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# Iron speciation in natural waters by sequential injection analysis with a hexadentate 3-hydroxy-4-pyridinone chelator as chromogenic agent

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ABSTRACT

A sequential injection method for iron speciation in various types of natural waters was developed using a synthesised hexadentate 3-hydroxy-4-pyridinone chelator (CP256). The denticity of the ligand that allow formation of the corresponding iron(III) complex in a 1:1 stoichiometry proved to be highly advantageous, in comparison with parent bidentate, hydroxy-4-piridinone chelators, with a two fold increase of reaction sensitivity and over 65% decrease of the LOD. A solid phase extraction approach was employed to attain matrix elimination, facilitating iron(III) determination and application to high salinity waters. The combination with the total iron determination obtained by the direct reaction of the ligand resulted in iron speciation. Two detection spectrophotometric cells were tested, a conventional flow cell (CFC) and a liquid waveguide capillary cell (LWCC). The dynamic concentration ranges were 0.1–2 mg/L with the CFC detection and 0.005–0.1 mg/L with the LWCC, with limit of detection of 30 μg/L and 6 μg/L, respectively. The developed method was successfully applied to a variety of natural waters.



## 1. Introduction

Although iron is a non-toxic metal, its speciation is a key parameter to assess due to its relationship with environmental bioprocesses. In fact, iron plays an important role in plant metabolism and in animal physiology. To improve the understanding of its role, it is necessary to develop robust analytical techniques with appropriate analytical characteristics to be employed as in-situ analysers [1]. Iron concentration in different water bodies is highly variable, ranging from milimolar concentrations values in estuarine waters to nanomolar concentration levels in open ocean waters. In natural waters, when aiming to measure metal ions at low levels, atomic absorption or emission techniques are some of the most popular choices. However, these techniques present an inherent low tolerance to physical/matrix interferences, making its application difficult, especially with the high salt contents expected in samples like sea or estuarine waters. In addition, atomic absorption spectroscopy is a relatively expensive technique and impairs in-situ analysis. In the environmental assessment of iron, speciation is more relevant than the total metal content, adding another disadvantage to atomic spectroscopy detection. In this context, molecular spectrophotometric techniques have been used as alternative methods for iron monitoring environmental samples. However, these techniques may require toxic reagents and so alternatives involving low toxic reagents are demanded, in a green chemistry approach. In this context, the use of newly synthesized iron ligands, bidentate 3-hydroxy-4-pyridinone (3,4-HPO) chelators, has been successfully described [2,3]. However, the main drawback reported by the authors was the low solubility of the ligand together with the need of using the ligand in a 3:1 ligand: iron stoichiometry [2], which implies a higher ligand solution volume, as the limited solubility prevents an increase in ligand concentration. So, to tackle this limitation, a more soluble and hexadentate 3-hydroxy-4-pyridinone ligand (Fig. 1) [4–6] was explored in this work.

In this scenario, to the already described advantages of using these ligands with high affinity for iron, the hexadentate ligand provides a 1:1 stoichiometry and a lower kinetic lability, making it a potential alternative as chromogenic reagent. With the hexadentate CP256 chelator, an increase in the determination

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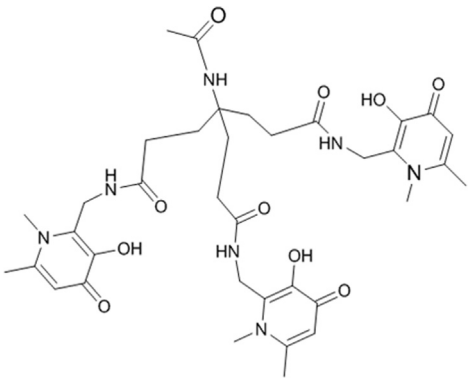

**Fig. 1.** Formula of the hexadentate chelator CP256, designated as compound 3 in Ref. [4].

sensitivity and a decrease in the limit of detection were expected.

The use of sequential injection analysis as the flow analysis technique aims to emphasise the green chemistry approach of reduced reagent and sample consumption and reduced waste volume. Since first described [7], sequential injection analysis has proven to be an efficient tool in water analysis [8]. The versatility of this technique enables to cope with the variability of water samples in dynamic aquatic systems, namely the need for implementing pre-concentration and/or cleaning up procedures [9,10]. Consequently, the variability of sample matrices, analyte concentration and speciation are problems effectively handled in-line with the sequential injection approach. In this context, a solid phase extraction column packed with a chelating resin was included for in-line matrix removal and iron(III) retention. Furthermore, flow methods enable automatic, miniaturised, reliable and real-time (possibility of in situ) analysis.

In the end, fully exploring the sequential injection flexibility, speciation of iron (determination of iron(III) and total iron) with an alternative complexing agent (CP256) as a new chromogenic reagent was attained. The described method was effectively applied to all types of natural waters and included four different approaches: the direct determination of total iron ($Fe^{3+}$ and $Fe^{2+}$) with the CP256; in-line solid phase extraction for iron(III) determination after its retention in NTA resin; in-line oxidation of iron(II) to iron(III) for total iron determination in high salinity samples; and use of a long path length flow cell (liquid waveguide capillary cell) for measuring low levels of iron. The developed methodology can be an effective tool to contribute for a better understanding of the iron distribution in dynamic aquatic systems and through the entire water column.

