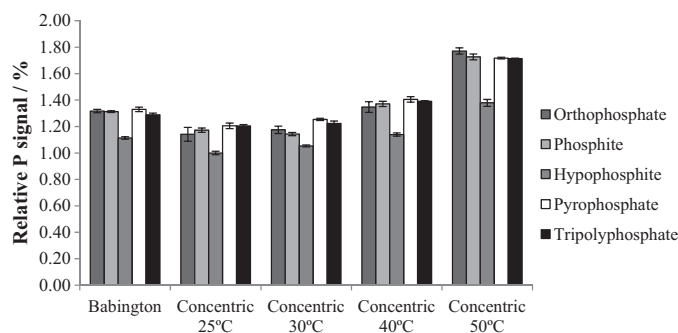

Fig. 2. Relative phosphorus signal with two different nebulizers at different temperatures. Bars correspond to emission signal divided by phosphorus concentration in each species. The result is corrected with the smallest value (hypophosphite).

the ICP-AES response [30]. To investigate this, five calibration curves, corresponding to each phosphorus species, were obtained. The obtained slopes (mean ($\pm tS_b$)) were 820.2(± 9), 837.3(± 14), 848.6(± 8), 824.9(± 10) and 811.5(± 17) corresponding to orthophosphate, phosphite, hypophosphite, pyrophosphate and tripolyphosphate, respectively. The calculated slopes are very similar with confidence intervals overlapped in the majority of cases. This result can be explained by the fact that the studied species are not volatile and their behaviour on ICP-AES detector does not showed differences.

3.1.3. Nebulizer system

To perform this study, we have compared the behaviour of a Babington and a concentric nebulizers. The Babington nebulizer was evaluated at room temperature (25 °C) and the concentric nebulizer, which has a thermostatic chamber, was evaluated at four temperatures (25 °C, 30 °C, 40 °C and 50 °C). In Fig. 2 it can be observed that an increase in temperature caused an increase in intensity. This can be explained in terms of an increase in the aerosol transport efficiency caused by an enhancement in the solvent evaporation. However, background also increased. At low temperatures (25–30 °C) the sensitivity improvement was not important whereas above 40 °C the plasma became highly unstable. Under these conditions, the obtained sensitivities were slightly lower than those furnished by the Babington nebulizer adapted to the double pass spray chamber. For these reasons and for simplicity Babington nebulizer was used at room temperature.

3.2. Chromatographic separation

The first point to achieve the chromatographic separation was the selection of the column. Checking previous work, we found that PRP-X100 was an anion exchange column commonly used in speciation, of either phosphorus or other anions [4,31–33]. In addition, its polymeric support allows working at a wider pH range, from 1 to 13. For all these reasons we decided to use this column in our study. Flow rate was another important variable for the chromatographic separation. Two flow rates were tested. At 1.5 mL min^{-1} peak shape was better, total chromatogram time was smaller and resolution was not worse than at 1 mL min^{-1}, so we finally chose this higher flow rate.

3.2.1. Choice of the mobile phase

The retention of anion species on HPLC anion exchange column is dependent on the pK_a of the species and mobile phase variables (pH, ion strength, flow rate and temperature). We test four different mobile phases with different pH. As show in Table 2, with acetic-acetate buffer (pK_a 4.75) and the injection of a sample with the five

Table 2
Comparison of eluents for the separation of orthophosphate, phosphite, hypophosphite, pyrophosphate and tripolyphosphate.

Eluent	pH	Orthophosphate R_T (min)	R_s Orthophosphate/phosphite	Phosphite R_T (min)	R_s Phosphite/hypophosphite	Hypophosphite R_T (min)	R_s Hypophosphite/pyrophosphate	Pyrophosphate R_T (min)	R_s Pyrophosphate/tripolyphosphate	Tripolyphosphate R_T (min)
50 mM acetic–acetate	4.75	4.9	–	4.9	–	4.9	–	–	–	–
100 mM formic–formiate	3.30	4.8	0.43	5.0	1.05	5.7	–	–	–	–
15 mM citric–citrate	2.30	5.4	1.79	7.3	1.44	9.3	–	–	–	–
10 mM ammonium nitrate	2.00	2.5	1.30	3.6	0.85	4.4	9.67	16.2	–	–

R_T: retention time; $R_s = (R_{Ta} - R_{Tb})/((1/2)(W_a + W_b))$ (resolution); W: peak width.