## 2. Experimental

### 2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and boiled Milli-Q water. The hexadentate 3,4-hydroxypiridinone ligand (a tripodal hydroxypyridinone) was synthesised according to the methods published in the literature [4,5] and characterized by elemental analysis, $^{1}H$ and $^{13}C$ NMR and mass spectrometry. Ligand solutions were obtained by dissolution of approximately 10.0 mg of the synthesised ligand in 20.0 mL of water corresponding to a concentration of 0.5 mg/L (521 μM).

A 0.60 M carbonate buffer solution was prepared by dissolving 2.5 g of sodium hydrogen carbonate in 50.0 mL of water. The pH was set to 10.6 with 2 M sodium hydroxide.

A 10.0 mg/L iron(III) stock solution was obtained by dilution of the atomic absorption standard of 1000 mg/L. An intermediate solution of 5.0 mg/L was obtained by appropriate dilution of the 10.0 mg/L stock solution and used to prepare the $Fe^{3+}$ working standards in the range: 0.10–2.00 mg/L, and was weekly prepared from dilution of the stock solution in 0.03 M nitric acid.

The solid phase extraction (SPE) column consisted of Nitrilotriacetic Acid Superflow resin (Qiagen, Netherlands), highly cross-linked 6% agarose and bead diameter 60–160 μm, packed in PVC tubing (2.3 mm i.d. and 2 cm length) by means of a syringe. Ordinary dishwashing sponge was placed at both ends of the column to entrap the resin. The 0.03 M nitric acid solution, used for the standard preparation, and 0.5 M nitric acid solution, used for reconditioning the column, were prepared from suitable dilution of the concentrated acid ($d=1.4$; 65%).

A 12.8 mM hydrogen peroxide solution was prepared from dilution of the concentrated reagent ($d=1.11$; 30%). A stock solution of iron(II) was prepared from the solid ammonium iron(II) sulphate hexahydrate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$) to a final concentration of 10.0 mg/L. This stock solution was diluted to obtain an intermediate solution of 5.0 mg/L, which was used to prepare the working standards in the dynamic range 0.1–2.0 mg/L (in 0.03 M of nitric acid).

### 2.2. Sequential injection manifold and procedure

The sequential injection manifold developed for iron speciation, with and without solid phase extraction (SPE), using the hexadentate 3,4-HPO ligand is depicted in Fig. 2.

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 59191-E8). All tubing connecting the different components of the flow system was made of PTFE (Omnifit) with 0.8 mm i.d.

An Ocean Optics USB 4000 charged coupled device detector (CCD) spectrophotometer, equipped with a pair of 400 mm fibre optic cable and a Mikropack DH-2000 deuterium halogen light source, was used as the detection system. A Hellma 178.710-QS flow-cell with 10 mm light path and 80 μL inner volume was used as a conventional flow cell (CFC) and a liquid waveguide capillary cell (LWCC 2100, World Precision Instruments, Sarasota, FL) with a 1.0 m path length, 250 μL inner volume and 550 μm inner diameter was used as alternative flow cell. Data acquisition of the

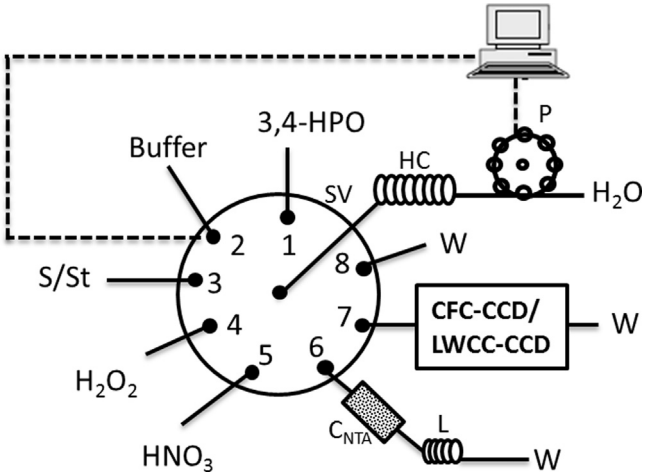


**Fig. 2.** Sequential injection manifold for iron speciation using the hexadentate 3,4-HPO ligand; SV, eight port selection valve; P, peristaltic pump; HC, 3 m holding coil; $C_{NTA}$, column packed with NTA resin; 3,4-HPO, Hexadentate hydroxypyridinone solution 0.6 g/L; Buffer, carbonate buffer at pH 10.6; S/St, sample or standard; $H_2O_2$, 5% peroxide solution; $HNO_3$, 0.03 M nitric acid solution; L, coil with 75 cm length; CFC-CCD/LWCC-CCD, spectrophotometric detector ($\lambda=460$ nm) with conventional flow cell or Liquid wavelength capillarity cell; and W, waste.

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**Table 1**
Protocol sequence for the developed sequential injection system (SI) for the iron speciation with 3,4-HPO hexadentate ligand as a colorimetric reagent without (steps A–D) and with SPE (E–M).

| Method | Step | SV position | Time (s) | Flow rate (μL/s) | Pump direction | Volume (μL) | Description |
|---|---|---|---|---|---|---|---|
| SI | **A** | 1 | 2.2 | 57.1 | a | 126 | Aspiration of ligand |
| | **B** | 2 | 1.5 | 15.8 | a | 24 | Aspiration of buffer |
| | **C** | 3 | 15.8 | 57.1 | a | 903 | Aspiration of sample/standard |
| | **D** | 7 | 42 | 57.1 | b | 1284 | Propelling to detector |
| SI-SPE | $\mathbf{A_{SPE}}$* | 4 | 1.5 | 15.8 | a | 24 | Aspiration of hydrogen peroxide for oxidation of $Fe^{2+}$ to $Fe^{3+}$ |
| | $B_{SPE}$ | 3 | 15.8 | 57.1 | a | 903 | Aspiration of sample/standard |
| | $\mathbf{C_{SPE}}$* | 4 | 1.5 | 15.8 | a | 24 | Aspiration of hydrogen peroxide for oxidation of $Fe^{2+}$ to $Fe^{3+}$ |
| | $\mathbf{D_{SPE}}$ | 6 | 42 | 43.6 | b | 1830 | Propelling to NTA resin column for $Fe^{3+}$ retention |
| | $\mathbf{E_{SPE}}$ | 1 | 2.2 | 57.1 | a | 126 | Aspiration of ligand solution |
| | $\mathbf{F_{SPE}}$ | 2 | 1.5 | 15.8 | a | 24 | Aspiration of buffer solution |
| | $\mathbf{G_{SPE}}$ | 6 | 4.7 | 43.6 | b | 205 | Propelling to NTA resin column for reacting with $Fe^{3+}$, removing it from the NTA resin |
| | $\mathbf{H_{SPE}}$ | 6 | 6.0 | 43.6 | a | 262 | Aspiration of the formed coloured complex |
| | $\mathbf{I_{SPE}}$ | 7 | 45/65** | 57.1/28.5** | b | 2569/1853** | Propelling to detector for absorbance measurement |
| | $\mathbf{J_{SPE}}$ | 5 | 3.0 | 57.1 | a | 171 | Aspiration of nitric acid |
| | $\mathbf{K_{SPE}}$ | 6 | 6.0 | 43.6 | b | 260 | Propelling through the column for conditioning |

* Steps included for the total iron determination with SPE.
** Values for the LWCC-CCD detection.

signal obtained at 460 nm and subtracting the blank at 800 nm was performed through the OceanOptics — Spectrasuite software running in a personal computer (Sony VAIO – C2DU7600 2 GB/100 GB) with Windows Vista Business OE MAct.

A computer (Samsung SD700) equipped with a PCL818L interface card, running a home-made software written in QuickBasic 4.5, was used to control the selection valve position and the peristaltic pump direction and speed. The sequence of steps with the respective time and volumes used for the methodology with and without the NTA column is shown in Table 1.

#### 2.2.1. Determination of total iron

The determination of total iron consisted upon the sequential aspiration of 3,4-HPO ligand, buffer and sample/standard (steps A–C). Then the mixing was promoted by the flow reversal while propelling the aspirated plugs towards the detector (step D).

#### 2.2.2. Determination of iron(III)

It was accomplished by using SPE with the NTA resin column; the first step consisted in the aspiration of the sample/standard (step $B_{SPE}$) and propelling it through the column (step $D_{SPE}$) for iron(III) retention. The propelled volume is about twice the sample/standard volume to ensure a complete washing of the column, minimising interferences. Afterwards, the 3,4-HPO ligand and buffer are aspirated (steps $E_{SPE}$ and $F_{SPE}$) and propelled to the NTA column (step $G_{SPE}$). The complex formation occurs removing the iron(III) from the NTA resin and then the formed coloured complex is aspirated from the NTA resin (step $H_{SPE}$) and propelled to the detector (step $I_{SPE}$) for absorbance measurement. Finally, nitric acid was aspirated and sent through the NTA resin column to prepare it for the following cycle by appropriate reconditioning (steps $J_{SPE}$ and $K_{SPE}$).

#### 2.2.3. Determination of total iron with SPE, for matrix removal

Two extra steps were included (steps $A_{SPE}$ and $C_{SPE}$) for aspirating the sample/standard between two plugs of hydrogen peroxide before sending to the NTA resin (step $D_{SPE}$). This ensured that iron was converted to iron(III) and retained in the NTA resin.

### 2.3. Sample collection and preparation

Water samples from inland and coastal bathing areas were collected in polyethylene plastic bottles of 0.5 L capacity at about 30 cm depth, and acidified at collection (pH ≈ 2), according to the collection reference procedure [11].

### 2.4. Accuracy assessment

For accuracy assessment, collected natural waters were analysed using the flame atomic absorption method (APHA 3111B) [11] and the results compared to those obtained with the developed SI method. An iron hollow-cathode lamp and an air-acetylene flame were used. The acidified water samples were filtered with glass fibre filters of 0.45 μm pore size prior to introduction in the atomic absorption spectrophotometer and calibration curves were made with the same standards of $Fe^{3+}$ previously described (Section 2.1).

Furthermore, four certified water samples, ERM-CA021a (soft drinking water) from LGC standard, NRC-CNR SLRS-4 (river water) and TM-27.3 (sea water) from the National Research Council Canada and NIST 1640 from National Institute of Standards and Technology, were also analysed with the developed SI method.

Recovery percentages were also calculated for several types of waters, for which a known concentration of iron(III) and/or iron(II) was added.

## 3. Results and discussion

The developed work aimed for iron speciation in various types of natural waters, including saline waters, which involved: the direct determination of total iron with the hexadentate ligand (CP256); matrix elimination with solid phase extraction (SPE), used either without and with peroxide for iron(III) and total iron determination, respectively; and a 1.0 m path length cell (LWCC) for expected low values. The reaction parameters were studied first, followed by the SPE operation parameters and then the set conditions were applied for the LWCC detection.