Table 3
Effect of the pH on the chromatographic separation with milliQ water and nitric acid as a pH modifier.

pH	R_s Orthophosphate/ phosphite	R_s Phosphite/ hypophosphite	R_T (min) Hypophosphite	R_T (min) Pyrophosphate
2.3	4.25	3.30	19.6	–
2.2	3.86	2.47	15.4	–
2.1	3.10	1.74	11.9	–
2.0	2.46	1.66	7.7	35.0
1.9	2.00	1.29	6.0	27.9
1.8	1.70	1.00	5.0	25.4

species we obtained just one peak. Then we tested formic–formate buffer (pK_a 3.75), a chromatogram with 3 peaks corresponding to orthophosphate, phosphite and hypophosphite was obtained. However, orthophosphate and phosphite appeared nearly overlapped. In previous works [18,34], the use of citric–citrate buffer (pK_a 2.94) was described for phosphorus speciation. With this buffer three separated peaks were obtained (orthophosphate, phosphite and hypophosphite) but it was not possible to elute the other two species (pyrophosphate and tripolyphosphate). Pyrophosphate and tripolyphosphate are the most acidic species (pK_1 = 0.8, pK_2 = 2.2, pK_3 = 6.7, pK_4 = 9.4 and pK_1 = 0.2, pK_2 = 1.4, pK_3 = 2.4, pK_4 = 6.6, pK_5 = 9.2, respectively) and at the working pH they are only partially protonated, so they are strongly retained in the column. Therefore we tested ammonium nitrate [17] as a mobile phase. The main advantage of this mobile phase was that retention times were lower than for the other mobile phases. Furthermore, it is easy and faster to prepare, there are not dissolved solids and lower pH can be obtained. With ammonium nitrate mobile phase pyrophosphate eluted efficiently however tripolyphosphate was still retained. For this reason, we decided to study additional variables affecting the chromatographic separation.

3.2.2. Effect of the pH of the mobile phase

During the methodology development we observed that pH had an important role in the chromatographic separation. As previously indicated, the studied species had a strong acidic character, and needed rather low mobile phase pH to elute efficiently from the column. A study of the effect of the pH was done. The study was performed from 1.8 to 2.3 with milliQ water and nitric acid as a pH modifier. At pH lower than 1.8 the polymeric support can suffer structural changes whereas at pH higher than 2.3 the chromatographic separation was degraded. The results shown in Table 3 demonstrated the importance of the pH in the chromatographic separation and that low pH allows the elution of the most retained species. However, the elution of tripolyphosphate was not achieved at this pH range and the orthophosphate, phosphite and hypophosphite resolution was worse at low pH. These results suggested that a gradient elution was necessary.

3.2.3. Gradient design

The main goal was the elution of tripolyphosphate, and the reduction on the retention time of pyrophosphate. Previous studies revealed that when mobile phase consisted in monovalent anions, high concentrations were required to elute highly retained anions [4]. In our case, NO_3^- was the exchanger anion and at the working pH the elution of tripolyphosphate was not possible. Based on this premise, a pH and an ionic strength gradient were investigated. First mobile phase consisted on 4 mM ammonium nitrate at pH 2.2 with 2% MeOH (A), lower pH and higher ionic strength was not desirable because it would be negative for the orthophosphate, phosphite and hypophosphite resolution. Mobile phase (B) (with

Table 4
Effect of ammonium nitrate concentration with gradient elution at an initial pH 2.2. Orthophosphate and phosphite retention times are not shown because the objective of this study was the elution of pyrophosphate and tripolyphosphate.

NH_4NO_3 (mM) concentration	R_T (min) Hypophosphite	R_T (min) Pyrophosphate	R_T (min) Tripolyphosphate
15	8.4	17.8	–
30	9.6	14.0	–
80	7.5	13.9	20.3
100	5.9	9.7	12.3
200	5.2	9.1	9.9

2% MeOH) had a pH value of 1.8 based on results in Table 3. Several gradient times and ammonium nitrate concentrations were tested. The results are shown in Table 4. It can be observed that with the increase of the ionic strength, the elution of tripolyphosphate was finally achieved. The selected concentration was 200 mM and the selected gradient was: 100% A from 0 to 6 min; 100% A to 100% B from 6 to 9 min; 100% B from 9 to 13 min; and 100% B to 100% A from 13 to 15 min.

3.3. Calibration

The linearity of the standard curves was investigated in the range from 5 to 40 mg P L^{-1} with the selected conditions. Peak area of the analyte was used for quantification. Linear regression curves ($n = 5$) were obtained with regression coefficients higher than 0.99 for all the analytes (Table 5). It may be observed that the slopes of the lines where similar for all the phosphorous compounds. This fact meant that a single standard could be used for obtaining the full calibration line. The so called Single Injection Calibration Approach [35] could be thus used. According to this methodology a standard containing the five phosphorous compounds would be prepared. Once injected, the peaks could be registered and the

calibration line would be obtained by plotting the peak area versus the phosphorous concentration. Unlike ICP-AES, other detectors such as the conductimetric one provided different results as a function of the phosphorous compound. The detection limits and quantification limits of the developed method were calculated as three and ten times, respectively, the regression residual standard deviation ($\widehat{\sigma}_{y/x}$) [36]. Limits of detection (LOD) in the range 1–5 mg L^{-1} and limits of quantification (LOQ) in the range 3–15 mg L^{-1} were obtained. The intra-day and inter-day precisions were evaluated with a 20 mg P L^{-1} solution of each analyte. RSD values between 1–6% and 2–7%, respectively, were achieved. A chromatogram at this level is displayed in Fig. 3.

3.4. Application to real samples

Finally, the HPLC-ICP-AES system has been evaluated with the analysis of real samples. Due to lack of reference material of phosphorus speciation we decided to use spiked samples. Tap water and effluent wastewater have been spiked at two levels (5 mg P L^{-1} at the low level and 20 mg P L^{-1} at the high level) ($n = 3$). The recoveries obtained are shown in Table 6. In the case of non spiked effluent wastewater, orthophosphate has been detected; however quantification has been not possible. Therefore intensity values from spiked effluent wastewater have been corrected with the intensity obtained from non spiked effluent wastewater before recoveries calculation. Recoveries ranged from 90.8 to 126.8 for tap water and from 91.5 to 142.1 for effluent wastewater. The results obtained at spiked level of 5 mg P L^{-1} were not good because in many cases they were below the LOQ. At spiked level of 20 mg P L^{-1} recoveries were closer to 100%, which indicates that complex matrices do not affect either the chromatographic separation or the ICP-AES detection. Therefore the developed system can be applied to water samples with different matrices at these concentration levels.

Table 5
Linearity, LODs and LOQs ($n = 5$).

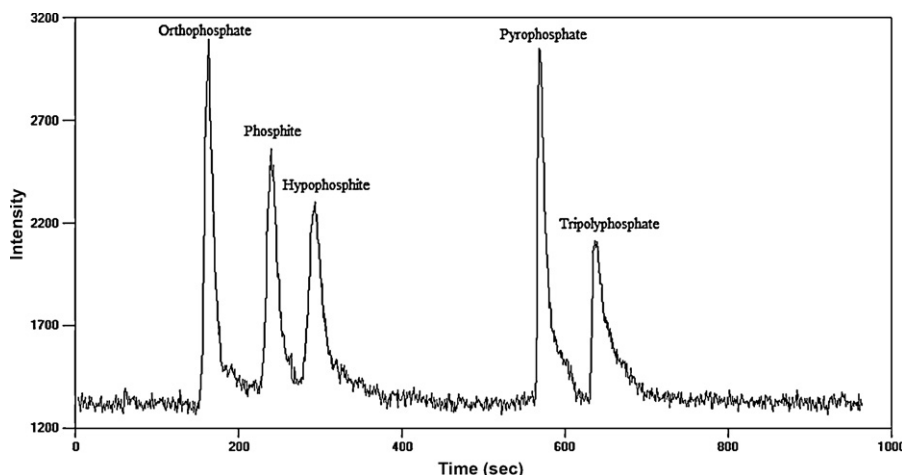| Analyte	Slope $\pm tS_b$	Intercept $\pm tS_a$	Regression coefficient (r^2)	LOD (mg P L^{-1})	LOQ (mg P L^{-1})
Orthophosphate	1164.3 ± 55	−615 ± 1507	0.9993	1	5
Phosphite	1136.6 ± 119	−829 ± 2911	0.997	3	9
Hypophosphite	1315.4 ± 240	−465 ± 5466	0.990	5	15
Pyrophosphate	1384.2 ± 66	−1186 ± 1617	0.9993	1	4
Tripolyphosphate	1383.2 ± 81	−5083 ± 2090	0.9996	1	3

Fig. 3. Chromatogram of milliQ water spiked at 20 mg P L^{-1} of each specie.

Table 6
Recoveries from spiked water at 5 and 20 mg P L^{-1} by HPLC-ICP-AES ($n = 3$).

Water type	Spiked level (mg L^{-1})	Recovery (RSD%)				
		Orthophosphate	Phosphite	Hypophosphite	Pyrophosphate	Tripolyphosphate
Tap water	5	111.7 (10)	120.0 (5)	100.3 (8)	90.8 (10)	126.8 (7)
	20	106.7 (5)	105.4 (6)	103.9 (1)	106.7 (4)	103.0 (3)
Effluent wastewater	0	<LOQ	n.d.	n.d.	n.d.	n.d.
	5	119.2 (7)	125.6 (7)	142.0 (5)	113.6 (3)	142.1 (4)
	20	114.1 (13)	111.2 (5)	91.5 (8)	112.5 (5)	103.5 (1)

4. Conclusions

A novel HPLC-ICP-AES method has been developed for the determination of orthophosphate, phosphite, hypophosphite, pyrophosphate and tripolyphosphate in a single run. This method would allow the determination of other elements present in the sample in a single run, when using a simultaneous ICP-AES. Various parameters affecting separation and detection have been studied. The chromatographic separation was achieved, with a gradient elution, in 12 min without tedious sample treatment. The phosphorus detection was carried out at 213.618 nm emission wavelength. The method has been validated using different water matrices spiked at the 5–20 mg L^{-1} level, obtaining recoveries in the 90.8–142.1% range. The developed method can be applied in some types of wastewater or landfill leachate. However, to apply the method in other environmental water samples, instrumental improvement is required to increase sensitivity.

Acknowledgements

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