### 3.1. Preliminary studies

The 3,4-HPO hexadentate ligand forms coloured complexes with iron(III) with a stoichiometry of 1:1 and a full description of its acid–base properties and affinity for iron has been performed by potentiometric and spectrophotometric methods and

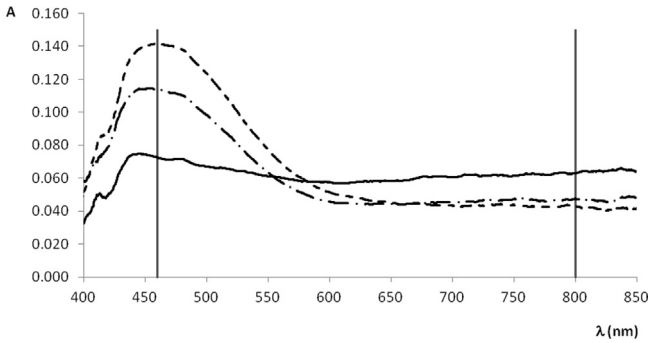


**Fig. 3.** Spectra of the hexadentate ligand; full line, the blank signal; dashed-dot line, 1 ppm $Fe^{3+}$; and dashed line, 2 ppm $Fe^{3+}$.

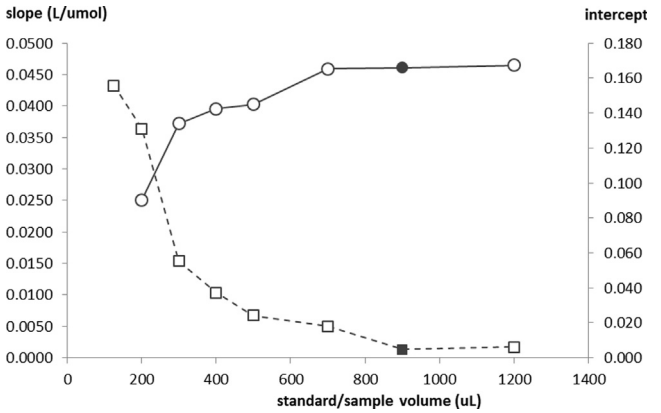


**Fig. 4.** Study of influence of the sample volume; circles, calibration curve slope representing method sensitivity; squares, calibration curve intercepts representing method limit; the points in black are the values obtained with the chosen sample volume.

previously described by one of us [4]. The values of $\log \beta(Fe^{3+})$ and $pFe^{3+}$ values are 34.4 and 29.8, respectively, and formation of the complex is fully achieved at pH above 3. In this work, and in order to compare it with the previous analytical study, pH was adjusted to pH ≈ 8. Considering that water sample collection for metal ions assessment requires acidification at collection to pH ≈ 2 and consequently involves standards also prepared at pH ≈ 2, there is a need for buffering the complex formation. Batch studies were performed to evaluate the buffer concentration to attain a final pH ≈ 8. A carbonate buffer was chosen, according to previous work by Mesquita et al. [3]. Because the buffer capacity is directly dependent on the sample/standard volume (set at pH=2) a combined study of the sample/standard volume, using the initial carbonate concentrations, was performed by measuring the pH of the solution after mixing (Supplementary information Fig. 1). As expected, the results showed that the increase of sample volume produced a decrease of pH and a final carbonate concentration of 16 mM was the minimal to ensure a final pH ≈ 8.

These studies also contributed to set the aspiration order in SI: ligand solution, buffer and sample, this way ensuring a good mixture of the sample with a buffered ligand solution. The buffer volume was set to the minimal reproducible amount of 24 µL [12], to ensure good mixing of the three plugs.

Analysis of the complex spectrum showed that the maximum absorption was observed at 460 nm, so this was the chosen wavelength for iron(III) quantification (Fig. 3). Furthermore, to minimise the influence of the schlieren effect in SI, as well as to effectively suppress the blank signal, the absorbance value at 800 nm was subtracted from the absorbance measured at 460 nm. This approach explored the CCD detector software which enabled to register the absorbance signal at two wavelengths, 460 nm and 800 nm.

The kinetics of the complex formation was also evaluated: the absorbance of a buffered mixture of 540 µL hexadentate ligand solution (0.05 g/L) and 960 µL of 1.25 mg/L iron(III) standard was measured for 1 min. Colour was observed almost immediately after mixing and there was no significant absorbance increase ( < 10%) during the measured time (1 min).

The preliminary studies enabled to set the basic conditions for the determination: the measurement wavelength of 460 nm (with subtraction of the signal at 800 nm); the use of a carbonate buffer with a final concentration (after overlapping of the plugs) above 16 mM; the aspiration order; and the minimal length of connection to detector due to the immediate complex formation.

### 3.2. Study of physical–chemical parameters for the complex formation

#### 3.2.1. Ligand solution

The volume of ligand solution was the first parameter to be studied; a ligand solution of 50 mg/L and a standard/sample volume of 300 µL were used. From the tested volumes within the range 68–171 µL, the volume of 125 µL was chosen as the calibration curve slope increased up to this value.

The ligand (CP256) solution concentration was studied up to 600 mg/L, and 500 mg/L (521 µM) was the chosen concentration. For this value, there was no significant difference when compared to the one obtained for 600 mg/L (in fact slope ≈ 5% lower) and still ensured an excess of ligand for all the iron(III) standards.

#### 3.2.2. Sample volume and buffer concentration

The sample volume was studied within the range 125–1200 µL (Fig. 4). According to the preliminary studies, the final concentration of carbonate buffer has to be 16 mM so the study of the influence of the sample volume on the sensitivity was made, simultaneously adjusting the initial concentration of carbonate buffer. The calibration curve slope increased with the increase of the sample volume up to 700 µL, and the intercept decreased with the sample volume increase up to 900 µL. So, to benefit from the highest sensitivity with the lowest detection limit the volume of 900 µL was chosen.

As the final concentration of 16 mM had to be attained with the set volume of 24 µL, for the chosen sample volume of 900 µL, the initial hydrogen carbonate buffer solution must be 0.60 M.

In the established conditions, the chosen approach to overcome the influence of the schlieren effect and the blank signal (Section 3.1, preliminary studies) was to subtract the absorbance signal at 800 nm to the absorbance signal at 460 nm. To test the efficiency of this approach, two calibration curves were compared: one obtained from the signal registered at 460 nm and another one obtained from the subtraction of the two signals. The subtraction approach resulted in an improvement of the calibration curves parameters: a slight increase in the slope ( > 6%) and a decrease in the intercept of approximately 1.5 fold.

### 3.3. Solid phase extraction

In order to attain matrix elimination, an in-line solid phase extraction (SPE) procedure was included. The chelating resin was packed in a PVC tube, 2.3 mm i.d. 2 cm length, entrapped with ordinary dish washing sponge. The size of the column was adopted from previous work [9] as the best option for sequential injection application.

#### 3.3.1. Resins

Three chelating resins were tested for iron(III) retention: Chelex 100, styrene divinylbenzene copolymers containing paired

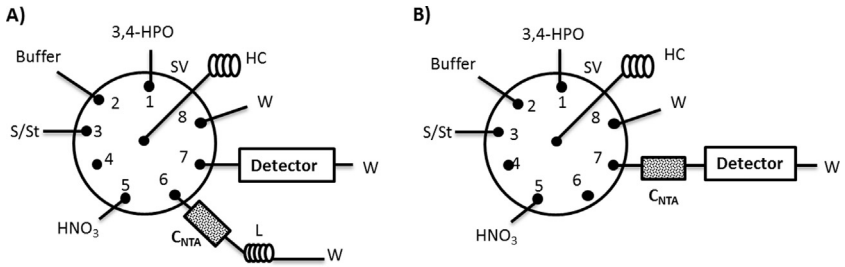


**Fig. 5.** Scheme of the two column positions tested: (A) column in a lateral port; (B) column in the way to the detector.

iminodiacetate ions, 200–400 mesh, 50% suspension in sodium acetate 0.5 M; Dowex 50X8, 20–50 mesh, 50% suspension in 30% ethanol; and NTA Superflow, highly cross-linked 6% agarose, 60–160 μm of bead diameter, 50% suspension in 30% ethanol. Similar columns were prepared for each resin and calibration curves established (Supplementary information Fig. 2). The conditioning of the columns was attained in-line: sodium acetate 0.5 M, for the Chelex column, and nitric acid 0.5 M for the Dowex and NTA columns.

The NTA resin was the chosen resin as it provided the highest sensitivity, over 75% higher than with the Chelex 100 and over 50% higher than the Dowex 50. Furthermore, the Chelex 100 required an additional conditioning buffer.

### 3.3.2. Position in the manifold

Having chosen the resin to assemble the SPE column, it was necessary to assess its location in the manifold. Two possible configurations were tested: the column placed on the way to the detector and the column in a lateral port of the selection valve (Fig. 5).

Calibration curves were established with both configurations and, although no major differences were observed, the column placed at a lateral port (Fig. 5A) provided an increase in sensitivity of 16% and a five-fold decrease in the limit of detection (calibration intercept). Furthermore, the NTA column in a lateral port facilitated iron speciation: total iron quantification attained without the NTA column; and iron(III) quantification using the NTA column.

Again, as for the direct determination (without SPE), the approach for removing signal refraction interference was evaluated. There were no differences observed in the calibration curve parameters ($<5\%$) for the two calibration curves (one obtained from the signal register at 460 nm and one obtained from the same signal but subtracting the signal registered at 800 nm).

### 3.3.3. SPE operation volumes

The sample volume, previously set at 900 μL (Section 3.2.2), was propelled through the NTA column with an equivalent volume of carrier, total volume $>1800$ μL, to ensure the washing out of any remaining sample in the column.

The volume of CP256 ligand solution, also previously set to 125 μL (Section 3.2.1), was propelled through the NTA column to complex the retained iron(III), removing it from the column. The volumes for propelling and aspirating the CP256 were assessed. First the propelling volume was studied, in the range 151–305 μL. The propelling volume of 205 μL was chosen as it corresponded to the highest sensitivity. Afterwards, calibration curves were established with different aspiration volumes and 261 μL was chosen from the range 205–305 μL as it also provided the highest sensitivity. Having set these operation volumes, and as the aspiration volume is higher than the propelling volume, it was necessary to include an extra tube after the NTA column to ensure that no air would go through the column. A length of 14 cm was enough for this purpose.

### 3.3.4. Column breakthrough

The column breakthrough corresponds to the maximum amount of iron(III) retained in the beads. Using iron(III) standards up to 10.0 mg/L and the set sample volume, different mass quantities of iron(III) were loaded into the packed NTA resin. The absorbance values increased significantly ($>35\%$) up to 4.5 μg of iron (III) and then started to stabilise ($<20\%$), indicating the maximum amount retained in the NTA resin column.

## 3.4. Iron speciation

As previously stated, the direct determination with the hexadentate CP256 ligand, without SPE, resulted in the determination of total iron. The use of the NTA column for SPE enabled the retention of iron(III) with consequent iron speciation.

In order to determine the total iron content with sample matrix elimination, it was necessary to oxidise Fe(II) to Fe(III), prior to the SPE step, as Fe(II) was not retained in the beads. This feature was tested and calibration curves with iron(III) and iron(II) standards were established and compared. When using the SPE approach with the NTA resin column, no calibration curves could be traced as the absorbance signal for the individual standards of iron(II) was statistically the same, with a standard deviation between the standards of 1.6%. So, hydrogen peroxide was chosen as the oxidising agent for the determination of total iron with SPE and two steps were added to the analytical cycle (Table 1), in order to sandwich the sample between two oxidant plugs [4]. The volume of oxidant was 24 μL per plug, the reported minimum volume to attain an effective overlapping [12] and the concentration of 12.8 mM was adopted from previous work [4]. Again, calibration curves with iron(III) and iron(II) standards were established and compared. Using the peroxide plugs, the slopes of the two calibration curves were practically the same (relative deviation 5%).

## 3.5. Interference assessment

### 3.5.1. Salinity

The evaluation of salinity interference in the determination was an essential parameter to assess considering the primary aim of applying the developed method to various types of natural waters, including seawater. The salinity interference studies were carried out using synthetic seawater. Iron standards were prepared in ultrapure water and in synthetic seawater and the established calibration curves compared.

For the direct determination without SPE, we could not establish a calibration curve because the absorbance value obtained for the different standards prepared in synthetic seawater was the same (relative deviation 7%) thus indicating a major interference in the determination.

Using the SPE for matrix elimination, the established calibration curves with the two sets of standards overlapped. The estimated slopes of the curves were assessed at the confidence intervals at 95%. The quality of the regression was tested by residual analysis (i.e. randomness and normality) and by the coefficient of

correlation, $R^2$, which was above 0.98 in both cases. No statistical difference, at 95% confidence level, was observed between the calibration curves with complete overlapping of the slope values thus indicating no salinity interference when using SPE.

#### 3.5.2. Other ions

Due to the nature of the colorimetric reaction, the possible interference of several bivalent and trivalent cations was assessed. Also, the application to water samples justified testing the influence of other major ions commonly present in waters, namely nitrate, nitrite and sulphate. The tested concentrations were based on maximum values mentioned in both Portuguese [13] and international legislation [11]. The solutions of the tested cations were obtained from dilution of atomic absorption standards except for nitrite and sulphate, which were obtained by dilution of a stock solution prepared from solid sodium nitrite and sodium sulphate. As for the anions, the tested concentrations were obtained from dilution of stock solutions prepared from the respective solids: sodium chloride, sodium nitrate, sodium nitrite and sodium sulphate. Several standards, with 0.60 and 0.30 mg/L of iron(III) and the tested concentration of interfering ions were prepared and analysed with the developed method, with and without SPE. The obtained absorbance values of the standard with and without interfering ion were registered and the interference percentage was calculated (Table 2).

Overall, for expected values in natural waters, no significant interferences were observed as most of the interference percentages were below 9%. Exceptions were observed for the phosphate without the SPE. However the tested concentration represented the worst case scenario and was not expected in natural waters.

### 3.6. Use of a LWCC

The expected values in seawater and other natural waters, namely river, are extremely low so an alternative detection cell was also tested. For low iron concentrations, the conventional flow cell (CFC) of 1 cm path length was replaced for a 100 cm path length liquid waveguide capillary cell (LWCC). With this detection system, a 10 fold decrease in the dynamic range was obtained together with a 100 fold increase in sensitivity. Although the LWCC could be used with and without the SPE approach, due to its capillarity characteristic, it was mainly used with SPE, to ensure the integrity of the LWCC.

**Table 2**
Interfering ions with the developed SI methodologies using CFC detection, with and without SPE.

| Possible interfering ion | Legislation maximum values | | Tested concentration of interfering ion (mg/L) | Interference (%) | |
|---|---|---|---|---|---|
| | UNFAO (mg/L) | Portugal (mg/L) | | SI | SI-SPE |
| $Al^{3+}$ | 5.0[a] | 20[b] | 2.5 | −5 | 3 |
| $Ca^{2+}$ | 15[b] | 50[b] | 15 | −1 | 5 |
| $Co^{2+}$ | 0.1[a] | 10[a] | 1.0 | 2 | −2 |
| $Cu^{2+}$ | 1.3[a] | 5[a] | 1.0 | −5 | −3 |
| $Mg^{2+}$ | 5.0[b] | 50[b] | 20 | −7 | −2 |
| $Mn^{2+}$ | 0.2[a] | 10[a] | 10 | 7 | 2 |
| $Ni^{2+}$ | 0.2[a] | 2.0[a] | 2.0 | −3 | 3 |
| $Zn^{2+}$ | 2.0[a] | 10[a] | 10 | −9 | 5 |
| $Cl^-$ | – | - | 20 | −1 | −4 |
| $NO_3^-$ | – | 50[a] | 50 | −1 | 0 |
| $NO_2^-$ | – | 0.1[b] | 0.05 | 2 | 2 |
| $SO_4^{2-}$ | – | – | 25 | 3 | 0 |
| $PO_4^{3-}$ | – | – | 5.0 | −1 | 10 |
| $Cd^{2+}$ | – | – | 0.01 | 0 | 1 |
| $Pb^{2+}$ | – | – | 0.015 | 0 | 0 |

[a] Irrigation waters.
[b] Streams waters.

### 3.7. Figures of merit

After all the studies for the speciation of iron based on the coloured complex formed with hexadentate ligand CP256, the characteristics of the developed method were summarised (Table 3).

The limits of detection and quantification, LOD and LOQ, were calculated according to IUPAC recommendations [14,15]. For three (LOD) and 10 (LOQ) times, the standard deviation of 10 consecutive injections of deionised water was used for the calculation. The determination rate was calculated based on the time spent per cycle. A complete analytical cycle took about 4 min for the SI method without SPE and 7 min for the SI with SPE, both with CFC. 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. So, the rate cycle is the time needed to complete one analytical cycle, in order to have one value. The consumption values of effluent production, per determination, was also calculated.

### 3.8. Application to natural waters

#### 3.8.1. Accuracy assessment for total iron determination

*3.8.1.1. Certified samples.* For accuracy assessment of the developed sequential injection (SI) method, three certified water samples were analysed and the results were compared to the certified value. The certified water samples were analysed with both detection systems, the conventional flow cell (CFC) and the 1 m liquid waveguide capillary cell (LWCC). The certified value corresponded to the total iron content, and the samples were not saline waters, so the developed SI method without the SPE was used for the analysis. The certified sample ERM-CA021a, a soft drinking water, with certified values in iron content of $197 \pm 2$ μg/L was analysed with the developed SI-CFC method and the obtained concentration was $203 \pm 42$ μg/L, corresponding to a relative deviation of 3%. A certified river water, NRC-CNR SLRS-4, with an iron content of $103 \pm 5$ μg/L was analysed with both detections systems the SI-CFC and the SI-LWCC and relative deviations of −9% and −7% were obtained corresponding to attained concentrations of $93.6 \pm 3.0$ μg/L and $96.0 \pm 0.0$ μg/L, respectively. The certified sample NIST 1640, with a certified value of $36.5 \pm 0.2$ μg/L, resulted in a relative deviation of 8% with the obtained concentration with the developed SI-LWCC method of $39.5 \pm 0.5$ μg/L.

An extra certified sample was assessed using the SI-LWCC method but with SPE, the sea water sample TM-27.3, with an iron content of 10.9 μg/L. The concentration obtained was 10.0 μg/L resulting in a relative deviation of 7%.

*3.8.1.2. Atomic absorption spectrometry.* For further accuracy assessment, 10 water samples were analysed with the developed SI method and the results were compared with the results obtained by the reference procedure consisting of the atomic absorption spectrometry (AAS) measurement (Supplementary information Fig. 3). The various combinations of the developed system were used: the determination with the CFC with and without the SPE and the LWCC with SPE. The linear relationship established between the sets of results was: $[Fe]_{SI} = 0.898(\pm 0.168) \times [Fe]_{AAS} - 0.005(\pm 0.019)$, where the values in parenthesis are 95% confidence limits. These figures show that the estimated slope and intercept do not differ statistically from values 1 and 0, respectively. Therefore, there is no evidence for systematic differences between the two sets of results [16].

*3.8.1.3. Recovery studies.* Additionally, several samples of inland bathing waters were collected at pH=2 [11] and spiked with iron (III) and/or iron(II) to final concentrations of 0.30 mg/L, volumes of

**Table 3**
Features of the developed method for iron speciation in water samples using CP256 ligand as a colorimetric reagent.

| Method | Flow cell | Dynamic range (mg/L) | Typical calibration curve[a] $A=S\times$ mg $Fe^{+3}/L+b$ | LOD (μg/L) | LOQ (μg/L) | Determination rate ($h^{-1}$) | Effluent production (mL) |
|---|---|---|---|---|---|---|---|
| SI | CFC | 0.10–2.00 | $A=0.050\pm0.001\times[Fe^{3+}]+0.000\pm0.002$ $R^2=0.997\pm0.001$ | 33 | 109 | 58 | 2.398 |
| SI | LWCC | 0.005–0.10 | $A=5.56\pm0.39\times[Fe^{3+}]+0.074\pm0.009$ $R^2=0.996\pm0.003$ | 3 | 6 | 42 | 1.852 |
| SI-SPE | CFC | 0.10–1.00 | $A=0.084\pm0.010\times[Fe^{3+}]+0.010\pm0.005$ $R^2=0.996\pm0.003$ | 27 | 91 | 28 | 4.605 |
| SI-SPE | LWCC | 0.005–0.10 | $A=6.17\pm0.08\times[Fe^{3+}]+0.215\pm0.081$ $R^2=0.993\pm0.002$ | 3 | 5 | 24 | 3.888 |

[a] Total of four calibration curves.

**Table 4**
Recovery percentages calculated from spiked natural waters assessed with developed method without solid phase extraction (SPE); SD, standard deviation; RSD, relative standard deviation.

| Sample ID | Initial SI | | | Added (mg/L) | | Found SI | | | Recovery (%) |
|---|---|---|---|---|---|---|---|---|---|
| | mg Fe/L | SD | RSD (%) | Fe(III) | Fe(II) | mg Fe/L | SD | RSD | |
| Tap 2 | 0.010 | 0.002 | 16 | 0.300 | – | 0.310 | 0.013 | 4 | 100 |
| | 0.010 | 0.002 | 16 | 0.300 | 0.300 | 0.638 | 0.058 | 9 | 105 |
| River 2 | 0.107 | 0.011 | 10 | 0.300 | – | 0.382 | 0.013 | 3 | 92 |
| | 0.107 | 0.011 | 10 | 0.300 | 0.300 | 0.789 | 0.018 | 2 | 114 |

**Table 5**
Recovery percentages calculated from spiked natural waters assessed with the developed method with solid phase extraction (SPE); SD, standard deviation; RSD, relative standard deviation.

| Sample ID | Initial SI | | | Added (mg/L) | | Found SI-SPE | | | Recovery (%) |
|---|---|---|---|---|---|---|---|---|---|
| | mg Fe/L | SD | RSD (%) | Fe(III) | Fe(II) | mg $Fe^{3+}/L$ | SD | RSD | |
| Tap 1 | 0.231 | 0.020 | 9 | 0.300 | – | 0.510 | 0.051 | 10 | 93 |
| | 0.231 | 0.020 | 9 | 0.300 | 0.300 | 0.521 | 0.008 | 2 | 97 |
| River 1 | 0.221 | 0.004 | 2 | 0.300 | – | 0.517 | 0.081 | 16 | 99 |
| | 0.221 | 0.004 | | – | 0.300 | 0.224 | 0.051 | 23 | –[a] |
| Estuary 1 | < LOD | – | – | 0.300 | – | 0.311 | 0.009 | 3 | 104 |
| | < LOD | – | – | 0.300 | 0.300 | 0.287 | 0.060 | 21 | 96 |
| Well 1 | 0.099 | 0.068 | 69 | 0.300 | – | 0.390 | 0.068 | 17 | 97 |
| | 0.099 | 0.068 | 69 | – | 0.300 | 0.111 | 0.034 | 31 | –[a] |
| | 0.099 | 0.068 | 69 | 0.300 | 0.300 | 0.388 | 0.000 | 0 | 96 |
| Sea 1 | 1.060 | 0.190 | 18 | 0.300 | – | 1.360 | 0.060 | 4 | 100 |
| | 1.060 | 0.190 | 18 | – | 0.300 | 1.040 | 0.090 | 9 | –[a] |
| | 1.060 | 0.190 | 18 | 0.300 | 0.300 | 1.350 | 0.280 | 21 | 97 |
| Spring 1 | 0.063 | 0.000 | 0 | 0.300 | – | 0.353 | 0.000 | 0 | 97 |
| | 0.063 | 0.000 | 0 | – | 0.300 | 0.051 | 0.000 | 0 | –[a] |
| | 0.063 | 0.000 | 0 | 0.300 | 0.300 | 0.389 | 0.000 | 0 | 109 |

[a] Recovery percentage not calculated because only iron(II) was added which is not determined with the SPE approach.

0.6 mL of 5.0 mg/L iron(III) and/or iron(II) standard were used. Then, the spiked samples were assessed with the two developed approaches, with and without SPE, to attest the efficiency of the iron speciation. The recovery percentages were calculated according to IUPAC recommendations [17] for both approaches without SPE (Table 4) and with SPE (Table 5). The overall average was 99.5% with a standard deviation of 5.9%. A statistical test (*t*-test) was used to evaluate if the mean recovery value did significantly differ from 100% and for a 95% significance level the calculated *t*-value was 0.168 with a correspondent critical value of 2.510. The statistical results indicate the absence of multiplicative matrix interferences.

The results obtained proved that iron speciation could be achieved by making the determination of total iron using the developed method without SPE and iron(III) determination using the SPE approach. Furthermore, because different natural waters were used, it confirmed that the developed methodology was applicable to different types of water samples.

## 4. Conclusions

The use of the hexadentate 3,4-HPO ligand, CP256, as an iron colorimetric reagent proved to be an environmental friendly effective alternative for the iron determination in all types of natural waters. As far as we know, only bidentate 3,4-HPO ligands had been used before as chromogenic reagents for iron, so this is the first application of an hexadentate 3,4-HPO ligand.

The advantages over the bidentates were various, as a consequence of the 1:1 stoichiometry: increased sensitivity of the determination (2 fold); lower detection limit (-65%); higher affinity for iron, facilitating the use as both chromogenic and eluent agent in the solid phase approach; higher solubility enabling reduction of the volume use which results in a better in-line mixture.

The complex formation proved to be almost immediate; no absorbance increase was observed after the initial colour

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formation, which is an excellent characteristic for a flow analysis application. The choice of sequential injection as a flow technique enabled to perform combined in the same manifold different determination approach and detection systems with a fast and automatic method. The developed method was successfully applied to a wide range of natural waters, mineral water, river waters, estuarine waters, tapwaters, and sea waters, after accuracy validation. The application to the sea waters was made possible by using a solid phase extraction approach with a NTA resin for retaining iron(III) and eliminating the sample matrix. The developed work enabled to prove the effectiveness of 3,4-HPO ligands as a selective, low toxicity reagents for iron speciation as a "more sustainable" alternative.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.05.062.

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